Functional analysis of ATM and ATR in the moss *Physcomitrella patens*

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Summary

Homologous recombination (HR) is an essential biological process, which plays a pivotal role in mechanisms such as meiotic crossover and the reparation of DNA double strand breaks. In addition to being a fundamental process of life, it is also the basis of gene targeting, a technical application, which utilizes the cells HR apparatus for precise genome editing. While GT has been successfully employed for functional genome analysis in mice and yeast, higher plants are innately inefficient in GT. This is because they repair DNA double strand breaks preferentially with another pathway, non homologous end joining (NHEJ), which does not lead to targeted insertions. On the other hand, the moss *Physcomitrella patens* preferentially uses HR for DSB repair as well as transgene integration. This feature makes *P. patens* a suitable model organism for studying HR in general, which could also provide a better understanding of its outstanding GT properties.

In order to extend our knowledge on this subject, the present thesis focused on two major topics. In the first part the inventory of DNA repair and recombination genes in *P. patens* was assessed via a BLAST based approach, which was made possible by the recent release of the *P. patens* genome draft. Intriguingly, *P. patens* possesses all important NHEJ genes, therefore its bias for HR over NHEJ could not be explained based on simple presence or absence of known NHEJ genes. On the other hand, there were similarities regarding the absence/presence of HR genes between *P. patens* and the also highly HR biased and GT efficient yeast. For example, BRCA1, BRCA2 and BARD1 are neither found in *P. patens* nor in yeast. Even more intriguingly they are available in *Arabidopsis* and also humans, where NHEJ is the dominant pathway. A further remarkable observation was that for 33 of the DNA repair and recombination related genes available in *P. patens* additional paralogs were identified. These paralogs are certainly interesting candidates for further studies.

The second part of the thesis focused on the ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) kinases, two key upstream signaling components of HR mediated DNA DSB repair. Utilizing the proficiency of *P. patens* in gene targeting *ATM* and *ATR* single as well as double mutants were generated by disrupting their kinase domains. These mutants were then used to characterize the function of the *P. patens* ATM and ATR homologs. Observing the mutant lines it was found that the loss of ATR function severely compromised vegetative and reproductive development while ATM disruption had just a mild effect on vegetative development. Testing the sensitivity of the mutant lines to various DNA DSB inducing agents it was found that in *P. patens* mainly ATR is required to facilitate the repair of these lesions, a situation similar to yeast but different to human cells and *Arabidopsis*, where also loss of ATM markedly affects sensitivity to these genotoxic agents. Similarly, another response to DNA DSB, the arrest of the cell cycle is also mediated by largely ATR in *P. patens* as well as yeast, while in *Arabidopsis* and human cells ATM also plays an important role. As part of the mutant phenotype analysis, the effect of *ATM* and ATR loss of function on the mode of transgene

integration was tested. It was found that removal of ATM function significantly increased NHEJ mediated random integrations while targeted integrations were unaffected, the latter were reduced only in the ATM and ATR double mutant.

Another major part of the thesis was to assess the global transcriptional change in response to bleomycin mediated DNA DSB induction in wild type as well as in mutant lines. Although *P. patens* is known to be biased towards HR, unexpectedly, in addition to HR genes also NHEJ genes were found transcriptionally induced in the wild type. Comparing the transcriptomes of wild type and mutant lines large differences in transcript levels were already observed without bleomycin treatment. Additional bleomycin treatment showed that a large number of DNA damage responsive transcripts could not be induced or repressed in the mutant lines. Another interesting finding was that while the transcriptional DNA damage response in Arabidopsis appears to largely rely on ATM function, in *P. patens* ATR seem to be more important in this regard.

Zusammenfassung

Als Homologe Rekombination (HR) wird ein essentieller biologischer Vorgang bezeichnet, der im Zuge des meiotischen Crossovers sowie in der Reparatur von DNA Doppelstrangbrüchen Anwendung findet. Darüber hinaus ist dieser Vorgang auch die Basis von Gentargeting (GT), eine technische Anwendung, welche den zellulären Rekombinationsapparat nutzt, um gezielt genomische Mutationen zu erzeugen. GT wird mit großem Erfolg in der funktionellen Genomanalyse in Hefe und Maus eingesetzt. In höheren Pflanzen jedoch ist diese Methode generell ineffizient, was dadurch erklärt wird, dass in Diesen DNA Doppelstrangbrüche (DSB) hauptsächlich mittels eines anderen Mechanismus repariert werden, der nicht-homologen Rekombination. Das Moos *Physcomitrella patens* hingegen nutzt vorzugsweise HR, sowohl für die Reparatur von DNA DSB als auch zur Integration von Transgenen. Diese Eigenschaft macht *P. patens* zu einem geeigneten Modellorganismus um grundlegende Aspekte der HR zu studieren und um das Verständnis seiner außergewöhnlichen GT Eigenschaften zu vertiefen.

Um die Wissensgrundlage auf diesem Gebiet zu erweitern, wurden in der vorliegenden Arbeit zwei Themengebiete bearbeitet. Im ersten Teil wurde der Bestand an DNA-Reparatur und –Rekombinationsgenen mittels eines BLAST basierten Ansatzes ermittelt, ein Unterfangen welches durch die kürzlich geschehene Veröffentlichung der Genomsequenz von *P.patens* ermöglicht wurde. Wie sich herausstellte besitzt *P. patens* alle bekannten wichtigen Gene der nicht-homologen Rekombination wodurch dessen Präferenz für HR nicht durch die An- oder Abwesenheit von NHEJ Genen zu erklären ist. Andererseits wurden Ähnlichkeiten bezüglich des HR Gen Bestandes zwischen *P. patens* und Hefe festgestellt, einem Organismus welcher ebenfalls bevorzugt HR nutzt und eine hohe GT Effizienz aufweist. Während zum Beispiel BRCA1, BRCA2 und BARD1 weder in *P. patens* noch in Hefe gefunden wurden, sind sie sowohl in Arabidopsis als auch im Mensch vorhanden, zwei Organismen in denen NHEJ dominiert. Eine weitere bemerkenswerte Feststellung war, dass für 33 der in *P. patens* detektierten DNA Reparatur- und Rekombinationsgene zusätzliche Paraloge gefunden wurden. Dies sind interessante Kandidaten für weitere Studien.

Der zweite Teil der Arbeit fokussierte sich auf ATM (ataxia telangiectasia mutated) und ATR (ATM and Rad3-related), zwei Kinasen die eine Schlüsselfunktion in der Signalkaskade zur Reparatur von Doppelstrangbrüchen via HR innehaben. Unter Nutzung der hohen GT Effizienz in *P. patens* wurden ATM und ATR Einzel- und Doppelmutanten erzeugt, indem ein Teil der Kinasedomäne durch eine Selektionskassette ersetzt wurde. Diese Mutanten wurden zur Untersuchung der Funktion der ATM und ATR Homologe von *P. patens* eingesetzt. In Bezug auf Wachstum und Entwicklung wurde beobachtet, dass der Verlust der Funktion von ATR zu schwerwiegenden Defekten in späteren Entwicklungsstadien sowie Sterilität führte, während ein Verlust der ATM Funktion lediglich geringfügige morphologische Veränderungen hervorrief. Um zu testen inwiefern *P. patens* ATM und

ATR ebenfalls eine Rolle in der Reparatur von DNA Doppelstrangbrüchen spielen wurden die Mutanten auf ihre Sensitivität gegenüber verschiedenen DNA DSB induzierenden Substanzen getestet. Es wurde festgestellt, dass in *P. patens* hauptsächlich ATR und nicht ATM für die Reparatur von DNA DSB benötigt wird, ähnlich wie in Hefe, aber im Unterschied zu Arabidopsis und menschlichen Zellen, wo ATM und ATR von ähnlicher Relevanz sind. Weitere Untersuchungen ergaben dass ein zentraler Schritt in der Reparatur von DNA DSB, der Arrest des Zellzyklus, in *P. patens* ebenfalls hauptsächlich durch ATR vermittelt wird. Dies stellt abermals eine Paralelle zu Hefe dar während in *Arabidopsis* und menschlichen Zellen ATM abermals eine mindestens ebenso wichtige Rolle spielt. Ein weiterer Aspekt der Phänotyp Analyse war es zu ermitteln ob der Verlust von ATM oder ATR einen Effekt auf Gentargeting haben würde. Es stellte sich heraus dass der Verlust der ATM Funktion zu einem signifikanten Anstieg zufälliger, durch nicht-homologe Rekombination vermittelte, Integrationen führte. Überdies wurde auch ein deutlicher Rückgang gezielter Integrationen beobachtet, allerdings nur in der ATM/ATR Doppelmutante.

Im Zuge der Analyse des globalen Transkriptionsmusters in Antwort auf Bleomycin induzierte DNA DSB im Wildtyp als auch in den Mutanten wurden einige interessante Beobachtungen gemacht. Zum einen wurde festgestellt dass, obwohl *P. patens* dafür bekannt ist eine Prädisposition bezüglich HR aufzuweisen, die Behandlung mit Bleomycin ebenfalls Gene die der nicht-homologen Rekombination zugeordnet sind induzierte. Beim Vergleich der Transkriptlevel von Wildtyp und Mutanten, zunächst im ungestressten Zustand, wurde festgestellt dass eine Vielzahl von Transkripten eine bereits erhöhte/erniedrigte Expression aufwies. Darüber hinaus zeigte in den Mutanten eine Vielzahl von Transkripten der DNA Schadensantwort keinerlei Responz auf die Bleomycin Behandlung. Letztlich ist noch zu vermerken, dass während die transkriptionelle Antwort auf DNA Schäden in *Arabidopsis* hauptsächlich unter Kontrolle von ATM zu sein scheint, in *P. patens* ATR die wichtigere Rolle spielt.

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Abbreviations:

µ	Micro
°C	Degree Celsius
A	Adenosine
aa	Amino acid
ATP	Adenosine-5’-triphosphate
bp	Base pairs
BSA	Bovine serum albumine
C	Cytidine
cDNA	Complementary DNA
CDS	Coding sequence
DMF	N,N-dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EST	Expressed sequence tag
EtOH	Ethanol
g	Gram
G	Guanosine
GUS	ß-glucuronidase
h	Hours
HU	Hydroxyurea
kb	Kilo base
l	Liter
LB	Lysogeny broth
LC-MS	Liquid chromatography coupled with mass spectrometry detector
m	Meter
M	Molar concentration (mole/liter)
MCS	Multi cloning site
mm	Milimeter
MMS	Methyl methanesulfate
MNU	N-nitroso-N-methylurea
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
n	Nano
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Negative logarithm of the proton concentration
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RT	Reverse transcription
RT	Room temperature
s	Seconds
SDS	Sodium dodecyl sulphate
ssDNA	Single stranded DNA
T	Thymidine
UTR	Untranslated region

UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
wt	Wild type
X-gluc	5-bromo-4-chloro-3-indolyl-b-D-glucuronide

1 Introduction

1.1 Genetic recombination, an essential aspect of life

Genetic recombination is a molecular event which is defined by the exchange of genetic information between two distinct DNA molecules, or between different regions on the same molecule. Recombination can be the result of different processes. Programmed recombination occurs during meiosis, facilitating the exchange of genetic information between homologous chromosomes during crossover (Smith and Nicolas 1998). This generates novel allele combinations in the resulting gametes that may affect the fitness of the resulting offspring. Another example for the importance of genetic recombination is the adaptive immune system as it is found in vertebrate organisms. There recombination is responsible for recombining the genes for antibody specificity, thus providing a highly diverse set of antigen recognition sites (Schatz 2004). In the previous examples, recombination is utilized to provide long term benefits for either a whole organism or an entire species. However, there is a much more frequent and acute event on the level of individual cells which requires the cells recombination machinery. That is the occurrence of DNA DSBs (double strand breaks) which are a potentially lethal DNA lesion. According to studies performed in yeast and mammalian cells, improperly repaired DNA DSBs may produce genomic deletions or rearrangements (Richardson and Jasin 2000, Honma et al. 2003). In addition, unrepaired DSBs may induce apoptotic cell death (Game 1993, Rich et al. 2000). It is assumed that there are two basic types of DNA DSBs which differ in the specifics of how they are generated as well as their structure (**Figure 1.1-1**). The first type of DNA DSB, termed direct or double ended, can be caused by ionizing radiation, radiomimetic chemicals or reactive oxygen species (ROS) as these agents are capable of causing breaks in the sugar-phosphate backbone of the DNA (Povirk et al. 1977, Bradley and Kohn 1979, Ward 1990). These DNA breaks are characterized by having two opposing ends and their formation is independent of the cell cycle stage (Povirk et al. 1989, Mahaney et al. 2009). The second type of DNA DSB has been designated as indirect, single ended, or replication specific (Helleday 2003). Contrary to the previously described DSB type these indirect DNA DSBs arise as a result of unrepaired minor DNA lesions such as UV induced DNA crosslinks or base alkylations which are caused by known carcinogenic substances such as nitrosamines or ethyl methanesulfonate (EMS) (Swann and Magee 1968, Swann and Magee 1971, Sega et al. 1976). If these lesions are not repaired prior to onset of DNA replication they block progression of the replication fork which may eventually lead to its collapse, resulting in the formation of a DNA DSB (Bessho 2003, Niedernhofer et al. 2004, Groth et al. 2010, Nikolova et al. 2010). While the immediate cause of replication specific DNA breaks is still not entirely clear it is presumed to be due to the action of nucleases (Petermann and Helleday 2010, Unno et al. 2013).

Owing to the fact that these DSBs originate from the collapse of a replication fork their structure is furthermore special in that they do not display two free DSB ends but just one (Helleday 2003, Shrivastav et al. 2007).

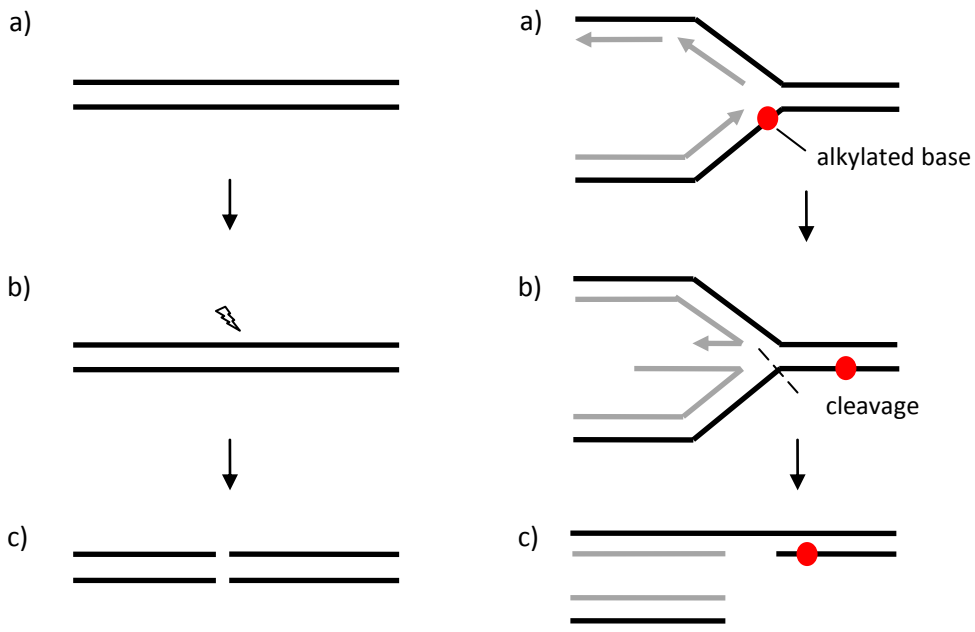

Figure 1.1-1: The two basic types of DNA DSBs. 1a) Direct DNA DSBs can be induced at any stage of the cell cycle. **1b)** They are caused by a variety of agents such as ionizing radiation, radiomimetic chemicals or endonucleases by disrupting the sugar-phosphate backbone of both DNA strands in close proximity. **1c)** The resulting DNA double strand break is characterized by two opposing DNA ends. **2a)** Indirect DNA DSBs occur only during DNA replication and are initiated by replication blocking lesions such as base alkylations or DNA crosslinks. **2b)** If the replication fork collides with an alkylated base for example, fork progression will be blocked and fork regression will be initiated instead. Fork regression distances the alkylated base from the bulky replisome at the replication fork, allowing it to be accessed by the base excision repair machinery. If the aberrant base is not repaired in a timely fashion however the replication fork may collapse and, presumably due to nuclease action, a double strand break will occur. **3c)** Due to the specific structure of replicating DNA molecules this resulting DNA break will be single ended.

In order to repair these highly deleterious DNA lesions, two fundamentally different recombination based pathways exist, non-homologous end joining (NHEJ) and homologous recombination (HR), both of which will be introduced in the following chapter.

1.2 HR and NHEJ the two modes of DNA DSB repair

In principle, DNA double strand breaks can be repaired via two major pathways, homologous recombination (HR) and non-homologous end joining (NHEJ). The central difference between the two is that HR requires a homologous DNA molecule to repair the break site whereas NHEJ does not. This is due to the fact that HR based repair utilizes an intact homologous DNA molecule as a template to repair the break site in a DNA synthesis dependent fashion whereas the NHEJ pathway "simply" religates the two opposing ends of a DNA DSB. These fundamental differences in the mechanism of these pathways also imply however that NHEJ is insufficient to repair the replication specific DNA DSBs as these only feature a single DNA end (Shrivastav et al. 2007). While the HR based pathway is the one predominantly used for repairing DNA DSBs in Yeast (Paques and Haber 1999), the chicken DT40 cell line (Takata et al. 1998) and the moss *Physcomitrella patens* (Markmann-Mulisch et al. 2007), most other systems like somatic mammalian cells (Mao et al. 2008) and higher plants like Arabidopsis *thaliana* (Markmann-Mulisch et al. 2007) prefer the NHEJ pathway for DSB repair, only showing increased dependency on HR during late S/G2 phases of the cell cycle, when either a sister chromatid or a double set of chromosomes is available (Rothkamm et al. 2003, Abe et al. 2005, Hinz et al. 2005). The increased use of HR for DSB repair in the S-phase of the cell cycle may also be related to the fact that replication specific indirect DNA DSBs are presumably irreparable by NHEJ, as mentioned earlier.

While many processes in HR mediated DSB repair as well as the full extent of involved genes are still unclear, recent studies in mammalian model systems have aided in producing a preliminary but comprehensive model for the mechanism and its key components (Misteli and Soutoglou 2009, Rhind 2009, Adams et al. 2010). The following section including **Figure 1.2-1** provides an overview of this model and some of the key proteins involved.

If a double ended DNA DSB is generated, first the trimeric MRN (MRE11, RAD50 and NBS1) complex will locate to the break site. The MRN complex then recruits a central component of the DSB repair pathway to the DNA lesion, the ATM (ataxia telangiectasia-mutated) protein kinase. The ATM protein is consitutively expressed and usually present in an inactive dimeric state (Bakkenist and Kastan 2003). After being recruited to the site of DNA damage the ATM protein is transformed to its active monomeric state through direct interaction with the MRN complex (Lee and Paull 2004, Lee and Paull 2005, You et al. 2005). Furthermore, in order to achieve full activity, ATM was found to also require autophosphorylation as well as acetylation (Bakkenist and Kastan 2003, Sun et al. 2007). At this point two important steps follow. First, the MRN complex, partly through its own exonucleolytic capabilities, but also through the activities of other exonucleolytic proteins, begins to resect the DNA at the break site(Paull and Gellert 1998, Eid et al. 2010), generating 3' overhangs. Simultaneously, the ATM kinase phosphorylates a large number of downstream components of the DNA damage

response. One of these is the histone protein H2AX (Burma et al. 2001), the phosphorylation of which presumably facilitates the accumulation of repair factors at the DSB site (Fernandez-Capetillo et al. 2004). Another pivotal phosphorylation substrate is CHK2 (Smith et al. 2010), its phosphorylation triggering the arrest of the cell cycle which provides the time necessary for the repair process. ATM is also thought responsible for chromatin relaxation via phosphorylation of KAP-1 (Ziv et al. 2006), increasing accessibility to the DNA break for other repair factors. Once the resection of DNA at the break site has generated a large enough single stranded DNA overhang, ATM and the MRN complex loose affinity to the now single stranded region (Shiotani and Zou 2009, Geuting et al. 2013). The 3' overhangs are instead bound by the single stranded DNA binding protein, replication protein A (RPA) (Wold 1997, Wu et al. 2005). The RPA bound ssDNA thereafter acts as a scaffold to recruit additional components of the DNA damage response (DDR) which are required to further process the DNA DSB. One of them is the ATR (ATM and Rad3-related) protein kinase, which through interaction with ATRIP binds to RPA covered ssDNA (Zou and Elledge 2003, Ball et al. 2005). Similar to ATM, ATR is another central signaling component whose kinase activity is presumed pivotal in initiating the further steps in the HR mediated pathway for DSB repair (Cimprich and Cortez 2008, Adams et al. 2010). Among its phosphorylation substrates are for example BRCA1 and Rad51, two components with important roles in the subsequent recombination process itself (Powell and Kachnic 2003, Verma and Rao 2013). In addition ATR mediated phosphorylation of the checkpoint kinase CHK1 is essential to uphold the cell cycle arrest induced by ATM and Chk2 during the initial stage of the repair process (Zhao and Piwnica-Worms 2001, Huang et al. 2008). Subsequent to ATR activation the RAD51 protein, in conjunction with recombination mediators such as BRCA2 (San Filippo et al. 2006), forms a nucleoprotein filament with the 3' single stranded DNA overhangs at the break site and initiates the search for a homologous DNA molecule as well as the subsequent strand invasion (Baumann and West 1998, Sneeden et al. 2013). The term strand invasion designates the process of the 3' single stranded DNA overhang of the damaged DNA molecule annealing to one of the intact strands of the homologous DNA molecule, thereby displacing the other. Subsequently, the sequence information of the damaged DNA molecule at the break site is restored by utilizing the intact homologous DNA molecule as a template in a DNA synthesis dependent process (Helleday et al. 2007).

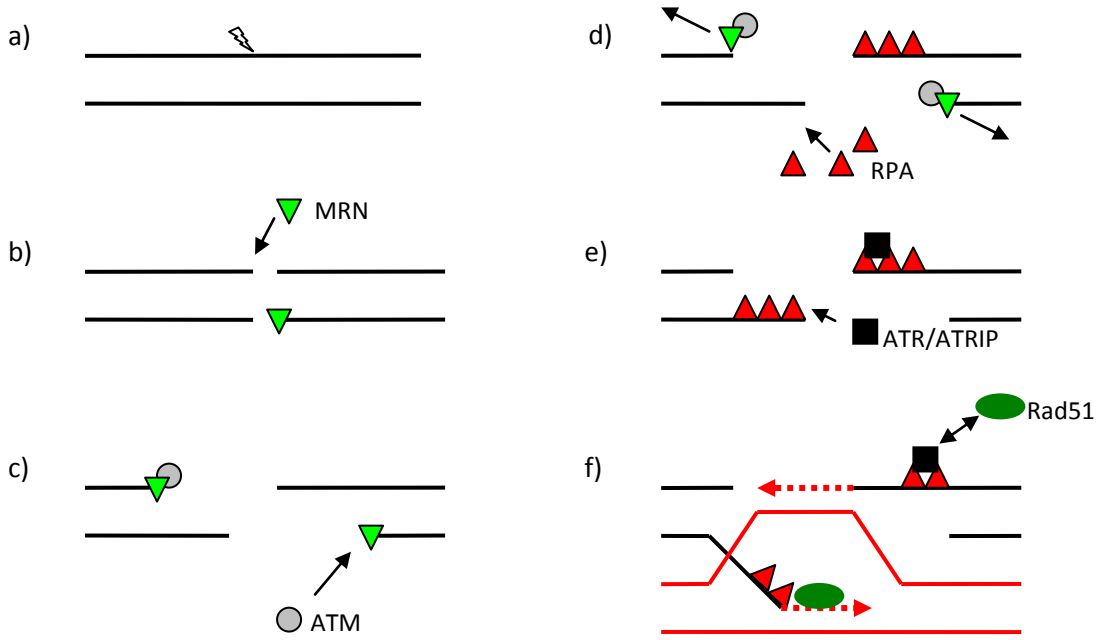

Figure 1.2-1: Outline of homologous recombination mediated repair of a direct double strand break (Misteli and Soutoglou 2009, Rhind 2009). a) A DNA DSB is induced through ionizing radiation or radiomimetic chemicals. **b)** The break site is detected and bound by the MRN complex which acts as a DNA damage sensor. **c)** ATM then binds to the MRN complex initiating strand resection and in addition phosphorylates downstream targets like Chk2 leading to cell cycle arrest. **d)** With increasing length of the ssDNA region the affinity of the breaksite for the MRN/ATM complex is diminished, leading to its replacement with replication protein A (RPA) **e)** Now ATR can, with the help of ATR interacting protein (ATRIP), bind to the RPA covered stretch of ssDNA at the breaksite and initiate the search for a complementary stretch of DNA. **f)** With the help of Rad51, which replaces RPA and ATR at the ssDNA terminus, the strand of the damaged DNA molecule invades into the intact homologue. Using the intact sequence as a template, the damaged part of DNA is repaired.

The alternative recombination based pathway for DSB repair is NHEJ. The NHEJ pathway, contrary to HR, does not require a homologous DNA molecule to repair DNA breaks and utilizes a largely different set of components (Beucher et al. 2009). As the name implies, it does not rely on extensive sequence homologies to repair a break site, but promotes “simple” religation of the free DNA ends at the break site. While this reduces the complexity of the repair process, it also increases the potential of generating small insertions or deletions at the break site which might be necessary to prepare the ends for ligation. This is especially the case if nucleotides at the break site contain additional lesions due to oxidative DNA damage (Mahaney et al. 2009). In the following section and **Figure 1.2-2** the essential steps and components of NHEJ will be discussed (Xu 2006). The beginning step in NHEJ is the recognition of the free DNA ends at the break site by the Ku70/KU80 heterodimer (Yaneva et al. 1997, Bekker-Jensen et al. 2006). The Ku70/Ku80 heterodimer forms a ring shaped structure, enclosing the free DNA ends, preventing their dissociation (Walker et al. 2001, Downs and Jackson

2004). In a next step this protein dimer recruits the DNA dependent protein kinase catalytic subunit (DNA-PKcs) to the break site (Spagnolo et al. 2006). Together with KU70 and KU80 the DNA-PKcs forms the heterotrimeric DNA-PK complex. Similarly to ATM and ATR in HR mediated DSB repair, DNA-PK acts as a transducer of the DNA damage signal via its kinase domain. It phosphorylates a large variety of NHEJ components such as the previously mentioned KU70 and KU80 as well as downstream effectors such as the Artemis nuclease, modulating their activity (Collis et al. 2004). DNA-PK is very likely also responsible for inducing cell cycle arrest via phosphorylation of Chk2 (Li and Stern 2005, Tomimatsu et al. 2009). Before the break site can be religated it may be necessary to fill in nucleotide gaps or remove overhangs. This task is performed by the DNA polymerases μ and λ (Lieber et al. 2010) and the exonuclease Artemis (Moshous et al. 2001). The final step in the repair process is the ligation of the break site. Carried out by the DNA ligase IV, this step is also dependent on the phosphorylation and binding of the DNA ligase IV by DNA-PK (Critchlow et al. 1997, Leber et al. 1998).

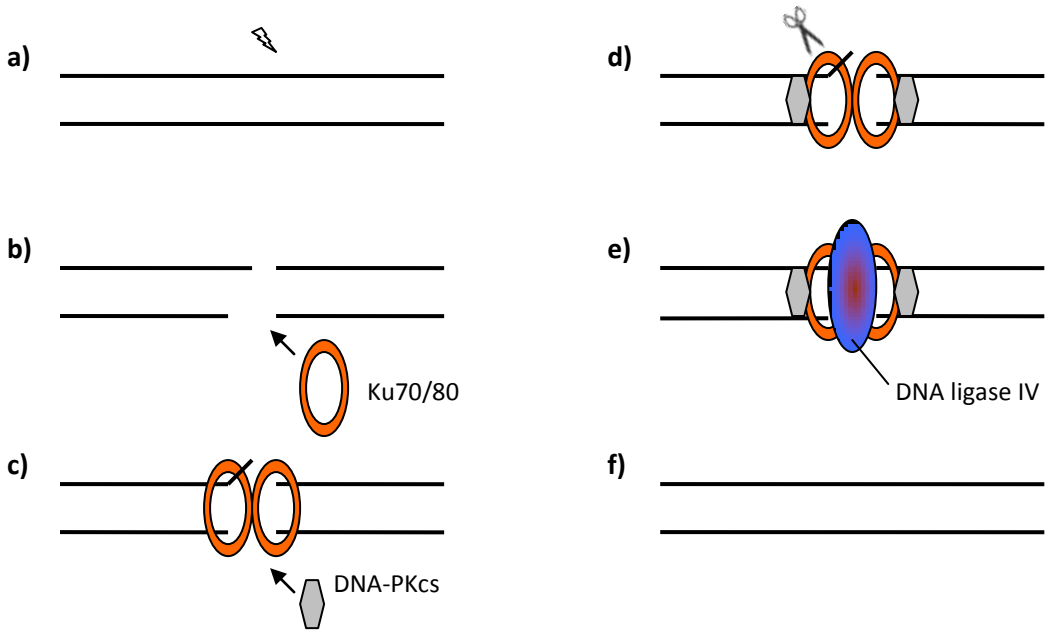

Figure 1.2-2: Outline of NHEJ mediated repair of a direct DNA DSB (Xu 2006). a) A double strand break is generated via ionizing radiation or oxidative stress. **b)** The break site is sensed by the Ku70/Ku80 Heterodimer which binds and tethers the DNA ends. **c)** This DNA-Protein structure promotes the localization of DNA-PKcs to the DSB site which together with Ku70 and Ku80 forms the DNA-PK heterotrimer. **d)** Gaps/overhangs are removed via nuclease or polymerase activity to prepare the break site for religation. **e)** DNA-PK promotes the ligation of the blunt DNA ends through DNA ligase IV. **f)** The DNA DSB is repaired.

As mentioned previously, due to the fact that ligation of broken DNA ends via NHEJ may require end processing and unlike in HR based DSB repair no homologous DNA molecule is used as a template for repair, there is a chance that genetic information may be lost (Burma et al. 2006). This is the reason

why this mechanism of DSB repair is often referred to as error-prone (Filkowski et al. 2004, Mandal et al. 2011)**.** However there is also substantial evidence that the previously described NHEJ pathway, also referred to as classical NHEJ, is largely precise in repairing DNA breaks (Difilippantonio et al. 2000, Ferguson et al. 2000, Guirouilh-Barbat et al. 2004). Instead there appears to be another DSB repair process, similar to NHEJ and therefore termed alternative NHEJ, which appears to be much more prone to producing errors (Deriano and Roth 2013, Ghezraoui et al. 2014). While it is still up for debate whether alternative end joining represents a wholly independent pathway from classical NHEJ there are a number of proteins which have been suggested to function primarily in alternative NHEJ such as PARP1, CtIP and DNA ligase III (Wang et al. 2005, Wang et al. 2006, Bennardo et al. 2008, Robert et al. 2009). Therefore, the classification of classical NHEJ as being overly prone to producing errors during DSB repair may be a misconception.

1.3 ATM and ATR, key components of the DNA damage response

As was previously described, ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) are key regulative components of the homologous recombination mediated pathway for DSB repair. Through their kinase activity they are capable of amplifying as well as diversifying the initial DNA damage signal, transducing it to the large number of substrates involved in the complex task of DNA DSB repair (Cimprich and Cortez 2008, Shiotani and Zou 2009, Shiloh and Ziv 2013). While many of their substrates are known, recent large scale proteome analysis suggest that there are probably many more which remain to be identified (Matsuoka et al. 2007). ATM and ATR are central components of the DNA damage response and thereby they are functionally and structurally highly conserved (Abraham 2001). Their function in DNA DSB repair has been found principally conserved in for example mammals (Cliby et al. 1998, Wright et al. 1998), yeast (Morrow et al. 1995) and Arabidopsis (Garcia et al. 2003, Culligan et al. 2004, Culligan et al. 2006).

ATM and ATR are huge proteins, the human homologs having a length of 3056 and 2644 amino acids respectively. They are members of the PIKK (phosphatidyl inositol 3' kinase-related kinases) family of serine/Threonine protein kinases. In humans this family also includes the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), mammalian target of rapamycin (mTOR), suppressor of morphogenesis in genitalia (SMG-1) and transformation/transcription domain-associated protein (TTRAP) (Lempiäinen and Halazonetis 2009). ATM and ATR both share a similar structure which is characteristic for PIKK type protein kinases (**Figure 1.3-1**).

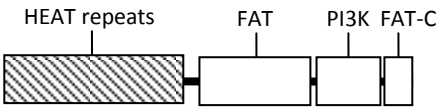

Figure 1.3-1: Schematic domain structure of human ATM and ATR. HEAT, Huntingtin, Elongation factor 3, protein phosphatase 2A and Target of rapamycin; FAT, FRAP, ATM and TTRAP; FAT-C, FRAP, ATM and TTRAP C-terminal; PI3K, Phosphoinositide 3-kinase, catalytic domain.

The N- terminus is defined by a large number of HEAT repeats which are known for their capacity to mediate protein-protein interactions (Perry and Kleckner 2003, Brewerton et al. 2004). Downstream to the HEAT repeats there is a FAT domain whose exact function is not yet fully established though available experimental evidence suggests a role for it in regulating the activity of the adjacent kinase domain (Lempiäinen and Halazonetis 2009). The PI-3 like kinase domain is located next to the FAT domain and is supposed to be essential for ATM and ATR function (Cliby et al. 1998, Park et al. 2001). At the C-terminal end of ATM and ATR a FAT-C domain is located. This comparably small domain is thought to be critical for the function of the PI3K kinase domain (Jiang et al. 2006, Lempiäinen and Halazonetis 2009).

1.3.1 The importance of ATM and ATR homologs varies in different organisms

The function of ATM and ATR in the repair of DNA damage is principally conserved in yeast, mammals and Arabidopsis. As a consequence of their important role in maintaining genome integrity loss of function mutants are expected to produce severe phenotypes. Humans carrying mutations in the ATM genes display a number of disease symptoms, the nature of which have lead to the designation of this gene, ataxia telangiectasia mutated (ATM). Humans suffering from this disease show neurodegeneration (ataxia) which impairs movement and coordination as well as abnormal dilation of blood vessels (telangiectasia) (Smith and Paterson 1980). Other prominent phenotypes include a predisposition to cancer, increased radiosensitivity, immune system deficiency, growth retardation and infertility (McKinnon 1987, McKinnon 2004, Lavin 2008). Generating a mouse ATM mutant via targeted disruption of the ATM kinase domain, the human disease symptoms could be largely replicated (Barlow et al. 1996, Xu et al. 1996). This also shows that ATM does indeed exert its main functions via its kinase domain. In Arabidopsis the effect of ATM loss of function however is far less severe, a reduction in seed set being the only observed phenotype while vegetative growth and development are seemingly completely unaffected. Yeast is even less affected by loss of function of its ATM homolog Tel1 as no effects on growth or development are observed (Greenwell et al. 1995, Morrow et al. 1995).

Compared to ATM, ATR (ATM and Rad3 related) appears to be much more important for basic growth and development in mammalian organisms. This is related to the fact that cre recombinase mediated deletion of ATR in a human somatic cell line resulted in cell death (Cortez et al. 2001). A

similarly severe effect was observed in mice as deletion of either the 5' region, including the start codon, or the kinase domain of *atr* were found to cause early embryonic lethality (Brown and Baltimore 2000, De Klein et al. 2000). Mec1, the yeast homolog of ATR was also found to be equally essential as its targeted deletion lead to a loss of cell viability (Weinert et al. 1994). Arabidopsis however appears to hardly rely on ATR as a T-DNA insertion into the *ATR* gene did produce neither a vegetative nor reproductive phenotype (Culligan et al. 2004).

1.3.2 Functions of ATM and ATR beyond HR mediated repair of somatic DNA DSBs

ATM and ATR are usually associated with their key role in the homologous recombination mediated repair of somatic DNA DSBs. However both protein kinases have also been found involved in a variety of other processes. ATR, as an example, has been attributed with stabilization of the replication fork during S-phase. This can help avoid its collapse in the event of replication blocking DNA lesions, preventing the formation of replication associated indirect DNA DSBs in the first place (Trenz et al. 2006, Ammazzalorso et al. 2010). ATR and its yeast homolog Mec1 have been found involved in affecting dNTP production in response to DNA damage by inducing the expression of ribonucleotide reductase genes (Huang et al. 1998, Zhang et al. 2009, Tsaponina et al. 2011). This is likely an important function as dNTPs are required in most DNA damage repair pathways including HR mediated DSB repair, nucleotide excision repair (NER) and base excision repair (BER). Another S-phase specific function of ATM and ATR is the control of origin firing, not only in response to DNA damage but also in the unperturbed cell cycle (Shechter et al. 2004). Another important function of ATM and ATR in maintaining genome integrity is to prevent the degradation of telomere ends as has been observed in a variety of model organisms including mammals, yeast and Arabidopsis (Silva et al. 2004, Vespa et al. 2005, Pennarun et al. 2010, Muraki et al. 2013, Di Domenico et al. 2014). Recent studies have also provided support for the notion that ATM and ATR both are directly involved in the regulation of components of crosslink repair (Kim and D'Andrea 2012) whereas only ATR was found to play an important role in facilitating nucleotide excision repair (NER) (Auclair et al. 2008, Shell et al. 2009). There is even evidence which supports the claim that specifically ATM (Segal-Raz et al. 2011, Zha et al. 2011) but possibly even ATR (Zhang et al. 2004) play a role in classical NHEJ. While all of the previously mentioned presumed functions of ATM and ATR take place in the nucleus there is also mounting evidence that suggest a cytoplasmic role for these proteins. A number of studies for example ascribe ATM with a function in insulin signaling (Yang and Kastan 2000, Ching et al. 2012) and others imply ATM to be important in maintaining mitochondrial homeostasis (Valentin-Vega and Kastan 2012, Valentin-Vega et al. 2012), possibly even due to involvement in mitochondrial DNA repair (Sharma et al. 2014).

1.4 Gene targeting, a technical application of homologous recombination

Homologous recombination is not only essential to maintain genome integrity, but is also employed as a tool to generate specific mutations via a process called gene targeting. Gene targeting is a technique which has been used to great effect in the field of reverse genetics. In principle it allows for the precise modification of any genomic locus whose sequence information is available. This is achieved by integrating an artificial DNA molecule, often referred to as targeting construct, at the genomic target locus to generate insertions, deletions or single nucleotide exchanges. The key feature of every gene targeting construct is a stretch of sequence which is homologous to the genomic locus to be modified. After the targeting construct has been introduced into the nucleus, this region of homology guides it to the target locus. Subsequently, this locus is modified in a recombination based process according to the sequence information provided by the targeting construct.

The assumption that gene targeting does rely on the cells homologous recombination machinery, as it is also employed in the repair of DNA DSBs, is founded on a number of things. The first and most obvious argument is that all targeting constructs require a region of sequence homology to the target locus just like the repair of a DSB requires a homologous chromosome. Secondly, it was found that linearization of the initially circular targeting constructs, by cutting inside the region of homology, thereby effectively creating double strand breaks, increased transformation efficiencies 4-3750 fold in yeast (Orr-Weaver et al. 1981) and 10-100 fold in mammalian cells (Lin et al. 1984). Direct evidence for at least partially conserved pathways for gene targeting and DSB repair were found when gene targeting efficiency was measured after key components of the HR mediated DSB repair machinery had been knocked out. Disabling the functions of Rad52 in Yeast or Rad51 in the moss *Physcomitrella patens* for example decreased the efficiency of HR mediated DNA DSB repair (Ayora et al. 2002, Markmann-Mulisch et al. 2007) as well as the capacity for gene targeting (Schiestl et al. 1994, Schaefer et al. 2010).

Gene targeting is a technique which has been applied with great success in functional studies in yeast (Hinnen et al. 1978) or vertebrate model systems such as mouse embryonic stem cells (Thomas and Capecchi 1986, Thomas et al. 1986) and the chicken DT40 B cell line (Buerstedde and Takeda 1991). In higher plants such as Arabidopsis however gene targeting has never been a feasible option to generate specific mutants as only 0.01 % - 0.001 % of transformants display successful gene targeting (Puchta and Fauser 2013). While overexpression of HR genes taken from gene targeting efficient organisms such as yeast, or mutation of NHEJ components were at times found to provide small improvements in targeting efficiency (Even-Faitelson et al. 2011, Qi et al. 2013) gene targeting remained a difficult and time consuming effort in higher plants. Instead, much more promising results have been obtained by applying an entirely different strategy. Based on the fact that

recombination is naturally induced by DNA double strand breaks in order to repair said lesions, specific nucleases were designed which would induce a DNA DSB at a predetermined target site. These “Zinc finger nucleases” (ZNF) or the more advanced “transcription activator-like effector nucleases” (TALEN) are based on combining two distinct domains, a DNA binding and a nuclease domain. While the nuclease domain is usually obtained from the *Fok*I restriction endonuclease, the DNA binding site has to be tailored according to the targeted genomic locus and subsequently fused with the nuclease domain. Transforming cells with this specific nuclease as well as a gene targeting construct, both designed to target the same locus, would in theory increase homologous recombination at the targeted locus via introduction of DNA DSBs. Indeed, utilizing the ZNF based approach gene targeting efficiencies of 2 and 10 % were obtained in tobacco protoplasts and embryogenic maize cells respectively (Townsend et al. 2009)(Shukla et al. 2009) and use of the TALEN system yielded 14 % gene targeting efficiency in tobacco protoplasts (Zhang et al. 2013). While these novel techniques are a huge step towards accessible gene targeting in plants, their recent emergence also means that not much is known about any negative side effects which may accompany them. It has for example been mentioned that the introduction of nucleases may lead to nonspecific cleavage of DNA in the transformed cells, introducing unwanted secondary mutations (Puchta and Fauser 2013). An alternative approach to try and improve gene targeting in higher plants involves the moss *Physcomitrella patens*. This plant has been found innately efficient in gene targeting (Schaefer and Zryd 1997) and due to that has been labeled as “green yeast” on occasion (Schaefer 2001, Shakirov et al. 2010). If one could learn why gene targeting is so efficient in *P. patens* one could possible use that knowledge to improve gene targeting in higher plants too. However, up until now there is no definite answer as to why gene targeting is so efficient in *P. patens* (Puchta and Fauser 2013).

1.5 The model plant *Physcomitrella patens*

P. patens is a non-vascular land plant belonging to the phylum of bryophyta. It is evolutionary distant from the premiere model plant Arabidopsis, as it diverged from the early predecessors of vascular plants approximately 430 - 700 million years ago (Nishiyama et al. 2003, Hedges et al. 2004). Besides the absence of a vascular system and the reduced complexity of its body plan, one of the main differences of *P. patens* compared to higher plants like Arabidopsis is the dominance of the haploid gametophytic stage. In Arabidopsis, the haploid gametophytic stage is reduced to only the embryo sac and pollen grains while the whole of the assimilatory tissue up until the formation of these structures is defined by the diploid sporophyte. This situation is reversed in *P. patens*. There the assimilatory tissue and the major developmental processes are restricted to the haploid gametophytic tissue while the diploid sporophyte consists of only the seta and spore capsule. This dominance of the haploid stage simplifies mutational studies as the disruption of a gene will not be

counterbalanced by a second allele (Reski 1998). Another interesting attribute of *P. patens* is its remarkable capacity for regeneration. Small pieces of gametophytic or sporophytic tissue are capable of developing into healthy and adult plantlets, similar to a germinating spore (Cove 2005). This property allows for easy vegetative propagation and thereby also facilitates the preservation of sterile mutants for prolonged periods of time. A further interesting trait of *P. patens* is its innate high efficiency in gene targeting approaches as was mentioned previously. Coupled with the recent release of its genome sequence (Rensing et al. 2008) this makes *P. patens* an excellent platform for reverse genetic studies. While the evolutionary distance to higher plants such as Arabidopsis thaliana may appear as a drawback in this regard, it has been shown that a number of processes are conserved between these two distantly related organisms. For example, the developmental pattern of *P. patens* has been found similarly responsive to the phytohormones ABA, auxin and cytokinin (Ashton et al. 1979, Knight et al. 1995, Imaizumi et al. 2002, Decker et al. 2006). Furthermore, large scale transcriptome and proteome studies have suggested that the response to salt stress, or more specifically the plant stress hormone ABA, is similar to what can be observed in higher plants (Richardt et al. 2010, Wang et al. 2010).

1.5.1 The lifecycle of *Physcomitrella patens*

The lifecycle of *P. patens,* as depicted in **Figure 1.5-1**, begins with the germination of a haploid spore **(a)**. The cells emerging from the spore proliferate via tip growth as well as side branching, forming a filamentous structure termed Protonema **(b)**. During initial protonemal growth one may observe two different types of cells, Chloronema and Caulonema. Chloronema type cells contain a large number of chloroplasts and their cell walls are perpendicular to the filament axis. The caulonemal cells on the other hand emerge only after the first few days of growth, arising out of some of the chloronemal cells which undergo a period of transitionary growth. Caulonemal cells contain less chloroplasts, proliferate faster (Menand et al. 2007)and are distinguished by the oblique orientation of their cell wall in respect to the filament axis. While chloronemal cells have been mainly attributed with assimilatory functions (Cove et al. 2006) the caulonema type cells are thought to mainly aid in substrate colonization and nutrient uptake (Menand et al. 2007). The growth of caulonema type cells is under auxin control (Johri and Desai 1973, Ashton et al. 1979) and may also be affected by exogenous cues such as light and nutrient availability (Thelander et al. 2005). After about 7 to 10 days the first buds start to emerge on caulonema type filaments, initiating the second stage of gametophytic growth whose induction relies on cytokinin (Reski and Abel 1985) (**c**). The buds proceed to develop into small leafy structures, the gametophores (**d**). Once mature the gametophores will give rise to the antheridia and archegonia, the respective male and female gametangia of *P. patens* (**e**). As *P. patens* is a monoecious plant each gametophore houses the male

as well as female gametangia. Once spermatozoids are released from the antheridia they proceed to fertilize the egg cell located in the archegonium. Successful fertilization will give rise to the sporophyte (**f**), the only diploid structure in the lifecycle of *P. patens*. Inside the sporophyte the next generation of haploid spores is generated via meiosis.

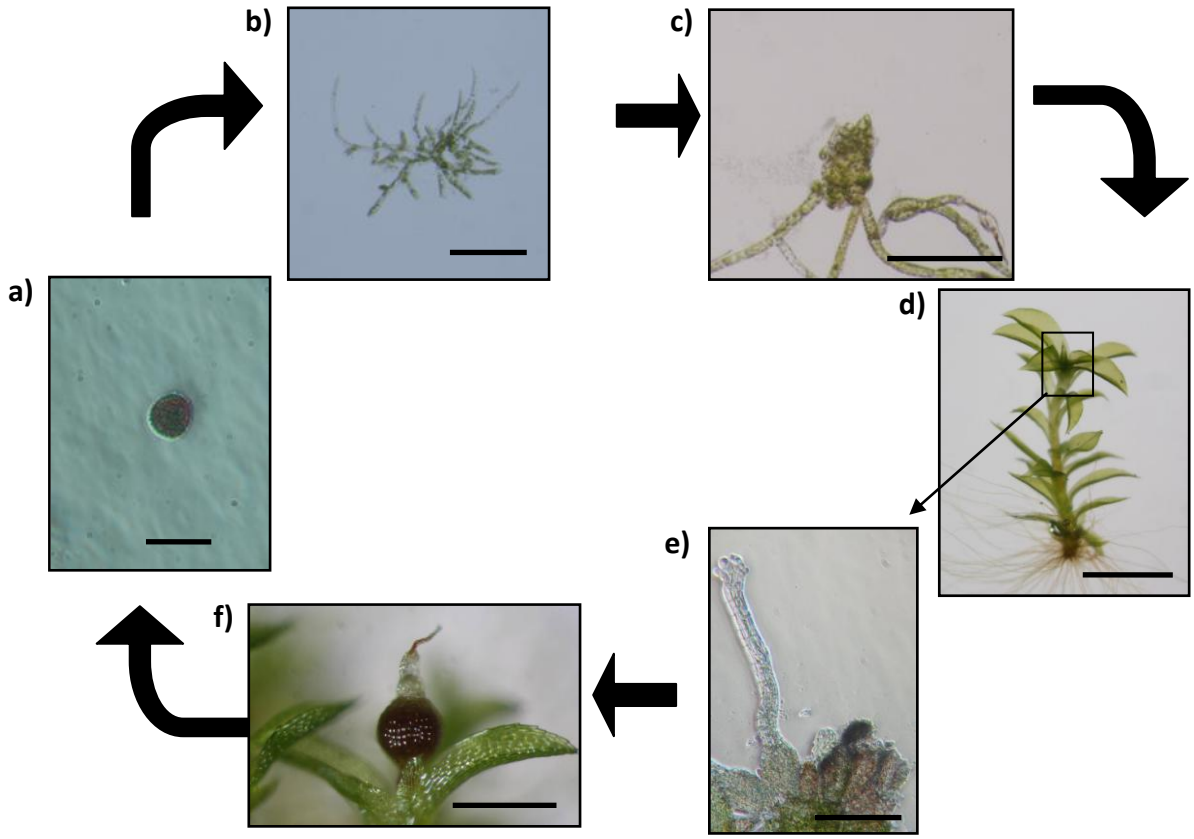

Figure 1.5-1: After germination of the spore (**a**), a filamentous tissue consisting of slow growing, chloroplast rich, chloronema cells and fast growing chloroplast poor caulonema cells is produced (**b**). After about one week buds begin to develop on the caulonema filaments (**c**). The buds mature into gametophores (**d**) which, having attained maturity, form the male (antheridium) and female (archegonium) reproductive organs (**e**). Following fertilization of the egg cell by the spermatozoa the only diploid structure in the lifecycle, the sporophyte (**f**), develops and subsequently produces the next generation of haploid spores via meiosis. Scale bars indicate, 10 µm (**a**), 500 µm (**b**), 200 µm (**c**), 1 mm (**d**), 50 µm (**e**), 500 µm (**f**).

1.6 Objectives of this work

The experimental work conducted in the course of this thesis is designed to adress two major topics. The first is to try and understand the basis of the remarkable gene targeting efficiency observed in *P. patens* (Schaefer and Zryd 1997). Speculating that the high gene targeting proficiency of *P. patens* may be reflected in a special set of DNA recombination/repair genes, the presence and absence of these genes in *P. patens* is determined via a BLAST based approach. To a certain degree this topic has already been explored in publication detailing the *P. patens* draft genome sequence in 2008 (Rensing et al. 2008). However, while nothing remarkable was announced this topic was not addressed in a meticulous fashion as it was only intended to provide a general overview.

The second part of the thesis is focused on the ATM and ATR kinases, two central components of the homologous recombination pathway. The first point here is to functionally characterize the ATM and ATR homologues of *P. patens*. For this purpose, it is intended to delete the kinase domain of *ATM* and *ATR* via gene targeting, in order to obtain loss of function mutants. The resulting mutant lines will then be subjected to a detailed phenotype analysis, including growth and developmental observations as well as investigating their response to genotoxic stress. This will provide information on the key aspects of ATM and ATR function in *P. patens* and allow a comparison with their counterparts in other organisms. In a next step the *ATM* and *ATR* mutant lines will be used to explore the transcriptional DNA damage response controlled by these two kinases. In plants this has so far only been done in Arabidopsis, utilizing a microarray based approach and *ATM* and *ATR* single mutants only. Our approach will utilize the more sensitive SuperSAGE technique and will expand on the Arabidopsis study by also including an *ATM* and *ATR* double mutant line into the analysis. This transcriptome approach is furthermore supplemented by a proteome analysis. Finally, again utilizing the innately high gene targeting efficiency of *P. patens*, it is planned to assess whether or not ATM and ATR are actually required for the homologous recombination process itself.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and Kits

The chemicals used in the experimental part of this work were supplied by Biozym (Hamburg), Duchefa (Haarlem, the Netherlands), Eppendorf (Hamburg), Merck (Darmstadt), New England Biolabs (Frankfurt), Serva (Heidelberg), Sigma-Aldrich (Steinheim), Roth (Karlsruhe), Gibco BRL (Karslruhe), and Invitrogen (Karlsruhe).

Kits used in this work were supplied by Invitrogen (Karlsruhe), Macherey-Nagel (Düren), Qiagen (Hilden) and Roche (Penzberg). Restricting and modifying enzymes were purchased from Roche (Penzberg), New England Biolabs (Frankfurt am Main), Takara (Saint-Germain-en-Laye, France) Fermentas (St. Leon-Rot), Stratagene (Heidelberg), and Invitrogen (Karlsruhe).
Oligonucleotides were synthesized by Metabion (Martinsried) and Invitrogen (Karlsruhe). Cloning vectors used were pGEM-T easy (Promega), pBLUESCRIPT SK- (Stratagene), pDONR-201 (Invitrogen), and pJAN33 (Weigel et al., 2003).

2.1.2 Software

Design of oligonucleotides and gene targeting constructs as well as basic sequence alignments and sequence assembly were carried out using the Vector NTI software package, version 11, from Invitrogen (Karlsruhe). Sequence alignments for the purpose of generating phylogenetic trees were done via ClustalX version 2.1, available at http://www.clustal.org/. Phylogenetic trees were inferred using the MEGA (Molecular Evolutionary Genetics Analysis) software tool version 4.1, downloadable at http://www.megasoftware.net/. Processing and visualization of experimental data as well as writing of this thesis was performed with the Microsoft Office software bundle.

2.1.3 Online resources

To conduct BLASTp analyses on various protein sequence databases the web based BLAST services at http://BLAST.ncbi.nlm.nih.gov/ and http://www.cosmoss.org/ were utilized. Protein query sequences for BLAST searches were obtained from the swissprot database accessible at http://www.uniprot.org/, TAIR release version 9 at http://www.arabidopsis.org/ and *P. patens* release version 1.6 at http://www.cosmoss.org/.

2.1.4 *P. patens* growth media

PPNH4 media for subculturing, phenotypic analysis and midterm storage:

0.8	g/l	$Ca(NO_3)_2 \times 4H_2O$
0.2	g/l	$MgSO_4 \times 7\ H_2O$
0.0125	g/l	$FeSO_4 \times 7H_2O$
0.5	g/l	$(NH_4)_2C_4H_4O_6$
1	ml/l	AltTES (stock solution)
1	ml/l	KH_2PO_4/KOH (stock solution)

PPNH4+ media for protoplast isolation and growth of protonema filaments in the dark:

PPNH4 medium with added Glucose, 5 g/l.

PPNH4-M media for regeneration of protoplasts

PPNH4 medium with an additional 66 g/l mannitol as osmoticum

PP- media, for induction of sexual propagation and phenotypic analysis:

PPNH4 medium omitting the ammonium tartrate as an additional nitrogen source.

AltTES (1000x stock):

55	mg/l	$CuSO_4$
614	mg/l	H_3BO_3
55	mg/l	$CoCl_2 \times 6\ H_2O$
25	mg/l	$Na_2MoO_4 \times 2\ H_2O$
55	mg/l	$ZnSO_4 \times 7\ H_2O$
389	mg/l	$MnCl_2 \times 4\ H_2O$
28	mg/l	KI

KH_2PO_4/KOH (1000x stock)

Dissolve 25g KH_2PO_4 with 4M KOH, adjust pH to 6.5 and add water up to a volume of 100 ml.

2.1.5 Bacterial strains

***E. coli* DH5α:**	supE44 PlacU169 (Q80 lacZPM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1
***E. coli* SCS110:**	rpsL (Strr) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44D (lacproAB) [F' traD36 proAB lacI q ZTM15]

2.1.6 Plasmids

Purpose/Name	Vector backbone	Reference
Plasmids used in assembly of *ATM* and *ATR* targeting vectors		
pUC18/Hyg	pUC	B. Reiss, unpublished
pUC/NPT	pUC	B. Reiss, unpublished
p1kbupATR	pGEM®-T easy	this work
p1kbdownATR	pGEM®-T easy	this work
p1kbupATM	pGEM®-T easy	this work
p1kbdownATM	pGEM®-T easy	this work
Plasmids containing constructs for ATM and ATR targeting		
pKOcol2	pBLUESCRIPT SK-	Zobell et al. 2005
pKoATR 2.1	pBLUESCRIPT SK-	this work
pKoATM 3.5	pBLUESCRIPT SK-	this work
Plasmids containing 5' and 3' RACE sequences of *ATM* and *ATR*		
M73_1	pGEM®-T easy	this work
M73_2	pGEM®-T easy	this work
M73_3	pGEM®-T easy	this work
M3_1	pGEM®-T easy	this work
M3_4	pGEM®-T easy	this work
M3_6	pGEM®-T easy	this work
R22_1	pGEM®-T easy	this work
R22_2	pGEM®-T easy	this work
R1_3	pGEM®-T easy	this work
R1_7	pGEM®-T easy	this work
Plasmids containing cDNA Sequence of *ATM* and *ATR*		
ATM5'_1	pGEM®-T easy	this work
ATM5'_2	pGEM®-T easy	this work
ATM3'_1	pGEM®-T easy	this work
ATM3'_2	pGEM®-T easy	this work
ATR5'1._1	pGEM®-T easy	this work
ATR5'1._2	pGEM®-T easy	this work
ATR5'2._1	pGEM®-T easy	this work
ATR5'2._2	pGEM®-T easy	this work
ATR3'_1	pGEM®-T easy	this work

Purpose/Name	Vector backbone	Reference
Plasmids containing template sequences for southern probes		
ATM_5'flank	pGEM®-T easy	this work
ATM_3'flank	pGEM®-T easy	this work
ATM_deleted	pGEM®-T easy	this work
ATM_Sul	pGEM®-T easy	this work
ATR_5'flank	pGEM®-T easy	this work
ATR_3'flank	pGEM®-T easy	this work
ATR_deleted	pGEM®-T easy	this work
ATR_NPTII	pGEM®-T easy	this work

2.1.7 Oligonucleotides

Purpose/Name	Length	Oligo Sequence 5‘ -> 3‘
Generation of probes for Southern blot		
sATR_3flank_fwd	19	CAGCGACAGCCACATCATT
sATR_3flank_rev	22	GCTTAGATTTACATGTGCGAGG
sATR_5flank_fwd	22	TGTCATCGAGCAATAGCCGAAC
sATR_5flank_rev	20	ACCGAGGCAGGGCTTGAAAC
sATR_del_fwd	18	AGGATGCCCGTATGATGG
sATR_del_rev	20	CTAGTGAGAGCCCTTTGTCG
sATR_NPT_fwd	18	TGGATTGCACGCAGGTTC
sATR_NPT_rev	20	CGATACCGTAAAGCACGAGG
sATM_del_fwd2	19	GTTGGACTTGGAGATCGAC
sATM_3flank_fwd	22	CTCTTACTATCGGAATGCTCAG
sATM_3flank_rev	21	GTGGAGACGTTAAGCAAGATG
sATM_5flank_fwd	21	GGCTTTAGAACACTTGTCCAT
sATM_5flank_rev	20	CTCTAGCACACCAGCACTTG
sATM_del_rev	21	CATAACAGATAGAGTGGCTTC
sATM_Sul_fwd	19	CAACAGTCAGCCGTGCCTC
sATM_Sul_rev	19	CATTGCCGATCGCGTGAAG

Purpose/Name	Length	Oligo sequence 5‘ -> 3‘
ATM and ATR transformant genotyping		
ATM_genome_fwd2	20	ACAGGATGCGGTAAGGCTTG
PpATM/ATR_p35S_rev	22	AATTGCCCTTTGGTCTTCTGAG
PpATM_genome_fwd	19	AACGCCATCTGTGCATTCG
PpATM_genome_rev	24	CAGTTCCAGACCTTAACCTTCATC
PpATM_locus_fwd	24	TGAAGGAAAACCAACGTGGGAAGG
PpATM_locus_rev	22	ATGCGTGGCTGATGTTTGTGTG
PpATM_tpSul_fwd	19	AAGCGCAATCACCATCTCG
PpATR_genome_fwd	19	TCGGTTGCTTACCCTTTGG
PpATR_genome_rev	19	AATGATGTGGCTGTCGCTG
PpATR_NPTII_fwd	19	TGGCTACCCGTGATATTGC
PpCol2 transformant genotyping		
GUS_out_4_rev	20	TGGTGTAGAGCATTACGCTG
Hyg_4_fwd	18	TCAGAGCTTGGTTGACGG
Ppcol2_3_3_rev	18	CCACAGGAGACTGCTTGC
Ppcol2_5_5_fwd	19	GATTGGTCTCCTGGATTGC
Ppcol2_del_3_fwd	22	GCATGGTTCGGTTACTCTCTAC
Ppcol2_del_5_rev	18	TCGTGTCGTGAAGCAAGC
ATM and ATR cDNA synthesis		
ATM_Ex3_1_rev	26	CCAGAACGTCCATTAGTATGCAATCG
ATM_Ex71_1_fwd	23	GGAGTGTCTGGTGTGGAAGGCAT
ATR_Ex1_2_rev	25	CCTAAGATTGGTAACACTGCATGGC
ATR_Ex20_1_fwd	24	ATCAATGGTTGACAGCACTATCGC
ATR_EX22_1_fwd	21	TCGAAATCCTAGCCTCGCTGC
ATR_Ex3_1_rev	23	GAGTCCATTTGGCTAAAGGCTGC

Purpose/Name	Length	Oligo sequence 5‘ -> 3‘
Cloning of ATM and ATR complete cDNA		
PpATM_3UTR_3_rev	19	CGTGACCTTGTGGAATTGG
PpATM_5UTR_4_fwd	21	GAAGCTAAGGTGAATCGGAAC
PpATM_Mid_1_rev	19	GCAAGATCACGCATGTCAG
PpATM_Mid_2_fw	20	ACCAGGTGGCGATGTAGTTC
PpATR_3UTR_3_rev	22	CTGACAGAGGTAGTGAAGGTGC
PpATR_5UTR_2_fwd	23	ATGTATCGCTGTCTGATAGTTCG
PpATR_5UTR_7_fwd	21	GAAGCTCTGGATCTCATGTTG
PpATR_5UTR_7_rev	21	CAACATGAGATCCAGAGCTTC
PpATR_Mid_1_fwd	21	GCTATTAGCCAGAAGTTGAGG
PpATR_Mid_5_rev	21	GAGTCCTACTTCCTGCCACTG
Sequencing of ATM and ATR cDNA		
ATM_ex12_rev	21	AGTAGACGAAATGCCTCATCC
ATM_ex15	20	TGTTCCACTGCGTCCTAACG
ATM_ex17	21	GGTCAAGCAGTGACGTTCTTC
ATM_ex17_rev	21	GTAAGGTAACGAGGTTGCCAG
ATM_ex18	22	CAAGAAGAGATTCCAGGCAGTC
ATM_ex18_rev	22	GCTCCTCAGTCAATGTTACAGG
ATM_ex20_rev	24	TAACTAGCTGCATATCTTTGAAGG
ATM_ex21	20	CTTTGCCTCAGTTGGCTTCT
ATM_ex23_rev	21	CACTTTGTCACTACACGCAGC
ATM_ex26	23	GTCATAGCACGTCTGTCAATACC
ATM_ex28_rev	22	CTCCAGCCTTATCCTGTTCATC
ATM_ex3_rev	19	TTTGTCTTCCGCTTTGTGG
ATM_ex31	21	TCCACCTTCACTACGAGATCG
ATM_ex32_rev	21	CTGGTCATCAAGGGTATTTGC
ATM_ex35	21	GCCTTCCACCTCAACTTCAAC
ATM_ex40	20	CTTGCTGCACGAAATGAATC
ATM_ex40_rev	21	AAGGTCTCCAAGAACATGAGG
ATM_ex45_rev	19	TGAGGCTTCCGAACTTGTC
ATM_ex46	21	TGCCTGCTCATATAGAGCTGC
ATM_ex47_rev	21	GCTCTCCCACTTGGTTACAGG
ATM_ex48	19	CTGGTCGAAGGTGATGCTG

ATM_ex5	21	GAGATTATACACACCCGCTCG
ATM_ex50_fwd	20	TGGTCCGTCTGATATTCAGG
ATM_ex51_fwd	21	TCTGCGTTTGAGGATTTATGG
ATM_ex52	22	TAGCAGAAACACGATCAGACAG
ATM_ex52_2_rev	21	GCTGTCTGATCGTGTTTCTGC
ATM_ex52_rev	21	CCATTTCTCGTTGATCCTGTG
ATM_ex56_rev	22	AGCTGCGATAACTTTCTAGTGC
ATM_ex57	18	GGACGCAATGCTGGAAAC
ATM_ex6_rev	22	TGGTCGAAAACTACAATGAAGC
ATM_ex61_rev	19	GGTTTCAAGCTCAGCAAGC
ATM_ex64	22	GTTATGAATGGAATCAACGCTC
ATM_ex66_rev	19	CCCACTCTAGCACACCAGC
ATM_ex7	22	TTGTATTGGAGTGCCAGTAAGC
ATM_ex70	20	CGGTTATGTCGTTGGACTTG
ATM_ex71_rev	19	TGGCTTCACAGCATCTTCG
ATM_ex9	20	GCTTTGGAACTCCTGTTTGG
ATR_ex10	22	AGCACAGAAGTTGACCAATCAC
ATR_ex10_rev	20	CGTTCGGATACTTCTTCCAG
ATR_ex11	20	AGAGACTGTTGCGGAGGTTG
ATR_ex11_rev	21	CACTTGCTGTTGAGAATCCAC
ATR_ex12	21	GATGGAGAAGAACTTTCTGCG
ATR_ex13_rev	19	CTTGGATCATGGGCACAAC
ATR_ex14	22	GTCAGATTGTGGTTTCGTTGAC
ATR_ex14_rev	21	CCTTCTGTGGCTTGTTTGAGC
ATR_ex15	21	CGATGGATGTATCTCTGGATG
ATR_ex15_rev	23	CGAAACTTAGATGTCAAACATGG
ATR_ex16	18	GCATCGTTCCGTTGTCAG
ATR_ex16_rev	20	TAGGGATAGCAGCAAGCAGC
ATR_ex17	21	CAGGAGCCGATGAAAATAGTC
ATR_ex17_rev	22	ATCGGCTCCTGTAACATATTCC
ATR_ex18_rev	19	TCATTGAACACCAGCCTGC
ATR_ex19	20	CAATGCTCACATGGAGATGG
ATR_ex2	20	TCTCTTTGCCGAACCTGAAC
ATR_ex20_rev	20	GCTTCAATCCACGAACTTGC
ATR_ex21	19	ATGCTCCAGCAGATGAACC

ATR_ex22_rev	21	CTGGAAGGTCTTTGAGACAGC
ATR_ex23	18	CGAGGAATGATGTGCAGG
ATR_ex23_rev	21	TTCACATTTGACAATCCATCG
ATR_ex24	23	CACTGAAGATTGTGGAATGATTG
ATR_ex24_rev	19	TGTTGAGAAACCAGCGGTG
ATR_ex4_rev	20	CACGAAGGAAGACCACACAG
ATR_ex5	21	TGGTACGTGCAGCAACAGTAG
ATR_ex6_rev	21	TCCAGACTGAGAGAATGCTCG
ATR_ex7	19	CTGCCACCAGCACATCATC
ATR_ex7_rev	22	GTTCTTCAGGATCAACTGCTTC
ATR_ex9_rev	20	TTCAGCGTTTCATCTTCACC

2.2 Methods

2.2.1 Nucleic acid techniques

2.2.1.1 Isolation of plasmid DNA from bacteria

To isolate plasmid DNA from bacterial cultures, Qiagen Mini-, Midi- or Maxi-Kits were employed according to the manufacturers instructions.

2.2.1.2 Isolation of *P. patens* DNA for genotyping

For the purpose of genotyping putative transgenic lines via PCR, a crude DNA extraction protocol was utilized (Berendzen et al. 2005). In short, a small piece of plant material, about 1-2 mm^2 in size, was transferred into a 2 ml Eppendorf tube holding 300 µl of lysis buffer. After adding two metal beads the samples were homogenized for 30 seconds with a Qiagen “Tissuelyser I” set to max frequency. Subsequently the lysate was incubated in a boiling water bath for 10 minutes after which the samples were centrifuged at 13.000 g for 10 min. Finally, 50 µl of the supernatant were transferred into a new tube and stored at -20°C.

Lysis buffer:

50	mM	Tris-HCl, pH 7.5
300	mM	Sucrose
300	mM	NaCl

2.2.1.3 Isolation of *P. patens* DNA for southern blot analysis

A modification of a standard CTAB extraction method (Scott and Bendich 1988) was used in order to obtain a large amount of high quality *P. patens* DNA for southern blot analysis. About 1 g of 7 day old protonema material was dried of excess water and ground up while it was kept frozen with liquid nitrogen. Powder was then immersed in 8 ml of CTAB B and incubated for 10 min at 65°C. Cell debris was removed via centrifugation at 5.500 g for 10 min and supernatant transferred to a new tube. One vol of $ChCl_3$/IAA (24:1) was added and samples were carefully mixed by inverting them repeatedly. After the following centrifugation at 5.500 rpm for 10 min, the aqueous supernatant was transferred to a new tube. To this, 2 vol of CTAB C were added and incubated for 30 min at RT to precipitate nucleic acids. After centrifugation of samples at 5.500 g for 30 min, supernatant was discarded and the pellet resuspended in 10 ml of 1M NaCl overnight. The next day, one vol of Isopropanol was added to the samples, incubating them at RT for 30 min for precipitation. Following centrifugation at 5.500 g for 30 min, the supernatant was removed and the pellet washed twice in 80% EtOH, centrifuging samples at 5.500 g for 10 min each time. Finally, the pellet was air dried and resuspended in 40µl of TE RNase (50 ng/ml RNase).

CTAB B:

2	%	CTAB
100	mM	Tris-HCl, pH 8
20	mM	EDTA
1.4	M	NaCl

CTAB C:

1	%	CTAB
50	mM	Tris-HCl, pH 8
10	mM	EDTA

2.2.1.4 Isolation of *P. patens* RNA

Approximately 100 mg of plant material were immersed in 6 ml of RNA lysis buffer supplemented with 18 µl DEPC. Using a homogenizer the sample was then ground for 15 seconds using a Miccra homogenizer D8 with the P8 blender attachment at 21000 rpm. Subsequently 6 ml of Roti©-Aqua-Phenol were added and the samples were homogenized for an additional 60 sat an increased speed of 35000 rpm. Afterwards, the samples were vortexed at max speed for about 30 s before they were centrifuged at 5.500 g for 15 min. After centrifugation the upper aqueous phase was transferred to a new tube containing 1 ml of $CHCl_3$. After thorough vortexing samples were centrifuged once again at 5.500 g for 15 min. Following centrifugation the aqueous phase was transferred once again to a new tube, this time already containing 1 ml of $CHCL_3$. After thorough shaking the samples were centrifuged as before. Repeating the latter $CHCl_3$ step once more, the clear supernatant was finally transferred to a new tube and 1/3 volume of DEPC treated 8M LiCl was added. For precipitation of RNA, samples were stored overnight at 4°C. On the following day the samples were centrifuged at

5.500 g and 4°C for one hour. After discarding the supernatant the pellet was dissolved in 85 µl of dH_2O, keeping the samples at RT for 30 min while vortexing them regularly. Subsequently, 5 µl of DNase (1U/µl Promega) and 10 µl of 10x DNase buffer were added to each sample. Samples were then incubated for 1 h at 37°C to remove the remaining DNA. Afterwards, 100 µl of phenol/$CHCl_3$ were added to facilitate removal of DNase. Following centrifugation of samples at 14.000 g, the aqueous phase was transferred to a new tube and 0.1 volumes of 3M Na-acetate as well as 2.5 volumes of 96% EtOH were added. Subsequently, samples were stored at -20°C overnight for RNA precipitation. The next day, samples were centrifuged at 14.000 g and 4°C for 30 min where after the supernatant was discarded. Washing the pellet twice with 80% EtOH, and intermittent centrifugation at 14.000 g for 10 min the pellets were finally dried and dissolved in 50µl of dH_2O. RNA was stored at -20°C.

Lysis buffer:

34.8	ml	dH_2O
8	ml	10% SDS
2	ml	1 M Tris pH 8.0

2.2.1.5 Quantification of DNA/RNA

To determine the concentration of dissolved DNA and RNA molecules in a solution, a peqlab microvolume spectrophotometer "Nanodrop™ ND-1000" was utilized. Due to the fact that photometric measurements of nucleic acid extracts may be disturbed by contaminating proteins, salts or organic compounds, concentrations were further estimated via gel electrophoresis. For this purpose, DNA or RNA samples were loaded on a gel together with several dilutions of known amounts of lambda phage DNA. By comparing the intensity of bands between samples and reference DNA after ethidium bromide staining, it was possible to determine the approximate DNA/RNA concentration in the samples.

2.2.1.6 Polymerase chain reaction (PCR)

The polymerase chain reaction (Kleppe et al. 1971, Mullis et al. 1986) allows for the amplification of a specific stretch of DNA sequence, utilizing a thermostable DNA polymerase and a pair of specific Oligonucleotides. This technique was applied for genotyping of *Physcomitrella patens* transformants, cloning of DNA fragments and synthesis of *ATM* and *ATR* cDNA. Composition of a standard PCR reaction was as follows:

5	µl	10x PCR Buffer (including $MgCl_2$)
1	µl	25 pM Primer forward
1	µl	25 pM Primer reverse
1	µl	10 mM dNTPs
1	µl	Taq Polymerase
1	µl	template DNA
ad 50	µl	dH2O

Reaction mixtures were incubated in a thermocycler which provided the required time and temperature profile for each PCR reaction. While the basic setup of a PCR program is similar, the temperature for primer annealing has to be adjusted for each individual primer pair and extension time is dictated by amplicon length (1kb/minute). A basic PCR program for the amplification of a 500 bp amplicon:

94	°C	3	min	denaturation	
94	°C	30	s	denaturation	} 36 cycles
56	°C	30	s	primer annealing	
72	°C	30	s	extension	
4	°C	∞		storage	

2.2.1.7 Gel electrophoresis

To analyze restriction digests and PCR amplificates or evaluate the quality of DNA and RNA preparations, the respective nucleic acid species were electrophoretically separated in an agarose gel. For this purpose, the samples were mixed with a gel loading buffer, loaded onto gels of 0.8 – 2 % agarose content and separated at 100 V. To be able to judge approximate size of bands, *Pst*I digested Lambda phage DNA was loaded alongside the samples on each gel. After the run gels were stained

for 15 min in a 0.025 % ethidium bromide solution and documented using a Polaroid CU-5 camera and a Fluo-Link FL 20-K UV transilluminator (Bachofer).

Gel loading buffer:

2	mg/ml	Xylencyanol
1	mg/ml	Bromphenol Blue
2	mg/ml	Orange G
50	% v/v	Glycerol
5	µl/ml	0.5M EDTA
2.5	µl/ml	10 % SDS

Running Buffer:

0.8	M	Tris
0.4	M	NaOAc
0.04	M	EDTA

adjusted to pH 8.3 with acetic acid

2.2.1.8 Recovery of DNA molecules after gel electrophoresis

To isolate distinct DNA species from an agarose gel, for downstram applications like cloning, DNA bands were cut out of the gel after ethidium bromide staining. To prevent damage to the DNA molecules during this procedure, a low intensity UV source was utilized for detection. Having obtained the agarose enclosed DNA, it was then purified via the "Nucleospin extract II" –kit from Macherey Nagel (Düren), according to the manufacturers instructions.

2.2.1.9 Southern blotting

To detect specific DNA fragments of restriction enzyme digested genomic DNA, the latter was transferred onto a nitrocellulose membrane and subsequently hybridized with a radioactively labeled DNA probe. For this purpose the digested DNA was first separated on a 0.8 % agarose gel at 250 mA for about 2.5 hours. Meanwhile, in preparation for the transfer, the blotting chamber was rinsed with 70 % EtOH, dH_2O and finally 0.4M NaOH. After the gel run the DNA was depurinated by incubating the gel in 0.25M HCl for 5 min. Subsequently the blotting sandwich was assembled by first off placing three layers of whatman paper atop the blotting chambers membrane. On top of these the gel was placed followed by the zetaprobe nitrocellulose membrane followed by three more layers of whatman paper. Care was taken to avoid any air bubbles in between of any layers as these might impede DNA transfer by disrupting the capillary flow of the blotting media. In addition, parafilm was placed around the gel to prevent the capillary flow bypassing the gel. Placing a stack of Kleenex atop the blotting sandwich, it was left overnight. The next morning the filter was baked at 80°C for 30 min and afterwards placed in hybridization buffer for overnight incubation at 65°C. The following day, a Sephadex 100-TE column was prepared by filling it into a glass capillary to the neck and rinsing it 2 – 3x with TE-buffer, sealing it with parafilm afterwards. In the following step the radioactive probe was prepared by first incubating 50 ng of the respective DNA fragment in a total of

35.5 µl dH_2O in a 100°C water bath for 5 min. Afterwards the denatured DNA was put on ice immediately for 5 min after which 5µl of 10x labeling buffer, 1.5 µl BSA as well as 3 µl klenow (2U/µl, Fermentas) were added. Transferring to the isotope lab, 5 µl of ^{32}P dCTP were added to the mix and it was incubated for 20 min at 37°C before being put back on ice. The hot labeling mix was then loaded onto the sephadex column. The column was subsequently rinsed once with 50 and thrice with 100 µl of TE-buffer, discarding the flowthrough. Afterwards 200 µl of TE-buffer were applied a total of three times and the flowthrough, now containing the radioactively labeled oligonucleotide probe, was collected in an eppendorf tube. After measuring the probes radiation level, they were incubated at 95°C for 10 min and subsequently placed on ice. Taking the nitrocellulose membrane out of the prehybridization buffer it was then sealed in a plastic bag and 8 ml of fresh hybridization buffer were added, via a small opening cut into one of the corners of the bag. Adding the 600 µl of radioactive probe the bag was sealed once more and incubated in a shaking waterbath at 65°C overnight. The next day, the hybridization buffer was discarded and the membrane washed 2 x in washing buffer 1 for 10 minutes each and 2 x in washing buffer 2 for 20 min each, each step carried out at 65°C. After washing, the wet membrane was tightly sealed in a plastic bag. It was subsequently placed in an x-ray cassette and a BioMax MS film (Kodak) was placed atop. The x-ray cassette was then stored for at least 24 hours at -80°C before the exposed film was developed.

2.2.1.10 Sequencing of DNA molecules

Sanger sequencing of DNA molecules was performed in-house by the Max Planck genome centre. The equipment employed for this purpose was either a 3730XL or 3130XL Genetic Analyzer from Applied Biosystems (Darmstadt).

2.2.2 Cultivation of *P. patens*

Physcomitrella patens protonema cultures were routinely grown in petri dishes on solid PPNH4 containing ammonium tartrate. For easy collection of plant tissue the medium was overlaid with a cellophane disc, preventing growth of protonema into the agar while still allowing for nutrient uptake. Growth conditions were set to 26°C and 4500 – 5000 Lux of continuous light in a Percival CU-365/D growth cabinet from CLF Laborgeräte (Emersacker). After learning that some of the installed bulbs emitted a low amount of UV light, which had a detrimental effect on some of our mutant lines, culture of plant material was restricted to UV free compartments. To maintain a stable supply of fresh plant material, plant tissue was subcultured every seven days. For this purpose, seven day old protonema tissue collected from two plates (diameter ≈90 mm) was immersed in 10 ml of dH_2O and homogenized with a Miccra homogenizer D8 equipped with a P8 homogenizer tool (ART-Moderne Laborgeräte, Hügelheim). For subculture purposes one ml of the ground up protonema filaments was

then dispersed on fresh PPNH4 plates, which were incubated for seven days in the growth cabinet before the procedure was repeated.

To induce sexual propagation and subsequent generation of spores, a different growth regime was required. Instead of growing plantlets on PPNH4, they were instead cultivated on PP- which lacks the ammonium tartrate present in PPNH4 medium. After cultivating plant material on PP- but otherwise standard growth conditions (permanent light, 26 °C) for 1 to 1.5 months, growth conditions were changed to 15 °C and 16 hours of light per day. These conditions induced the formation of male and female gametangia. Maintaining a constant level of moisture the first mature sporophytes were usually observed after about 2 more months, subsequent to the change in temperature and light conditions.

2.2.3 Generation of ATM and ATR mutant lines

2.2.3.1 Assembly of gene targeting vectors

The ATM and ATR targeting vectors consisted of a vector backbone into which a targeting construct was cloned. The targeting construct was made up from a 5' and 3' region of homology specific for the targeted gene with a selection marker cassette in between. The ATM specific gene targeting vector pKOATM was assembled from 4 fragments. The 5' region of homology was cut out of the plasmid p1kbupATM by *Xma*I and *Nco*I digest. The sulfadiazine resistance gene under 35S promoter control was cut from the plasmid pUC18/sul-2 via *Nco*I and *Sac*I. The 3' homologous region was released by digesting the plasmid p1kbdownATM with *Sac*I and *Spe*I. These three fragments were then cloned into a pBluescript plasmid linearised with the enzymes *Xma*I and *Spe*I. The ATR targeting vector pKOATR was constructed in an analogous manner. The 5' region of homology was released from p1kbupATR via *Cla*I and *EcoR*I digest. The kanamycin resistance gene NPTII under 35S promoter control was cut out from pUC/NPTII via *EcoR*I and *Hind*III. The 3' region of homology was released from the plasmid p1kbdown by *Hind*III and *Sal*I digest. These fragments were then cloned into the pbluescript plasmid which was linearised with *Cla*I and *Sal*I. Proper assembly of both targeting vectors was verified via restriction digest as well as Sanger sequencing.
To later on release the targeting constructs from the targeting vector for use in transformation pKOATM was digested with *Xma*I and *Spe*I and pKOATR was digested with *Cla*I and *Sal*I.

2.2.3.2 Isolation and transformation of *P. patens* protoplasts

For protoplast isolation 8-10 plates of densely plated, 7 day old protonema filaments grown on PPNH4 medium overlaid with cellophane were used as starting material. First, the protonema tissue

was scraped off the cellophane and immersed in 10 ml of 0.5 M mannitol. After adding 15 ml of 2 % (w/v) Driselase solution, prepared in 0.5 M mannitol, protonemata were incubated for 30 min at 21°C for cell wall digestion. Subsequently, the protoplast suspension was filtered in sequence through a 100 µm and 50 µm pore size sieve to remove cell wall debris. Afterwards, protoplasts were washed with 0.5 M mannitol to remove the driselase. For this purpose protoplasts were centrifuged at 100 g for 5 minutes, with deceleration and acceleration set to the slowest setting. After centrifugation the supernatant was removed and protoplasts were taken up in 50 ml of fresh 0.5 M mannitol. This procedure was repeated until protoplasts are immersed for the third time in 0.5 M mannitol. The total protoplast yield was then determined by counting them utilizing a hemocytometer and microscope. Afterwards, protoplasts were centrifuged once more and resuspended in an appropriate volume of MMM medium to achieve a protoplast concentration of $1.5*10^6$ per ml. Subsequently 180 µl (90µg) of targeting construct DNA were pipetted into an empty 50 ml Falcon tube and 1.8 ml of the protoplast suspension was then added. In the following, 1.8 ml of PEG was carefully mixed into the suspension by gentle tilting and turning of the tube. Having obtained a homogeneous suspension, protoplasts were subjected to heat shock treatment at 45°C for three minutes to facilitate PEG mediated DNA uptake. Following the heat shock, protoplasts were kept at room temperature for 10 min after which PPNH4-M was slowly added to the protoplast suspension up to a total volume of 50 ml. Tubes were then wrapped in aluminium foil and stored at 26°C overnight. The following day a 1 % solution of low melting agarose was prepared in PPNH4-M and kept at 42°C to prevent premature solidification. Protoplasts were unwrapped and centrifuged at 100 g for 5 min with deceleration and acceleration set to 0. After centrifugation supernatant was removed up until only 10 ml remained. An equal volume of the prepared 1 % low melting agarose was added and carefully mixed with the protoplast suspension which immediately afterwards was dispensed in 2 ml aliquots onto PPNH4-M agar plates overlaid with a cellophane disc. Plates were then transferred into a growth chamber with continous light and 26°C, where they were kept for six days for cell wall regeneration and proliferation. After 6 days the small protonema colonies which had formed from the initial protoplasts were subjected to the first antibiotic selection cycle. For this purpose, the small plantlets were transferred together with the cellophane disc onto PPNH4 plates supplemented with the selective agent corresponding to the resistance gene located on the targeting construct used in the transformation. After 14 days of growth resistant plantlets were picked and individually transferred onto new plates without the selection agent. There they were grown for a further 14 days before being subjected to selection once more. In total the switch between growth on selective and nonselective media was performed for a total of three cycles, in order to remove unstable transformants.

2.2.3.3 PCR analysis of putative mutant lines

To identify stable transformants carrying the intended genomic modification at the ATM/ATR locus, a PCR based screen was devised. PCR reactions were set up to amplify the 5' and 3' integration sites as well as the complete target locus (**Figure 2.2-1**). For ATM, the primers PpATM_genome_fwd2 and PpATM/ATR_p35S_rev were designed to check the 5' integration site while PpATM_tpSul_fwd and PpATM_genome_rev were used to check the 3' site of transgene integration. In addition to that, PpATM_genome_fwd2 and PpATM_genome_rev were used for an over the locus PCR, amplifying a stretch containing the complete target locus. For ATR, the primers PpATR_genome_fwd and PpATM/ATR_p35S_rev were used for checking the 5' integration site while PpATR_NPTII_fwd and PpATR_genome_rev were specific for the 3' site of transgene integration. Furthermore, PpATR_genome_fwd and PpATR_genome_rev were used for the "over locus PCR".

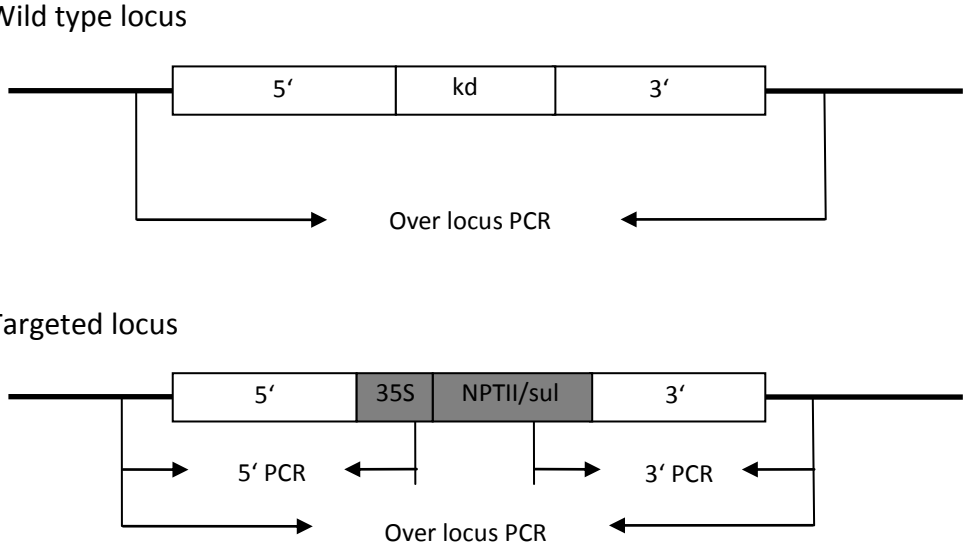

Figure 2.2-1: PCR strategy to distinguish between wild type (a) and targeted loci (b) in stably transformed lines. Vertical lines connected to arrows indicate the binding site of primers. **5'**, 5' region of homology; **3'**, 3' region of homology; **35S**, promoter region of cauliflower mosaic virus; **NPTII**, neomycin phosphotransferase gene; **sul**, mutated dihydropteroate synthase gene providing resistance to sulfadiazine..

2.2.3.4 Generation of probes for southern blot analysis of putative ATM and ATR mutants

For the southern analysis of transformant lines, a set of 3 distinct DNA probes was constructed for the characterization of putative *ATM* and *ATR* mutant lines each. The 5' probe was designed to be specific for the upstream flanking region of predicted targeting construct integration. For *ATM*, the 5' probe was amplified using the primers sATM_5flank_fwd and sATM_5flank_rev while for *ATR* sATR_5flank_fwd and sATR_5flank_rev were employed. The 3' probe was designed to be specific for

the downstream flanking region of the predicted site of targeting construct integration. For *ATM* the primer pair sATM_3flank_fwd and sATM_3flank_rev were used while for *ATR* sATR_3flank_fwd and sATR_3flank_rev were paired for amplification. The "del" probe was designed to be specific for the region of the *ATM*/*ATR* kinase domain which, upon successful targeting, would be replaced by the selection marker cassette. For *ATM* the DNA probe was generated with primers sATM_del_fwd2 and sATM_del_rev while for *ATR* sATR_del_fwd and sATR_del_rev were used. The Amplicons for DNA probe generation were between 500 - 1000 bp in size and were amplified using genomic wild type DNA as a template. Following gel electrophoretic separation, the PCR products were extracted via "Nucleospin Extract II"-kit from Macherey Nagel and ligated into the pGEM-T easy vector. After transformation of ligated plasmids into *E. coli* DH5α and blue/white selection, plasmids were extracted from a number of clones and their inserts sequenced for identification. Once verified, these DNA sequences were released from their respective vectors via restriction digest and separated from the vector backbone by gel electrophoresis and subsequent purification. Purified DNA was then stored at -20°C to be used for radiolabeling in the hybridization step of the southern analysis.

2.2.4 Determination of ATM/ATR transcript structure via Reverse transcription PCR

To determine the transcript structure of *ATM* and *ATR* we first performed a RACE. 5' and 3' RACE of *P. patens ATM* and *ATR* genes was performed with the Roche "5'/3' RACE Kit, 2nd generation" according to the manufacturers specifications. Reverse transcription was carried out with 2 µg of total RNA extracted from seven day old protonema subcultures. In the 5' RACE we employed the gene specific primers ATM_Ex3_1_rev and ATR_Ex1_2_rev for *ATM* and *ATR* respectively. The 3' RACE was conducted with gene specific primers ATM_EX73_1_fwd for ATM and ATR_EX22_1_fwd for *ATR*. PCR program for 3' RACE was set to 2 min initial denaturation at 94°C followed by 36 cycles of 30s denaturation at 94°C, 30s annealing at 64°C and 30s elongation at 72°C. For the 5' RACE PCR the PCR program consisted of 2 min initial denaturation followed by 36 cycles of 30s denaturation at 94°C, 30s annealing at 55°C and 1 min elongation at 72°C. PCR amplified cDNA products were separated on a 1% agarose gel, extracted via Macherey Nagel "Nucleospin© Extract II" and ligated into the "pGEM©-t Easy Vector System" (promega) according to the manufacturer's instructions. After blue/white selection a number of colonies were selected for mini preps. Plasmid inserts were finally sequenced and resulting sequences aligned to the genomic sequence of the respective ATM or ATR locus using the Vector NTI 11 software suite.

After obtaining the sequence information for the terminal regions of the *ATM* and *ATR* transcripts we proceeded to assess the remainder. Using 2 µg of total RNA, first strand synthesis of *ATM* and *ATR* were performed with gene specific primers PpATM_3UTR_1_rev and PPATR_3UTR_1_rev

respectively. Due to the large size of the full transcripts of approximately 10000 bp we opted to split both genes into smaller pieces after initial failed amplifications. The *ATM* cDNA was split into two parts, a 5' part defined by primers PpATM_5UTR_4_fw and PpATM_Mid_1_rev and a 3' part defined by PpATM_Mid_2_fw and PpATM_3UTR_3_rev. The *ATR* cDNA was divided into three segments for PCR amplification. The first segment was defined by primers PpATR_5UTR_2_fw and PpATR_5UTR_7_rev, the second by PpATR_5UTR_7_fw and PpATR_Mid_5_rev and the last one by PpATR_Mid_1_fw and PpATR_3UTR_3_rev. PCR amplified cDNA segments were ligated into the "pGEM©-t Easy Vector System" and sequenced in forward as well as reverse orientation utilizing unique primers as listed in **2.1.7**.

2.2.5 Analysis of growth phenotype of mutant lines

2.2.5.1 Starting material for growth experiments

Due to the sterility of all our *ATR* mutant lines, spores were no option as a starting point in phenotyping experiments. Instead, freshly homogenized protonema subcultures were utilized. For this purpose, 7 day old, subcultures were homogenized as per standard procedure for subculturing. Subsequently filament fragments were filtered through a 100 µm sieve. This produced a suspension of protonema fragments of mainly 1-5 cells, providing a more standardized pool of starting material for phenotyping experiments. Prior to plating, material was diluted 1:40 in dH_2O and 2 ml were dispensed per plate for the growth experiments.

2.2.5.2 Observation of early developmental stages

Protonema fragments were prepared as described previously and plated on PPNH4 agar plates. To prevent inhibition of growth petri dishes were furthermore not sealed with parafilm. Plates were then incubated under standard conditions at 26°C and permanent light. Development of individual filaments was observed on a regular basis using a stereo microscope for a total duration of 3 weeks.

2.2.5.3 Observation of late developmental stages and special growth conditions

Prior to the start of the actual growth experiment, small filament fragments, prepared as described above, were pregrown on PPNH4 agar overlaid with cellophane. After 6-7 days of growth, similar sized protonema colonies of 1-2 mm^2 were selected and transferred to large 128 mm x 128 mm plates which contained the specific media type for the actual experiment. Growth and development of individual plantlets was then observed for up to 2 months.

2.2.5.4 Observation of plantlets during the reproductive stage

To evaluate the reproductive capabilities of our mutant lines, small pieces of seven day old subcultured protonema material were transferred to PP- media. These plantlets were then grown for about 1-2 months until they had produced gametophores with 10 or more leaflets. To induce the formation of gametangia on the gametophores, temperature was lowered to 15°C and the previous permanent light regime switched to short day conditions (8 hours light, 16 hours dark). Gametophores were extracted on a weekly basis and dissected to observe the male and female gametangia during their various developmental stages up to the mature sporophyte. Albeit mature sporophytes tend to appear after about 2 months following change of growth conditions a number of cultures were kept for as long as 2 years to compensate for any possible delays in spore formation in mutant lines.

2.2.5.5 Assessment of plantlet growth speed

The rate at which protonema filaments spread on the growth media might be indicative of defects in cellular proliferation. To indirectly measure growth speed of individual filaments, the surface area of plantlets, stating from 1-5 cells, was determined after 10 days of growth in standard conditions. To measure the surface area of a large number of individual plantlets digital images were taken from the 10 day old plates. The surface area of individual plantlets was then evaluated by use of the 2d image analysis software “imageJ”. In a first step, digital images of plantlets obtained in the RGB format were split up with the “split channels” function to obtain an 8 bit grayscale image of the blue channel. This image was then converted into a binary format by applying a threshold. This function transforms each pixel of a picture into either black or white, depending on its prior intensity in the grayscale picture and the selected threshold value. Careful adjustment of the threshold value thus yielded a black & white picture in which the area of each plantlet was represented by a black shape. Applying the “analyze particles” function then measured the area of each of these shapes, yielding the approximate surface area of each individual plantlet.

2.2.5.6 Assessment of viability in young filaments during regeneration

To measure the viability of young filaments, their survival rate after the first six days of regeneration was assessed. This experimental timeframe was chosen as filaments seemed to die only during this early stage of regeneration. Once cells had begun to successfully proliferate once or twice the respective filaments did usually grow up to reach maturity, barring any additional biotic or abiotic stress factors. Starting filaments for the assay were prepared according to **2.2.5.1** and plated on PPNH4 agar. After a lag phase of 12 hours the initial number of living filaments was assessed via

stereomicroscopic observation. Filaments were scored as alive if at least one of its cells displayed a green or brown color as even the latter were found able to proliferate on some occasions. The initial lag before counting the base number of living cells was implemented to exclude those filaments from the analysis which would die due to physical damage introduced during mechanical homogenization of the starting material. Growing the filaments for a total of six days the number of living filaments was determined once more in order to determine the survival ratio of filaments.

2.2.5.7 Measuring proliferation in *P. patens* cells

The "Click-iT® EdU cell proliferation assay" from Invitrogen (Darmstadt) allows for the detection of cells actively replicating their DNA. It is based on the specifically modified thymine analogon EDU. In a first step this base analogon, if made available during cellular proliferation, is readily incorporated into DNA during replication. In a second step the EDU is then covalently bound by a modified alexa488 fluorophore, allowing for subsequent detection of cells having replicated their DNA while in the presence of EDU. Thus, by growing plantlets in the presence of EDU for a defined period of time, one could easily observe the pattern of proliferation during any stage of *P. patens* development. The plant material for proliferative analysis was pregrown under normal growth conditions. Once the desired developmental stage was reached, plant material was transferred to liquid PPNH4 media supplemented with 20 µM EDU. Plant material was then grown for two, six or twelve more hours, in constant light, on a shaker. Subsequently, plantlets were fixated in 3.7 % PFA and processed further according to the kit's instructions. After mounting of plant material on glass slides the fluorescent nuclei were detected using a fluorescence microscope.

2.2.5.8 Analysis of induced caulonemal growth

To analyse the growth of especially caulonema type filaments, a specific growth regime was applied. For this purpose, a clump of seven day old protonema tissue from a standard subculture was transferred onto PPNH4+ medium which compared to the standard PPNH4 medium also contains glucose. Petri dishes were then wrapped in aluminium foil and grown for 3 more weeks in a vertical position. These growth conditions promote the development of mainly caulonema type filaments. In addition these also grow unidirectionally due to their innate negative gravitropism in the absence of light, simplifying length comparisons of filaments.

2.2.5.9 Protoplast DNA damage assay

To assess the relevance of *P. patens* ATM and ATR in the repair of different types of DNA damage, an assay based on the survival rate of individual protoplasts was devised. Following isolation of protoplasts they were stored in the dark at 26°C overnight. On the next day, the total number of

visually healthy protoplasts was determined by counting them using a hematocytometer. Protoplasts were then centrifuged at 100 g for 5 min and resuspended in PPNH4-M to attain a final concentration of 200000 protoplasts per ml. For the treatment with the genotoxic agents bleomycin and mitomycin, slightly deviating experimental setups were utilized.

For bleomycin, 31 ml of PPNH4-M supplemented with the required concentrations of the genotoxic agent were provided in a 50 ml Polypropylene Tube to which 1 ml of protoplasts was added. Protoplasts were then incubated for 1.5 h at room temperature while carefully inverting the suspension every 10 minutes. After the allotted time protoplasts were directly plated, in 2 ml aliquots, on PPNH4-M agar plates without bleomycin.

In case of mitomycin, the required concentrations of the genotoxic agent were provided in 1 ml of PPNH4-M in a 2 ml reaction tube to which 1 ml of the protoplast suspension was added. Due to the fact that mitomycin induces DSBs indirectly, by generating DNA interstrand crosslinks which are transformed into DSBs during replication, protoplasts were incubated for a total of 16 hours before plating. For this purpose, tubes were placed in a slowly revolving rotator, located in a dark room to prevent photodegradation of mitomycin. Afterwards, the 2 ml of treated protoplasts were added to 30 ml of PPNH4-M and aliquots of 2 ml each were plated on PPNH4-M plates without mitomycin.

After protoplasts had been plated, they were regenerated for 10 days under standard growth conditions. Subsequently the number of regenerated plantlets was counted and the percentage of surviving protoplasts in the treated samples was calculated relative to the controls.

2.2.5.10 Small plantlet DNA damage assay

To complement the protoplast based DNA damage assay in assessing the sensitivity of transgenic lines regarding different types of genotoxic stress, an additional experimental approach was chosen. In this setup, small plantlets of wild type and knockout lines were pre-grown on PPNH4 overlaid with cellophane until they reached a size of about 1-2 mm^2. Plantlets were then transferred on PPNH4 agar supplemented with different concentrations of bleomycin, mitomycin, MMS and MNU. Subsequently, the growth of individual plantlets was observed for up to two weeks and plantlets grown on media containing the respective genotoxic agent were compared to mock treated plantlets in order to score sensitivity by plantlet size. The lines in which the size difference between mock treated and treated plantlets were highest were also assigned the highest sensitivity rating.

2.2.6 Bioinformatics

2.2.6.1 Assembly of list of known recombination genes

To evaluate the available set of recombination genes in *P. patens*, a list of recombination genes as they are known from human, yeast and arabidopsis was compiled first. These genes were obtained by consulting topical publications as well as online resources like http://www.uea.ac.uk/~b270/repair.htm or http://sciencepark.mdanderson.org/labs/wood/DNA_Repair_Genes.html. If a gene in the compiled list was available in only one or two of the chosen organisms, after initial data mining, the missing homologues were sought after utilizing various resources. First off the swissprot database at http://www.uniprot.org/ was queried for curated entries of the missing homologues. If that approach did not yield any positive result a BLASTp search was conducted utilizing the sequence of the known homologue versus the swissprot as well as NCBI refseq protein databases of the organism in which that homologue was missing. To verify the resulting hits, they were backBLASTed against the swissprot as well as NCBI refseq databases of the respective organism the initial query sequence was taken from. If the backBLAST returned the initial query as the best hit the respective homologue was added to the list.

2.2.6.2 BLAST search for recombination gene homologues in *P. patens*

Once the list of genes was complete, all the assembled queries of each organism were blasted versus the *Physcomitrella patens* protein model database version 1.6, utilizing the BLAST service at https://www.cosmoss.org/. All of the top hits, including the first hit where one could notice a marked e-value dropoff in relation to the previous hit were noted vor further verification. For that purpose these hits were backBLASTed against the swissprot non-redundant sequence database at http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi?PAGE=Proteins. If the best hit in the backBLAST matched with the initial query used in obtaining the putative *P. patens* the latter was deemed verified, independent of the e-value. Homologs with an e-value lower than e^{-20} however were labeled for later reference. Whenever the backBLAST result was not entirely clear, with different genes being closely tied for the top hit, a further analysis was performed for the respective gene. The protein sequence of the questionable *P. patens* homolog was then used to construct a phylogenetic tree together with the top backBLAST hits from yeast, human and Arabidopsis. This was done by first using ClustalX version 2.1 available at http://www.clustal.org/ to generate an alignment. The alignment was then imported into the MEGA software version 4.1 available at http://www.megasoftware.net/ to generate a phylogenetic tree using the Neighbour Joining algorithm of clustering. Bootstrapping was performed utilizing replications set to 5000 and random seed to 64238. In addition to the generation

of phylogenetic trees a domain search on each of the protein sequences, utilizing the interpro domain prediction software located online at http://www.ebi.ac.uk/interpro/, was performed. If both of the resulting datasets supported the notion of the Physcomitrella hit being a putative homologue, the latter was added to the list of verified homologs.

2.2.7 SuperSAGE based transcriptome analysis

2.2.7.1 Preparation of plant material and genotoxic stress treatment

Standard subcultures of 7 day old wild type and the three mutant lines Pp *atm-1* Pp *atr-1* and Pp *atmatr-1* were homogenized and plated on PPNH4 overlaid with cellophane. After 5 days of growth under standard conditions the plant material was collected. For the stress treatment approximately 5 gram fresh weight per line were either mock treated or treated with 0.3 U of bleomycin. For the duration of the treatment plant material was immersed in 300 ml of PPNH4 medium in a 500 ml DURAN bottle. Subsequently plant material was washed and transferred to 300 ml of fresh PPNH4 medium. After 3 hours of incubation the plant material was collected and RNA extraction was immediately started. Subsequently RNA quality was controlled via gel electrophoresis and stored at -20°C.

2.2.7.2 SUPERSage analysis of bleomycin and mock treated wild type and mutant lines

The SuperSAGE analysis, including the experimental as well as the bioinformatics part, were conducted at GenXPRO GMBH (Frankfurt am Main). The approach followed the fundamental methodology as it has been developed by Matsumura et al. (Matsumura et al. 2008). In short, 26 bp tags were isolated from RNA preparations and sequenced via next generation sequencing technology. The obtained sequence dataset was then cleared of PCR-artifacts using the TrueQuant software suite. In the following the verified tags were annotated via BLAST. To be able to compare transcript levels in between different samples, the number of tags for each transcript was normalized to number of tags per million (tpm). GenXpro software tools were used for the statistical analysis and comparison of tag libraries. To calculate the probability of a tag being differentially expressed was calculated as described by Audic and Claverie (Audic and Claverie 1997).

3 Results

3.1 The DNA repair/recombination genes of *P. patens*

P. patens unlike Arabidopsis but similar to yeast has been found innately efficient in gene targeting (Schaefer and Zryd 1997, Puchta 2002, Britt and May 2003), the precise modification of a select genomic via homologous recombination (HR). It has been proposed that high gene targeting efficiencies as observed in *P. patens* and yeast are related to HR being the dominant pathway for somatic DSB repair in these organisms (Markmann-Mulisch et al. 2007). Likewise it has been suggested that the lack of efficient GT in higher plants like Arabidopsis is related to non homologous end joining being the primary repair mechanism for somatic DSBs (Puchta 2002). A bias for either HR or NHEJ as observed in these organisms may be related to the presence or absence of genes known to be involved in these processes. To test this hypothesis, the complement of DNA recombination and repair genes as present in *P. patens* was assessed. This was done by initially assembling a list of DNA repair/recombination genes as known from studies in yeast, human and Arabidopsis by consulting scientific literature (Prakash et al. 1993, Wood et al. 2005) as well as online resources (http://www.uea.ac.uk/~b270/repair.htm). The protein sequences of these genes were then used in a BLAST search to find the respective *P. patens* orthologues. To increase the dependability of the obtained BLAST results a backBLAST was conducted. In case of an inconclusive backBLAST, the putative orthologues, initial queries and backBLAST hits were used in inferring phylogenetic trees. If the putative orthologues clustered with the initial query sequences instead of other backBLAST hits they were furthermore checked for the presence of hallmark domains via interpro software (http://www.ebi.ac.uk/interpro/). In case these two additional datasets supported the notion that the analyzed sequence might indeed be an orthologue of the initial query, it was added to the list. To increase accessibility of this large number of genes they were divided into various subcategories, indicating their main functionality. It should be noted however that this allocation is only superficial, as many of the listed genes would fit to more than one of the categories. ATM and ATR for example were filed under checkpoint while they also have a central function in homologous recombination.

For 120 (67 %) out of the total of 178 DNA repair genes in the list of human, A. thaliana and yeast DNA repair/recombination genes, at least one orthologue was found in *P. patens*(**Table 3.1-1**). Comparing the individual overlap in available DNA repair genes between *P. patens* and the other three organisms this was 72 % with yeast as well as with humans and about 92 % for *A. thaliana*. This demonstrates that the components of DNA repair are well conserved, even across phylogenetically distant organisms.

One of the initial motivations to analyze the set of available DNA repair genes in *P. patens* was to determine whether its preference for homologous recombination might be reflected in components of the non homologous end joining pathway being missing. Observing the results of the BLAST analysis, this does not seem to be the case. All of the central NHEJ components such as Ku70, Ku80, DNA ligase 4, XRCC4 and NHEJ1 (Hefferin and Tomkinson 2005), which are present in Arabidopsis, were also found in *P. patens*. Thus it is very likely that the preference for HR in *P. patens* is not a result of a lack in NHEJ genes.

Evaluating the BLAST results further, a number of interesting observations were made. First, a large number of DNA repair genes are at least duplicated in *P. patens*. For 33 out of the total of 120 orthologs, which were found in *P. patens*, additional paralogs were detected. Among those genes which were found at least in duplicate are central components of homologous recombination such as RAD54, RAD9, WRIP and PAXI1 in addition to genes involved in a wide range of other DNA maintenance processes.

Furthermore, although the availability of DNA repair/recombination genes in *P. patens* and *A. thaliana* is largely similar, there are a number of distinct differences. Out of the total of 120 DNA repair/recombination gene homologs found in *P. patens*, 6 are not present in *A. thaliana*. These are PKMYT1, WRN, Rad54B, DDX11, PRKDC and SIR2. Likewise, there are also 11 genes which while being present in Arabidopsis are missing in *P. patens*. This latter complement of genes consists of BRCA1, BRCA2 and BARD1 as well as SYCP1, SYCP2, HDAC2 and XRCC2, all of which are found in humans but not in yeast. The remaining two, RAD7 and MEK1 meanwhile have been indentified in yeast but not in humans. It is interesting to note, that yeast as well as *P. patens* do not contain BRCA1, BRCA2 and BARD1 whereas *A. thaliana* as well as mammals do. These three genes are known for their important role in homologous recombination mediated DSB repair (Welcsh et al. 2000, Yoshida and Miki 2004). These findings suggest that *P. patens* has a unique complement of recombination genes and also shares some interesting similarities with yeast in this regard.

Table 3.1-1: DNA repair/recombination genes and their availability in human, yeast, Arabidopsis and *P. patens*. "**+**" indicates that the gene is present in the respective organism, each additional "**+**" symbol denotes the presence of an additional paralog. "**-**" indicates that no homolog was found for the gene in the respective organism. A grey shaded background indicates that the e- value of the best homolog found with any of the used queries was above e^{-20}.

Gene	*H. sapiens*	*S. cerevisiae*	*A. thaliana*	*P. patens*	*P. patens* Accessions
checkpoint					
ATM	+	+	+	+	Pp1s135_65V6.1
ATR	+	+	+	+	Pp1s77_262V6.1
ATRIP	+	-	+	+	Pp1s60_281V6.1
ATRX	+	-	+	+	Pp1s34_370V6.1
BARD1	+	-	+	-	
BRCA1	+	-	+	-	
CHK1	+	+	-	-	
CHK2	+	+	-	-	
DDC1	-	+	-	-	
DUN1	-	+	-	-	
HUG1	+	+	-	-	
HUS1	+	+	+	+	Pp1s59_63V6.1
LCD1	-	+	-	-	
MAPK1	+	+	+	++	Pp1s207_63V6.1
					Pp1s138_117V6.1
MDC1	+	-	-	-	
MEC3	-	+	-	-	
MEK1	-	+	+	-	
P53	+	-	-	-	
PMYT1	+	-	-	+	Pp1s207_100V6.1
RAD17	+	+	+	+	Pp1s184_70V6.1
RAD24	-	+	-	-	
RAD9	-	+	-	-	
RAD9A	+	-	+	++	Pp1s130_202V6.1
					Pp1s124_86V6.1
SOG1	-	-	+	+	Pp1s251_11V6.1
TP53B	+	-	-	-	
WEE1	+	+	+	+	Pp1s197_56V6.1
Homologous recombination					
BRCA2	+	-	+	-	
BRCC36	+	-	+	+	Pp1s54_11V6.2
COM1	+	+	+	+	Pp1s242_87V6.1
GEN1	+	+	+	+	Pp1s391_28V6.1
INO80	+	+	+	++	Pp1s304_7V6.1
					Pp1s45_2V6.1
MRE11	+	+	+	+	Pp1s18_235V6.1
MUS81	+	+	+	+	Pp1s15_297V6.1
NBN	+	-	+	+	Pp1s219_52V6.1
PAXI1	+	-	++	++++	Pp1s97_25V6.2
					Pp1s160_107V6.1
					Pp1s35_92V6.1
					Pp1s232_74V6.1
R51A1	+	-	-	-	
RA51B	+	-	+	+	Pp1s129_197V6.1
RA51C	+	+	+	+	Pp1s236_47V6.1
RA51D	+	-	+	+	Pp1s137_214V6.1
RA54B	+	-	-	+	Pp1s212_41V6.1
RAD5	+	+	+	++	Pp1s41_174V6.1
					Pp1s73_179V6.1
RAD50	+	+	+	+	Pp1s51_220V6.1

Gene	*H. sapiens*	*S. cerevisiae*	*A. thaliana*	*P. patens*	*P. patens* Accessions
Homologous recombination					
RAD51	+	+	+	++	Pp1s42_140V6.1
					Pp1s31_236V6.1
RAD52	+	+	-	-	
RAD54	+	+	+	++	Pp1s341_67V6.1
					Pp1s236_78V6.1
RAD55	-	+	-	-	
RAD59	-	+	-	-	
RDH54	-	+	-	-	
RECQ1	+	-	+	+	Pp1s93_66V6.1
RECQ2	+	+	+++	+	Pp1s243_58V6.1
RECQ4	+	-	+	+	Pp1s232_56V6.1
RECQ5	+	-	+	-	
RMI1	+	+	+	+	Pp1s201_114V6.1
RMI2	+	-	+	+	Pp1s159_85V6.1
RQSIM	-	-	+	++	Pp1s222_3V6.1
					Pp1s152_88V6.1
RTEL1	+	-	+	+	Pp1s3_567V6.1
RUVB1	+	+	+	++	Pp1s4_73V6.1
					Pp1s40_79V6.1
RUVB2	+	+	+	+++	Pp1s255_64V6.1
					Pp1s402_30V6.1
					Pp1s88_18V6.1
SRS2	-	+	+	+	
TOP3A	+	+	+	+	Pp1s475_4V6.1
TOP3B	+	-	+	+	Pp1s474_3V6.1
WRIP1	+	+	+	+++	Pp1s16_272V6.1
					Pp1s455_6V6.1
					Pp1s97_156V6.1
WRN	+	-	-	+	Pp1s128_34V6.1
WRX	-	-	+	+++	Pp1s246_95V6.1
					Pp1s135_47V6.1
					Pp1s4_355V6.1
XRCC2	+	-	+	+	Pp1s45_271V6.1
XRCC3	+	-	+	-	
XRS2	-	+	-	-	
Non-homologous end-joining					
DCR1C	+	-	-	-	
KU70	+	+	+	+	Pp1s299_4V6.1
KU80	+	+	+	+	Pp1s121_27V6.1
NHEJ1	+	-	-	-	
PRKDC	+	-	-	+	Pp1s78_226V6.1
XRCC4	+	-	+	++	Pp1s34_261V6.1
					Pp1s147_88V6.1
Meiotic recombination					
DMC1	+	+	+	+	Pp1s9_248V6.1
HOP1	-	+	-	-	
HOP2	+	+	+	+	Pp1s335_13V6.1
MND1	+	+	+	+	Pp1s41_172V6.1
RE102	-	+	-	-	
RE104	-	+	-	-	
REC8	+	+	+	+	Pp1s351_7V6.1
RED1	-	+	-	-	

Gene	*H. sapiens*	*S. cerevisiae*	*A. thaliana*	*P. patens*	*P. patens* Accessions
Meiotic recombination					
SGO1	-	+	-	-	
SPO11	+	+	+	+++	Pp1s62_130V6.1 Pp1s14_84V6.1 Pp1s248_24V6.1
SYCP1	+	-	+	-	
SYCP2	+	-	+	-	
SYCP3	+	-	-	-	
ZIP1	-	+	-	-	
UV repair					
DDB1	+	-	+	++	Pp1s458_4V6.1 Pp1s203_55V6.1
DDB2	+	-	+	+	Pp1s114_132V6.1
FEN1	+	+	+	++	Pp1s456_8V6.1, Pp1s39_160V6.1
RAD18	+	+	-	-	
RAD23	++	+	++	++++	Pp1s58_148V6.1 Pp1s286_52V6.1 Pp1s3_105V6.1 Pp1s3_98V6.1
RAD7	-	+	+	-	
UBC2	+	+	++	+++	Pp1s91_88V6.1 Pp1s91_87V6.1 Pp1s219_106V6.1
Crosslink repair					
	+	+	+	++	Pp1s377_22V6.1
DCR1A					Pp1s120_12V6.1
DCR1B	+	-	+	+	Pp1s68_3V6.1
FACD2	+	-	+	+	Pp1s204_101V6.1
FANCA	+	-	-	-	
FANCB	+	-	-	-	
FANCC	+	-	-	-	
FANCE	+	-	-	-	
FANCF	+	-	-	-	
FANCG	+	-	-	-	
FANCJ	+	-	++	+	Pp1s95_66V6.1
FANCL	+	-	+	+	Pp1s156_74V6.1
FANCM	+	+	+	+	Pp1s9_477V6.1
Nucleotide excision repair					
ERCC1	+	+	+	++	Pp1s117_170V6.1 Pp1s117_181V6.1
ERCC2	+	+	+	+	Pp1s145_26V6.3
ERCC3	+	+	+	+	Pp1s177_124V6.1 Pp1s2991_1V6.1
ERCC4	+	+	+	+	Pp1s3_646V6.1
ERCC5	+	+	+	+	Pp1s31_24V6.1
ERCC6	+	+	+	++++	Pp1s66_144V6.1 Pp1s34_212V6.1 Pp1s84_259V6.1 Pp1s155_61V6.3
RAD1	+	-	+	+	Pp1s67_171V6.1

Gene	*H. sapiens*	*S. cerevisiae*	*A. thaliana*	*P. patens*	*P. patens* Accessions
Nucleotide excision repair					
RAD16	-	+	+	++	Pp1s3_639V6.1
					Pp1s132_19V6.1
SYF1	+	+	+	+	Pp1s139_28V6.1
XPA	+	+	-	-	
XPC	+	+	+	+	Pp1s12_235V6.1
Base Excision Repair					
PARP1	+	-	+	+	Pp1s114_181V6.1
PARP2	+	-	+	+	Pp1s324_39V6.1
PARP3	+	-	+	+	Pp1s59_305V6.1
PNKP	+	-	+	+	Pp1s240_31V6.1
XRCC1	+	-	+	++	Pp1s224_52V6.1
					Pp1s85_35V6.1
Mismatch repair					
DIN7	-	+	-	-	
EME1	+	-	+	+	Pp1s72_302V6.1
EXO1	+	+	+	++	Pp1s10_231V6.2
					Pp1s212_68V6.2
MLH1	+	+	+	+	Pp1s58_199V6.1
MLH2	-	+	-	-	
MLH3	+	+	+	+	Pp1s5_400V6.1
MSH1	-	+	-	-	
MSH2	+	+	+	+	Pp1s251_77V6.1
MSH3	+	+	+	+	Pp1s30_339V6.1
MSH4	+	+	+	+	Pp1s226_85V6.1
MSH5	+	+	+	+	Pp1s84_88V6.2
					Pp1s152_6V6.1
MSH6	+	+	+	++	Pp1s90_86V6.1
MUTS	-	+	-	+	Pp1s3_417V6.1
MUTYH	+	-	+	+	Pp1s151_27V6.1
PMS1/2	+	+	+	+	Pp1s474_7V6.1
General functions					
DDX11	+	-	-	+	Pp1s370_33V6.1
DNL1	+	+	+	+	Pp1s223_54V6.2
DNL3	+	-	-	-	
DNL4	+	+	+	+	Pp1s150_94V6.1
LIF1	-	+	-	-	
	+	+	+++	++	Pp1s152_136V6.1
PIF1					Pp1s300_57V6.1
	+	+	+	+++	Pp1s77_167V6.2
					Pp1s77_195V6.2
RAD21					Pp1s351_7V6.1
RNF4	+	-	+	++++	Pp1s491_21V6.1,
					Pp1s121_28V6.2,
					Pp1s3_270V6.1,
					Pp1s369_26V6.1
SCC2	+	+	+	+	Pp1s104_102V6.1
SIR2	-	+	-	+++	Pp1s68_122V6.2
					Pp1s272_33V6.1
					Pp1s15_90V6.2
SMC1A	+	+	+	+	Pp1s91_43V6.1
SMC1B	+	-	-	-	

Gene	*H. sapiens*	*S. cerevisiae*	*A. thaliana*	*P. patens*	*P. patens* Accessions
General functions					
SMC2	+	+	+	+	Pp1s52_57V6.1
SMC3	+	+	+	+	Pp1s410_17V6.1
SMC5	+	+	+	+	Pp1s274_85V6.1
SMC6	+	+	+	+	Pp1s61_278V6.1
STAG1	+	+	+	+	Pp1s199_157V6.1
STAG2	+	-	-	-	
STAG3	+	-	-	-	
TOF1	-	+	-	-	
TOPB1	+	-	+	+++	Pp1s1_250V6.1
					Pp1s47_22V6.1
					Pp1s208_91V6.1
ATM/ATR interactors					
4EBP1	+	-	-	-	
AATF	+	-	+	+	Pp1s186_46V6.1
ABL1	+	-	-	-	
AKT1	+	-	-	-	
BID	+	-	-	-	
CDC5L	+	+	+	+	Pp1s641_1V6.1
CHD4	+	-	+	++	Pp1s33_329V6.1
					Pp1s235_76V6.1
CLSPN	+	-	-	-	
CREB1	+	-	-	-	
E2F1	+	-	+	++++	Pp1s22_60V6.1
					Pp1s38_356V6.1
					Pp1s364_42V6.1
					Pp1s97_96V6.1
H2AX	+	-	+	+++	Pp1s55_112V6.1
					Pp1s452_4V6.1
					Pp1s188_35V6.1
HDAC1	+	+	+	+++	Pp1s223_52V6.1
					Pp1s351_29V6.1
					Pp1s180_68V6.1
HDAC2	+	-	+	-	
IKBA	+	-	-	-	
MCA3	+	-	-	-	
MCM2	+	+	+	+	Pp1s28_266V6.1
MCM3	+	+	+	+	Pp1s9_156V6.1
MCM7	+	+	+	+	Pp1s31_86V6.1
MDM2	+	-	-	-	
MDM4	+	-	-	-	
	+	+	+	++	Pp1s226_56V6.1
PTPA					Pp1s111_153V6.1
	+	+	+	++	Pp1s10_103V6.1
RENT1					Pp1s44_135V6.1
	+	+	+	+++	Pp1s222_133V6.1
					Pp1s192_40V6.1
RFA1					Pp1s257_1V6.1
RFA2	+	+	+	+	Pp1s357_53V6.1
SOSB1	+	-	+	+	Pp1s112_133V6.1
SP1	+	-	-	-	
	+	-	+	+++	Pp1s217_52V6.1
					Pp1s25_22V6.1
STRAP					Pp1s1020_4V6.1

Gene	*H. sapiens*	*S. cerevisiae*	*A. thaliana*	*P. patens*	*P. patens* Accessions
ATM/ATR interactors					
TERF1/2	+	-	++	++++++++	Pp1s74_197V6.1 Pp1s114_137V6.1 Pp1s176_113V6.2 Pp1s1_349V6.1 Pp1s176_88V6.1 Pp1s260_3V6.1 Pp1s49_258V6.1 Pp1s152_10V6.1
TIF1B	+	-	-	-	
TRIM1	+	-	-	-	

3.2 Functional analysis of ATM and ATR in *P. patens*

3.2.1 The ATM and ATR homologs of *P. patens*

To generate *P. patens ATM* and *ATR* mutant lines for a functional study, it was first required to identify their sequence homologs in *P. patens*. Utilizing the H. sapiens and Arabidopsis ATM and ATR protein sequences as queries, the *P. patens* sequence homologs were identified in a BLAST search against the *P. patens* protein database at www.cosmoss.org. In each case one remarkable hit with an e-value close to 0 was obtained. Cosmoss database gene identifiers are Pp1s135_65V6.1 for the ATM and Pp1s77_262V6.1 for the ATR homolog. As the gene models used to infer the ATM and ATR proteins present in the queried database were based on predictions, with very little EST data available to support them, it was decided to perform a full length cDNA sequencing of both genes. To obtain the full length cDNA of *P. patens ATM* and *ATR*, first a RACE approach was pursued to obtain the 5' and 3' terminal regions of both genes. Gene specific primers for the RACE were chosen according to the publicly available predicted gene models. After determining the beginning and end of the cDNA sequences the remainder was cloned and sequenced. Due to the large size of the two genes the *ATM* and *ATR* cDNA's were split up into 2 and 3 pieces respectively for amplification and cloning.

Including the 5' and 3' UTR regions the *ATM* cDNA was found to be 9895 bp in length, split into 78 exons , its full genomic sequence encompassing 29126 bp (**Figure 3.2-1**). Contrary to that the predicted models mRNA sequence was only 8995 bp, divided into 73 exons across a 28763 bp genomic sequence. The *ATR* cDNA amounted to a total of 9000 bp, divided into 25 exons with a 14652 bp genomic sequence. The predicted ATR mRNA model only consisted of 8364 bp, split among 26 exons, its genomic sequence stretching across 14136 bp.

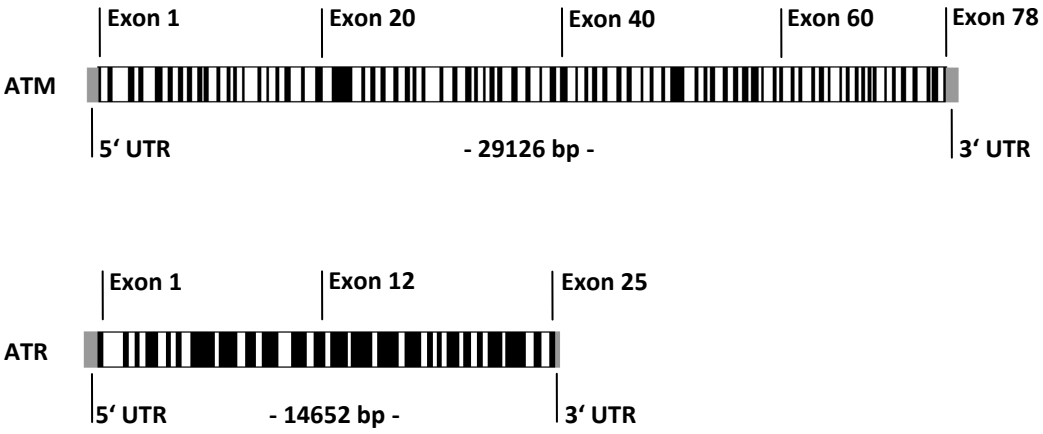

Figure 3.2-1: Genomic models of *P. patens ATM* and *ATR* based on RACE and cDNA sequencing. Grey boxes indicate UTR regions, black boxes indicate exons and white boxes designate introns.

Predicting the open reading frame (ORF) of the *ATM* cDNA, one major ORF extending from 372 – 9602 bp was found, producing a 2897 amino acid protein upon translation. The prediction of the ORF for our *ATR* cDNA produced an aberrant result. Instead of one big ORF, a small one extending from 400 – 2331 bp was obtained together with a larger one extending from 2404 – 8883 bp. This implied the presence of an extra stop codon in the cloned cDNA sequence. Performing a quick PCR analysis of the problematic region with newly generated cDNA produced a smaller PCR product as was to be expected from the sequencing results (data not shown). This indicated a possible splicing failure in the respective cDNA fragment which had been cloned for sequencing. As this fragment did not include the region intended for the later comparative analysis this phenomenon was not investigated further. Ignoring the additional stop codon the translation of the *ATR* cDNA yielded a protein of 2827 amino acids in size.

Having obtained the protein sequence of the *P. patens* ATM and ATR homologues their domain configuration was analyzed. The predicted domain structure of the *P. patens* ATM and ATR proteins as well as that of the human, yeast and Arabidopsis homologs are presented in **Figure 3.2-2**. As is evident, the characteristic C-terminal region with the FAT, PI3K and FATC domains is conserved in the ATM and ATR homologues of all organisms. However, differences are observed in the N-terminal region of ATM. ATM homologues of human and yeast display a TAN domain and the Arabidopsis homologue carries a domain named PWWP. Neither of these domain types was detected in our *P. patens* homologue. In addition, only the Arabidopsis and human ATM homologues displayed an N-terminal ARM domain.

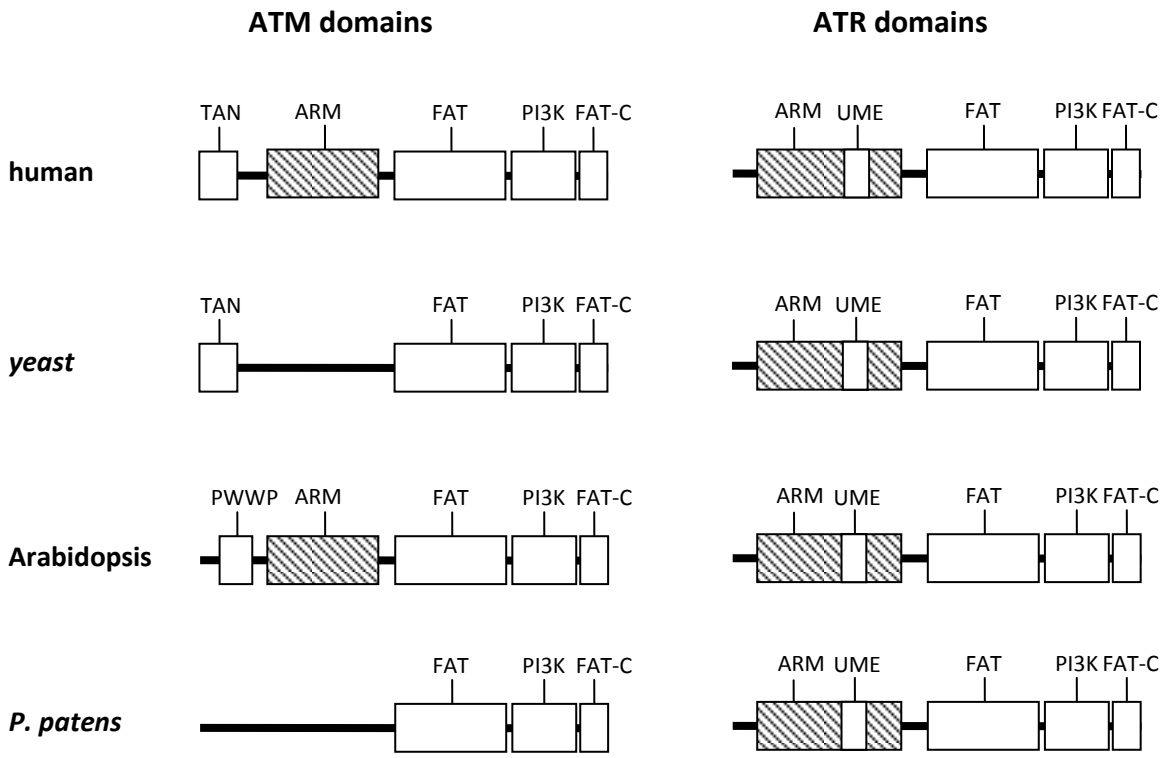

Figure 3.2-2: Domain structure of ATM and ATR homologues of human, Arabidopsis, yeast and *P. patens*. Presence of domains was determined by scanning protein sequences against the InterPro collection of protein signature databases. **ARM**, Armadillo repeat; **FAT**, FRAP, ATM and TTRAP; **FAT**-C, FRAP, ATM and TTRAP C-terminal; **PI3K**, Phosphoinositide 3-kinase, catalytic domain; **PWWP**, Pro-Trp-Trp-Pro; **UME**, UVSB PI-3 kinase, MEI-41 and ESR1.

In ATM and ATR homologs of different species the PI3K kinase and adjacent FAT-C domain have been found to display the highest degree of evolutionary conservation (Culligan et al. 2004, Templeton and Moorhead 2005), a phenomenon likely related to the fact that PIKK family kinases exert their functionality through mainly protein phosphorylation (Cliby et al. 1998, Park et al. 2001). **Table 3.2-1** displays the percent identity matrix for the PI3K kinase regions of *H. sapiens*, *S. cerevisiae*, *A. thaliana* and *P. patens* ATM and ATR homologs. As would be expected from the closer phylogenetic proximity *P. patens* PI3K regions of ATM and ATR were found to be most similar to these of Arabidopsis.

Table 3.2-1: Homology of ATM and ATR PI3K domains of various organisms. Percent identity matrix of the ATR and ATM Pi3K domains of *P. patens* (Pp), *A. thaliana* (At), *H. sapiens* (Hs), and S*. cerevisiae* (Sc). Pairwise comparisons were performed with the "Clustal Omega" software.

	*Sc*ATM	*Hs*ATM	*At*ATM	*Pp*ATM
*Sc*ATM	100	46.91	45.42	46.67
*Hs*ATM		100	60.98	59.76
*At*ATM			100	79.52
*Pp*ATM				100

	*Sc*ATR	*Hs*ATR	*At*ATR	*Pp*ATR
*Sc*ATR	100	44.67	42.66	42.81
*Hs*ATR		100	51.95	52.55
*At*ATR			100	83.17
*Pp*ATR				100

To further characterize the *P. patens* ATM and ATR homologs their C-terminal kinase region was evaluated for conservation of functionally critical residues. **Figure 3.2-3** shows an alignment between the PI3K and FAT-C domains of the *H. sapiens*, *S. cerevisiae*, *A. thaliana* and *P. patens* ATM proteins. The alignment is enhanced by highlighting a number of residues which were found to be important in the human ATM homologue. The marked residues are known to either be mutated in human cancer tissue (indicated by black arrows) or have been identified as being essential for the functionality of the kinase domain in mutagenesis experiments (indicated by white arrows). While only 12 out of the 23 residues which are known to be mutated in human cancer were conserved across all organisms, all 3 of the residues essential for the kinase functionality of human ATM were found conserved.

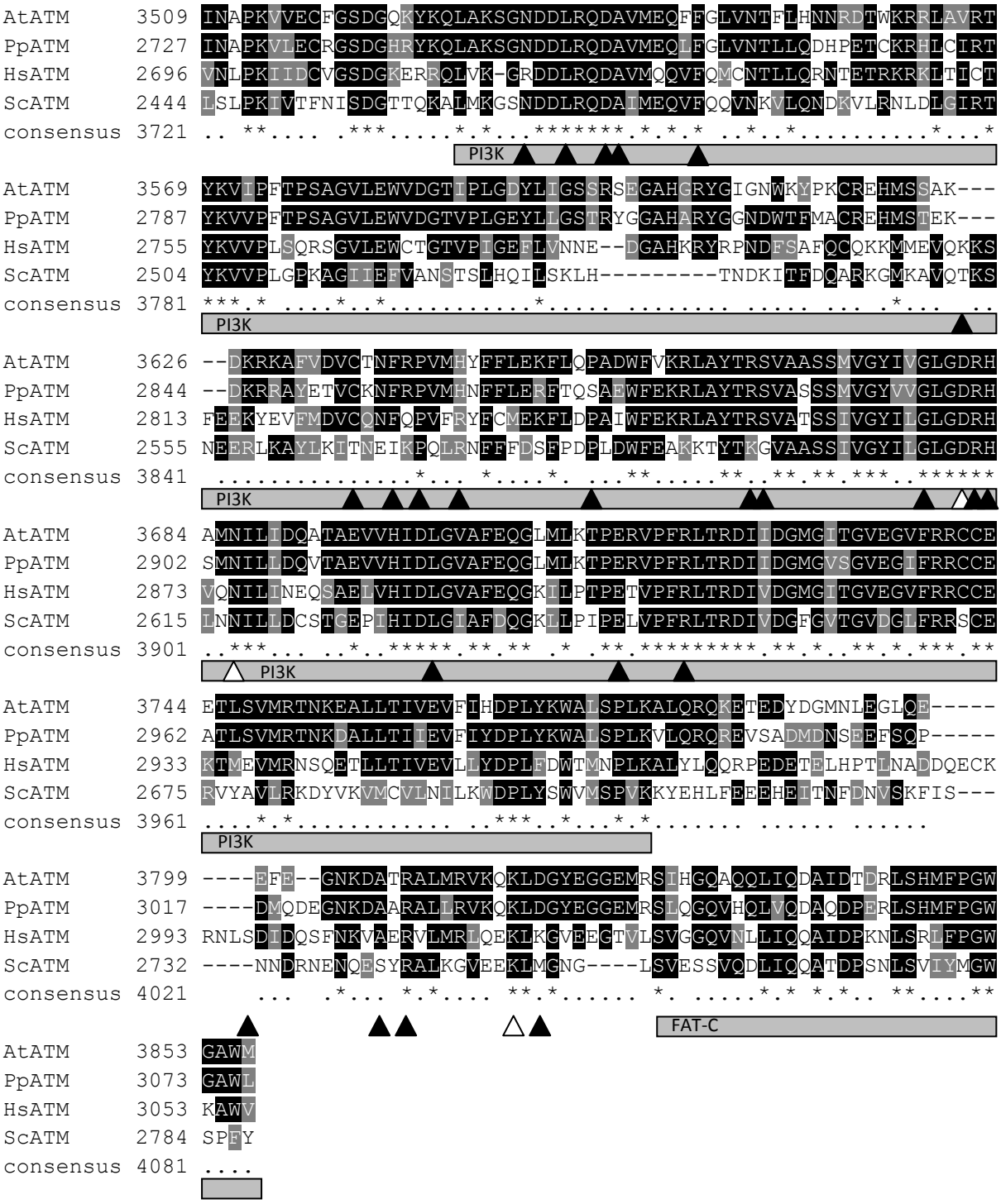

Figure 3.2-3: Alignment of the C-terminal region of ATM homologs from *P. patens* (Pp), *A. thaliana* (At), *H. sapiens* (Hs) and *S. cerevisiae* (Sc). The numbers on the left indicate the position of the first residue in each row for each protein. Grey bars delineate the PI3K and FAT-C domains. Black triangles indicate residues which have been found mutated in patients suffering from either ataxia telangiectasia or various forms of cancer. White triangles designate residues whose mutation has been found to lead to a loss of kinase activity. The symbols in the consensus sequence indicate whether a residue is conserved in all aligned sequences "*", conserved substitutions ":" or semi conserved substitutions ".". Alignments were performed utilizing the "Clustal Omega" software.

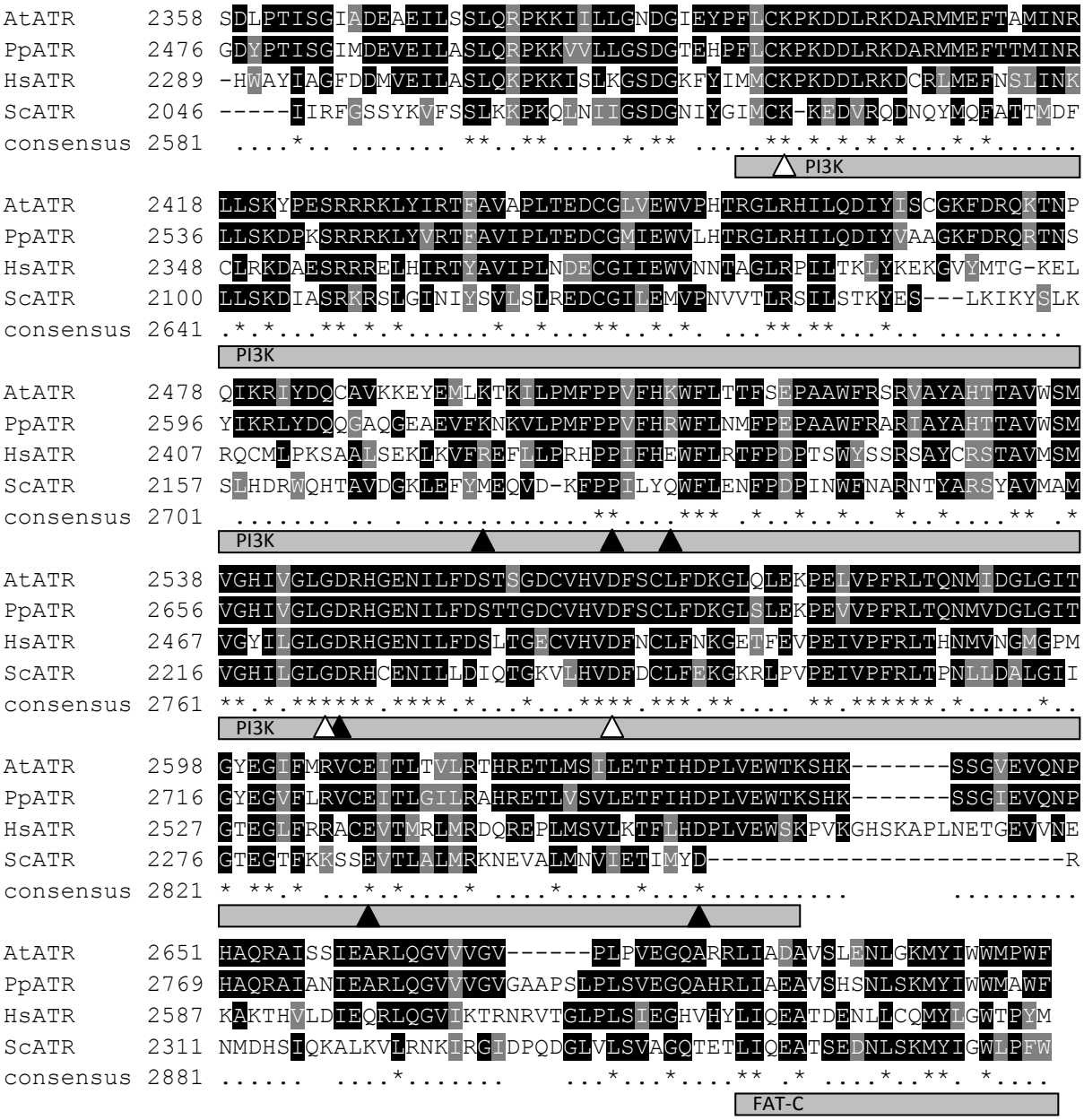

Figure 3.2-4: Alignment of the C-terminal region of ATR homologues from *P. patens* (Pp), A. thaliana (At), H. sapiens (Hs) and S. cerevisiae (Sc). The numbers on the left indicate the position of the first residue in each row for each protein. Grey bars delineate the PI3K and FAT-C domains. Black triangles indicate residues which have been found mutated in patients suffering from either ataxia telangiectasia or various forms of cancer. White triangles designate residues whose mutation has been found to lead to a loss of kinase activity. The symbols in the consensus sequence indicate whether a residue is conserved in all aligned sequences "*", conserved substitutions ":" or semi conserved substitutions ".". Alignments were performed utilizing the "Clustal Omega" software.

Figure 3.2-4 shows an alignment between the PI3K and FAT-C domains of the human, Arabidopsis, yeast and *P. patens* ATR proteins. Once again the alignment is enhanced by highlighting a number of residues found to be important for the human ATR protein functionality. The marked residues were found to be either mutated in human cancer tissue (indicated by black arrows) or found essential for the functionality of the kinase domain in mutagenesis experiments (indicated by white arrows). Out of 6 residues which were found mutated in various human cancers, 4 are conserved in all species. Similar to ATM, all of three residues which have been deemed essential for kinase functionality of the *H. sapiens* homolog are conserved in *P. patens* and others.

The fact that all residues of ATM and ATR which were found essential for kinase functionality were conserved in *P. patens* and the other three organisms attests to the high degree of conservation in this functional domain. However at the same time residues which were found to be involved in the formation of human cancers were not conserved that strictly. Thus even in this highly conserved region there is evidence for a possible difference in functionality between ATM and ATR homologues of different organisms.

3.2.2 Generation of *P. patens* ATM and ATR mutant lines

3.2.2.1 Transformation and selection of transformants

To generate *P. patens ATM* and *ATR* mutant lines, its high innate efficiency in gene targeting was utilized. Two specific targeting constructs were designed, pKOATR and pKOATM, to disrupt the kinase domains of ATM and ATR respectively, as these are presumed to be vital for the functionality of these proteins (Barlow et al. 1996, Cliby et al. 1998, Park et al. 2001). The targeting constructs were transported into the nuclei of *P. patens* wild type protoplasts via PEG mediated DNA uptake (Schaefer and Zryd 1997). It was also tested whether or not using lower concentrations of DNA in transformation may improve the ratio of targeted to random integrations. For this purpose, 4 batches of protoplasts were transformed with pKoATM and pKoATR each, one with 5 µg, one with 10 µg and two with 15 µg of targeting construct DNA. Transformed protoplasts were subsequently regenerated into small plantlets and subjected to 3 alternating cycles of growth on media with or without antibiotic selection. This selection regime was required to remove unstable transformants, a common byproduct of transformation experiments conducted in *P. patens* (Cove 2005, Kamisugi et al. 2006). In total, 42 and 40 stable transformants were obtained in the transformation experiments with the pKoATM and pKoATR targeting constructs respectively.

3.2.2.2 PCR analysis of putative ATM and ATR mutant lines

To evaluate whether or not stable transformants obtained in the gene targeting approach carried the intended genomic modification at the ATM/ATR locus, a PCR based screen was devised (**2.2.3.3**). PCR reactions were set up to amplify a region spanning across the 5' and 3' integration sites as well as the complete targeted locus (**Figure 3.2-5**).

All of the 42 and 40 stable transformants, obtained via transformation with the pKoATM and pKoATR targeting constructs respectively, were tested according to the previously described PCR strategy. Results of the PCR analysis are summarized in **Table 3.2-2** and **Table 3.2-3**.

Among the transformants obtained with the pKoATM and pKoATR constructs a total of 6 and 5 individuals were found respectively which displayed all three of the PCR products as expected from a

successful targeting event. PCR results for the remainder of transformants were suggestive of either random or complex integration patterns of the targeting constructs.

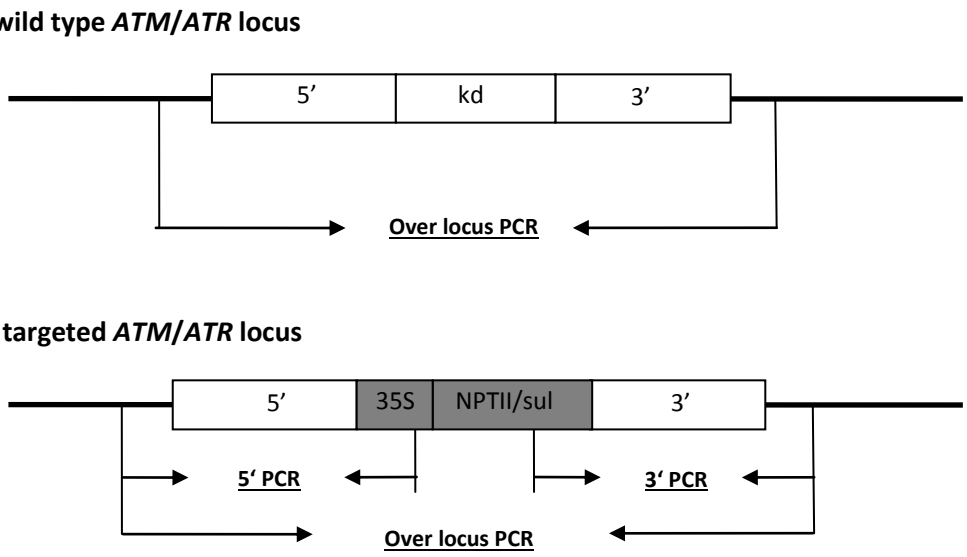

Figure 3.2-5: Schematic representation of PCR strategy used in analysis of ATM and ATR loci. (**a**) In our PCR approach, transformants which still retained the wild type locus with an intact kinase domain would generate a PCR product in only the over the locus PCR. (**b**) In transformants which were successfully targeted, the over the locus PCR as well as the 5' and 3' PCR's would generate a PCR product. Due to different lengths of the kinase domain and the selection marker cassette it is replaced by upon targeting, the over the locus PCR produces different sized PCR products in transformants with a wild type or targeted locus. Vertical lines connected to arrows indicate the approximate binding site of primers. **5'**, 5' region of homology; **3'**, 3' region of homology; **35S**, promoter region of cauliflower mosaic virus; **kd**, stretch of sequence coding for part of the kinase domain of ATM and ATR which will be replaced by selection marker cassette upon successful targeting; **NPTII**, neomycin phosphotransferase gene providing kanamycin resistance; **sul**, mutated dihydropteroate synthase gene providing resistance to sulfadiazine.

Table 3.2-2: **PCR screening results of all stable transformants obtained in gene targeting experiment with the pKoATM targeting construct.** Transformants are designated according to amount of DNA used in the respective transformation (**5**, **10**, **15** µg), the batch of transformed protoplasts in capital letters (**A** or **B**), the number of the plate the transformant was placed on after the initial selection step (**I-IV**) and the consecutive numbering for each individual transformant on a single plate (**1-10**). "**+**" indicates the amplification of a PCR product as expected from successful integration of the targeting construct. A "**–**" indicates that no PCR band was obtained. "**wt**" designates a PCR result which indicated the presence of a intact wild type locus. ***** indicates amplification of one or more PCR products of unexpected size.

transformant	5' PCR	3' PCR	over locus PCR
5 III 1	+	+	wt
10 I 1	+	+	-
10 I 2	+	-	wt
10 I 4	**+**	**+**	**+**
10 I 5	+	+	-
10 III 2	-	-	wt
15A I 1	**+**	**+**	**+**
15A I 2	+	+	-
15A I 3	+	-	-
15A I 4	-	+	-
15A I 7	+	-	-
15A I 8	+	+	-
15A II 1	+	+	wt
15A II 2	+	+	-
15A II 3	+	+	-
15A II 7	-	-	wt
15A II 8	+	-	-
15A II 10	+	+	-
15A III 1	+	*	-
15A III 2	-	+	-
15A III 4	+	+	-
15A III 5	-	+	-
15A III 6	+	*	-
15A III 7	+	+	-
15A III 8	+	+	-
15A IV	+	+	-
15B I 1	+	-	-
15B I 2	+	+	-
15B I 4	**+**	**+**	**+**
15B II 2	-	-	wt
15B II 3	-	+	-
15B II 4	-	-	wt
15B II 5	+	+	wt
15B III 1	**+**	**+**	**+**
15B III 2	-	+	-
15B III 3	-	-	wt
15B III 4	+	+	-
15B III 5	**+**	**+**	**+**
15B III 6	-	-	wt
15B III 7	**+**	**+**	**+**
15B IV 1	+	+	-
15B IV 3	+	+	-

Table 3.2-3: **PCR screening results of all stable transformants obtained in gene targeting experiment with the pKoATR targeting construct.** Transformants are designated according to amount of DNA used in the respective transformation (**5**, **10**, **15** µg), the batch of transformed protoplasts in capital letters (**A** or **B**), the number of the plate the transformant was placed on after the initial selection step (**I-IV**) and the consecutive numbering for each individual transformant on a single plate (**1**-**27**). "**+**" indicates the amplification of a PCR product as expected from successful integration of the targeting construct. A "**–**" indicates that no PCR band was obtained. "**wt**" designates a PCR result which indicated the presence of a intact wild type locus. ***** indicates amplification of one or more PCR products of unexpected size.

transformant	5' PCR	3' PCR	over locus PCR
5 II 3	+	+	+
5 III 13	+	+	-
10 I 10	-	-	wt
10 I 17	-	-	wt
10 II 6	-	-	wt
10 II 13	-	-	wt
10 II 14	-	-	wt
10 III 9	+	+	+
10 IV 9	+, *	-	wt
10 IV 10	+	+	+
15A I 1	+	-	wt
15A I 2	+	+	-
15A I 4	+	+	-
15A I 6	-	-	wt
15A I 7	+	+	*
15A I 8	+	+	-
15A I 15	-	-	wt
15A I 17	-	-	wt
15A I 18	-	-	wt
15A II 1	+	+	*
15A II 6	+	+	+
15A II 16	-	-	wt
15A III 13	+	+	-
15A IV 6	+	+	*
15A IV 14	+	-	+
15A IV 16	+	*	wt
15A IV 19	-	-	wt
15B I 2	-	-	wt
15B I 28	-	-	wt
15B II 3	-	-	-
15B II 7	-	-	wt
15B II 23	-	-	wt
15B III 22	-	-	wt
15B III 24	-	-	wt
15B III 25	-	-	wt
15B III 27	+	+	-
15B IV 4	-	-	wt
15B IV 8	-	-	wt
15B IV 10	+	+	*
15B IV 25	-	-	wt

3.2.2.3 Southern blotting analysis of select transformants

Having assayed all stable transformants via PCR, it was decided to conduct an additional southern blot analysis of a select subset of transformants. This was done for two reasons. First, a PCR based screen may be prone to produce artifacts as well as being limited in the depth of information it provides. It is for example unsuitable if one would like to find out whether or not additional targeting vector fragments may have integrated at random locations in the genome of the transformants. It would be important to know this as random integrations may disrupt other genes and thus affect the phenotype of mutant lines. Second, the additional information on transgene integration patterns obtained via the Southern approach may reveal peculiarities regarding the mode of transgene integration in *P. patens*. Therefore, the subset of transformants chosen for southern analysis included the most promising candidates, which are those testing positive for targeted integration in all three PCR reactions, as well as a variety of lines which tested out differently in the PCR analysis.

To analyse the *ATM* and *ATR* loci in the selected subset of transformants, genomic DNA was extracted and digested with the restriction enzymes *Sty*I and *BsoBI* respectively. These enzymes were selected as they would provide different sized fragments of the *ATM*/*ATR* loci, depending on whether or not targeting had occurred. To detect these fragments in the southern blot, three sequence specific DNA probes were generated for analysis of the putative ATM and ATR mutants each (**2.2.3.4)**. **Figure 3.2-6** showcases a schematic of wild type and targeted *ATM*/*ATR* loci, the fragment sizes obtained after restriction digest as well as the DNA probe hybridization sites. The 5' probe was generated from a stretch of sequence in close upstream proximity of the 5' region of homology of *ATM*/*ATR*. In case of the *ATM* locus this probe should detect a 5455 bp fragment if no targeting had taken place whereas a 2035 bp fragment would indicate targeting. For the ATR locus the detection of a 6440 bp sized fragment would indicate an unchanged locus whereas a 3360 bp fragment would be observed if targeting was successful. The 3' probe was designed in a similar fashion, being specific for a stretch of sequence in close downstream proximity of the 3' region of homology. In regard to the *ATM* locus, this probe would detect a 5455 bp sized fragment if no targeting had taken place and a 3653 bp fragment in case targeting had occurred. For the *ATR* locus the 3' probe would detect a 6440 bp fragment if no targeting had taken place and a 4199 bp fragment if the targeting construct had successfully integrated at the 5' site of homology. The third probe was designed to be specific for that stretch of sequence coding for the kinase domain which would be replaced with a selection marker cassette in case of successful gene targeting. For the *ATM* locus this probe would detect a 5455 bp fragment if gene targeting failed and no fragment at all if targeting had occurred. For *ATR* a 6440 bp fragment would be detected in untargeted lines whereas no fragment would be detectable in accurately targeted lines. In addition to these specifically generated probes the complete pKoATM

and pKoATR targeting constructs including the vector backbone were used as probes. These allowed the detection of all of the above mentioned fragments for the ATM and ATR locus respectively as well as any number of fragments carrying random integrations of any part of the targeting vector backbone. A total of 18 stable pKoATM transformant lines, with an additional wild type sample serving as an untransformed control, were subjected to Southern blot analysis (**Figure 3.2-7**). The summarized results for the Southern blot and PCR analysis are listed in **Table 3.2-4**.

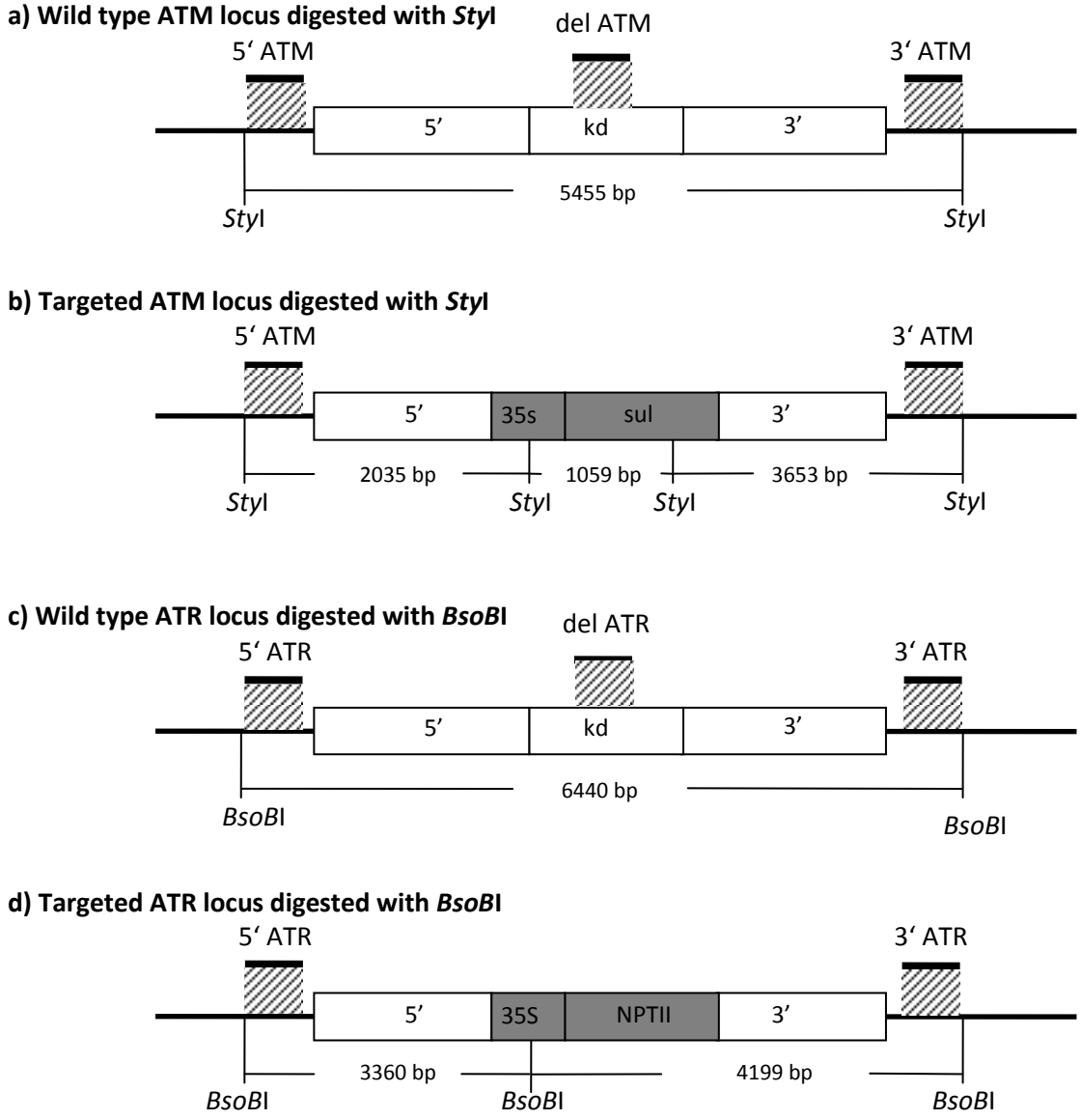

Figure 3.2-6: Schematic for Southern blot analysis of putative *ATM* and *ATR* mutants. Wild type as well as targeted loci are shown for *ATM* and *ATR*, with vertical lines designating cutting sites for the indicated restriction enzyme. The expected size of restriction fragments is noted in between the adjoined horizontal lines. Horizontal bars designated 5’ ATM/ATR, 3’ ATM/ATR and del ATM/ATR represent the DNA probes with their respective hybridization sites indicated by a rectangle of diagonal stripes. **5’**, 5’ region of homology; **3’**, 3’ region of homology; **35S**, promoter region of cauliflower mosaic virus; **kd**, stretch of sequence coding for part of the kinase domain of ATM and ATR which will be replaced by selection marker cassette upon successful targeting; **NPTII**, neomycin phosphotransferase gene II providing kanamycin resistance; **sul**, mutated dihydropteroate synthase gene providing resistance to sulfadiazine.

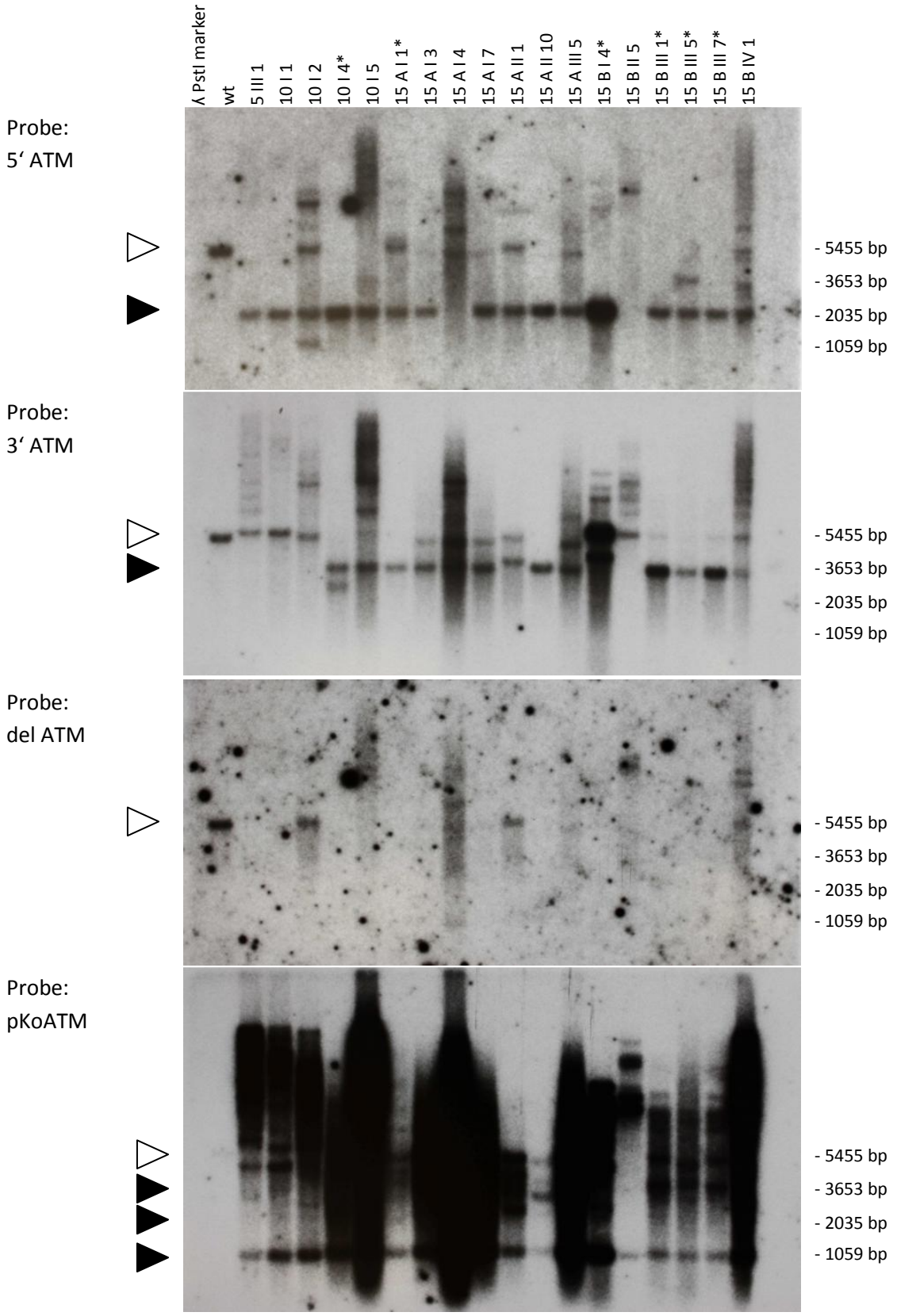

Figure 3.2-7: Southern blot analysis of selected stable pKoATM transformants. Genomic DNA of putative transformants was digested with *Sty*I, blotted and hybridized with four individual probes, **5’ ATM**, **3’ ATM**, **del ATM** and **pKoATM**. Bands corresponding to an untargeted locus are marked with a white triangle while a black triangle indicates the band size as expected from a successfully targeted locus. Lines which tested positive for accurate targeting in the previous PCR analysis are marked with a “*****” after their designation.

Table 3.2-4: Summary results of PCR and Southern blot analysis of select putative *ATM* mutant lines. a) To verify the findings of the initial PCR screen it was repeated with the higher quality CTAB DNA as it was extracted for the southern. -, indicates that no product was amplified in the PCR, suggesting that the ATM locus was altered, albeit not by accurate gene replacement as intended. **+**, indicates that the PCR product was of the size which indicated successful gene targeting. wt indicated that the PCR product was the same as in the wild type, proposing that the intact wild type ATM locus was still present. Lines in which the outcome of all three PCRs suggested targeted integration were shaded grey. **b) -**, indicates that no hybridization occurred, this result was only observed with the probe for the deleted region, suggesting that it had indeed been removed due to gene targeting. **+**, indicates that the fragment as expected from successful gene targeting was detected. **wt**, indicates that the detected fragment had the size as in the wild type, suggesting that the wild type ATM locus was still intact. **?**, indicates that one or more weak additional bands were observed whose size did not fit to the wild type or targeted locus, suggesting an incomplete digest or cross hybridization. *****, indicates additional strong bands were detected the size of which was not specific for either a wild type or targeted ATM locus, suggesting a complex integration pattern.

	a) PCR results			b) southern blotting results			
transformant	**5' PCR**	**3' PCR**	**locus PCR**	**5' ATM probe**	**3' ATM probe**	**ATM del probe**	**pKoATM**
5 III 1	+	+	-	+	*	-	**
10 I 1	+	+	-	+	*	-	**
10 I 2	+	-	wt	wt, +, *	wt, *	wt	**
10 I 4	+	+	+	+	+, *	-	**
10 I 5	+	+	-	+, ?	+, *	*	*
15 A I 1	+	+	+	+, *	+	-	*
15 A I 3	+	+	-	+, ?	+, ?	-	*
15 A I 4	+	+	-	wt, *	+, *	wt, *	*
15 A I 7	+	+	-	+, *	+, ?	-	*
15 A II 1	+	+	wt	+, wt	wt, *	wt	*
15 A II 10	+	+	-	+	+	-	*
15 A III 5	+	+	-	+, wt	wt, +	*	*
15 B I 4	+	+	+	+, ?	*	-	*
15 B II 5	+	+	wt	*	*	*	*
15 B III 1	+	+	+	+	+, ?	-	*
15 B III 5	+	+	+	+, *	+	-	*
15 B III 7	+	+	+	+	+, ?	-	*
15 B IV 1	+	+	-	+, *	+, *	wt, *	*

Evaluating the results in regard to finding suitable *ATM* mutant lines for the subsequent functional analysis, the 6 lines which were previously tested positive for transgene integration in all three PCR reactions were once again found to be the most promising. It was found that the stretch of sequence coding for a major part of the *ATM* kinase domain was indeed successfully deleted in all 6 of these lines. However, hybridization with the other probes, especially the whole targeting plasmid one, indicated that all of these lines may have sustained additional integrations besides those expected from a successful targeting event. A number of additional signals observed with certain probes however can be discarded as cross hybridizations, due to them being much weaker in intensity than the expected bands. This phenomenon is for example very obvious in the transformants 15BIII1 and 15BIII7 when hybridized with the 3' probe. As especially the probe generated from the complete pKoATM targeting plasmid produced extra signals of non negligible intensity it could not be ruled out

that even the most promising transformants may have sustained additional genomic alterations. This was problematic as in a worst case scenario this may affect the results of the later phenotypic analysis. It was therefore decided that while the bulk of the experiments would be conducted with the 15BIII1 line, the 10I4 and 15BIII5 lines would be used as auxiliaries to verify the results of key experiments. To standardize the naming of these selected mutant lines they were redesignated as *atm-1* (15BIII1), *atm-2* (10I4) and *atm-3* (15BIII5) and will be addressed in that manner from this point onward.

One additional interesting observation could be made when comparing the signals obtained with the pKoATM targeting plasmid derived probe. It appears as if the 6 lines which tested out ok in the PCR analysis are comparably "clean" in their band pattern using this probe. Those in which the PCR gave mixed results however very often display a large number of additional high intensity bands. The latter phenomenon is very likely indicative of a large number of random integrations of sequences derived from the targeting vector backbone. This hinted at the possibility that *P. patens* cells may periodically favor random, Non-homologous-end-joining mediated over targeted, homologous recombination based, integrations of transgenes.

Analogous to the southern analysis of putative *ATM* mutants, the same procedure was performed with 18 pKoATR transformants, with an additional wild type sample serving as an untransformed control (**Figure 3.2-8**). Summarized results for the Southern blot and PCR analysis are listed in **Table 3.2-5**.

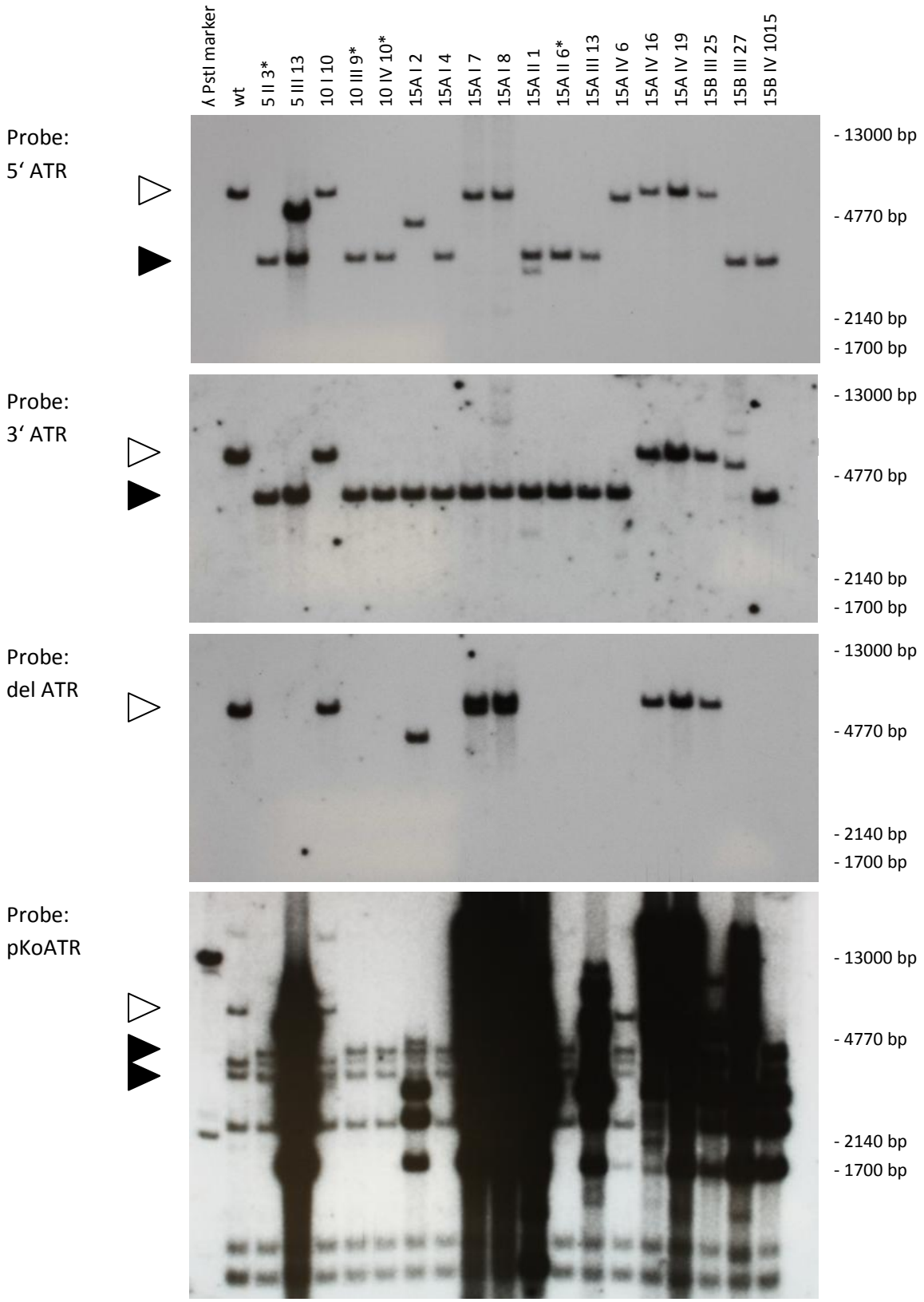

Figure 3.2-8: **Southern blot analysis of selected stable pKoATR transformants.** Genomic DNA was digested with BsoBI, blotted and hybridized with four individual probes, **5’ ATR**, **3’ ATR**, **del ATR** and **pKoATR**. Bands corresponding to an untargeted locus are marked with a white triangle while a black triangle indicates the band size as expected from a successfully targeted locus.

Table 3.2-5: Summary results of PCR and Southern blot analysis of select putative *ATR* mutant lines. a) To verify the findings of the initial PCR screen it was repeated with the higher quality CTAB DNA as it was extracted for the southern. -, indicates that no product was amplified in the PCR, suggesting that the ATM locus was altered, albeit not by accurate gene targeting as intended. **+**, indicates that the PCR product was of the size which indicated successful gene targeting. **wt**, indicates that the PCR product was the same as in the wild type, proposing that the intact wild type ATM locus was still present. Lines in which the outcome of all three PCRs suggested targeted integration were shaded grey. **b) -,** indicates that no hybridization occurred, this result was only observed with the probe for the deleted region, suggesting that it had indeed been removed due to gene targeting. **+**, indicates that the fragment as expected from successful gene targeting was detected. **wt**, indicates that the detected fragment had the size as in the wild type, suggesting that the wild type ATM locus was still intact. **?**, indicates that one or more weak additional bands were observed whose size did not fit to the wild type or targeted locus, suggesting an incomplete digest or cross hybridization. *****, indicates additional strong bands were detected whose size was not specific for a wild type or targeted ATM locus, suggesting a complex integration pattern. ******, indicates a very large number of additional fragments were detected.

	a) PCR results			**b) Southern blotting results**			
transformant	**5' PCR**	**3' PCR**	**locus PCR**	**5' ATR probe**	**3' ATR probe**	**del ATR probe**	**pKoATR**
5 II 3	+	+	+	+	+	-	+
5 III 13	+	+	-	+, *	+	-	**
10 I 10	-	-	wt	wt	wt	wt	wt, *
10 III 9	+	+	+	+	+	-	+
10 IV 10	+	+	+	+	+	-	+
15A I 2	-	+	-	*	+	*	+, *
15A I 4	+	+	-	+	+	-	+
15A I 7	+	+	-	wt	+	wt, *	**
15A I 8	+	+	-	wt	+, ?	wt, *	**
15A II 1	+	+	-	+, ?	+, ?	-	**
15A II 6	+	+	+	+	+	-	+
15A III 13	+	+	-	+	+	-	**
15A IV 6	+	+	*	wt	+	-	wt, +, *
15A IV 16	-	-	wt	wt	wt	wt	**
15A IV 19	-	-	wt	wt	wt	wt	**
15B III 25	-	-	wt	wt	wt	wt	**
15B III 27	+	+	-	+	*, ?	-	**
15B IV 10	+	+	*	+	+	-	+, *

Focusing on the four transformants whose PCR analysis suggested accurate gene targeting events had taken place, the Southern blot was found to confirm these findings. In these lines each of the probes used for hybridization produced the signals as they were expected from an accurate gene targeting event. The only probe which produced extra signals in these lines was that which was derived from the pKoATR targeting plasmid. The extra signals spotted in these lines however are most likely the result of nonspecific hybridization as they can be observed in every single transformant as well as the wild type. Consulting the picture of the ethidium bromide stained gel made prior to blotting (data not shown) these signals were found to coincide with positions where there were high concentrations of DNA present. This supported the notion that these bands were indeed most likely a result of nonspecific hybridization, the signal merely showing up due to the large

amount of DNA available for cross hybridization at these locations.
Also, just like with the pKoATM transformants it was observed that transformants whose PCR and southern analysis suggested they arose from an accurate gene targeting event did not display the huge number of seemingly random additional signals often observed in other transformants. As mentioned previously this suggests that individual protoplasts may have been biased towards either random or targeted integration of the targeting DNA construct during transformation.
A further phenomenon apparent from the hybridization signals obtained with the pKoATR probe was that a number of lines, which displayed a marked increase in hybridization signals, appeared to contain the same set of additional bands. Upon closer examination it was found that in the lines 5III13, 15AI7, 15AI8, 15AII1, 15AIII13, 15BIII25, 15BIII27 and 15BIV10 three additional bands of high intensity were detected. Elucidating the approximate size of these fragments it was revealed that they corresponded to a digested pKoATR targeting plasmid. This highly suggests that even after three rounds of selection, copies of the targeting plasmid used for transformation may still be retained extra-chromosomally. Similar to the ATM mutants described earlier 3 promising ATR mutant lines were selected for the subsequent phenotypic analysis. 5II3 was selected as the standard mutant line used for the majority of phenotyping experiments while 10III9 and 15AII6 were used to verify some of the key phenotypic phenomena. To follow standard mutant naming procedures these lines were redesignated as *atr-1* (5II3) *atr-2* (10III9) and *atr-3* (15AII6) and for all subsequent parts of this thesis will be addressed in that manner.

In conclusion, the PCR and Southern based analysis of putative *ATM* and *ATR* mutant lines revealed that the gene targeting approach had produced a sufficient number of mutant lines, suitable for the subsequent functional analysis. However, albeit *P. patens* is generally assumed to be highly efficient in homologous recombination mediated gene targeting (Schaefer 2001), random integration events via NHEJ appeared to also be a frequent occurrence in many of the transformants. This highlights the importance of a robust assay system and suggests that a solely PCR based approach may not be entirely sufficient to select the most suitable transformant lines.
Furthermore, a secondary objective of the gene targeting experiments was to determine whether the amount of DNA used in transformation may affect the ratio of targeted to random integrations. The results obtained in this regard were inconsistent. While with the pKOATR targeting construct less DNA used in transformation generated a greater percentage of accurately targeted lines, this phenomenon was not observed with the pKOATM construct.

3.2.2.4 Flow cytometric analysis of select transformant lines

After transformants had been preselected according to PCR and Southern analysis they were furthermore checked for their ploidy level. This was necessary due to the fact that the method used in the transformation of protoplasts may result in protoplast fusion, effectively doubling the ploidy level of affected transformants. Since diploidization of the normally haploid *P. patens* has been found to produce growth and reproductive phenotypes (Grimsley et al. 1977, Schween et al. 2005), this would greatly complicate the later phenotypization of ATM and ATR mutant lines.
To determine the ploidy level of transformants, crude extracts of nuclei were prepared from 7 day old protonema cultures. The lines tested comprised those which were previously selected for the phenotyping, *atm-1, atm-2, atm-3, atr-1, atr-2, atr-3* as well as a wild type and known diploid line serving as controls. After staining crude nuclear extracts with DAPI, ploidy levels of samples were determined via flow cytometric measurement (**Figure 3.2-9**). As it turned out, none of the tested mutant lines were found to be diploid.

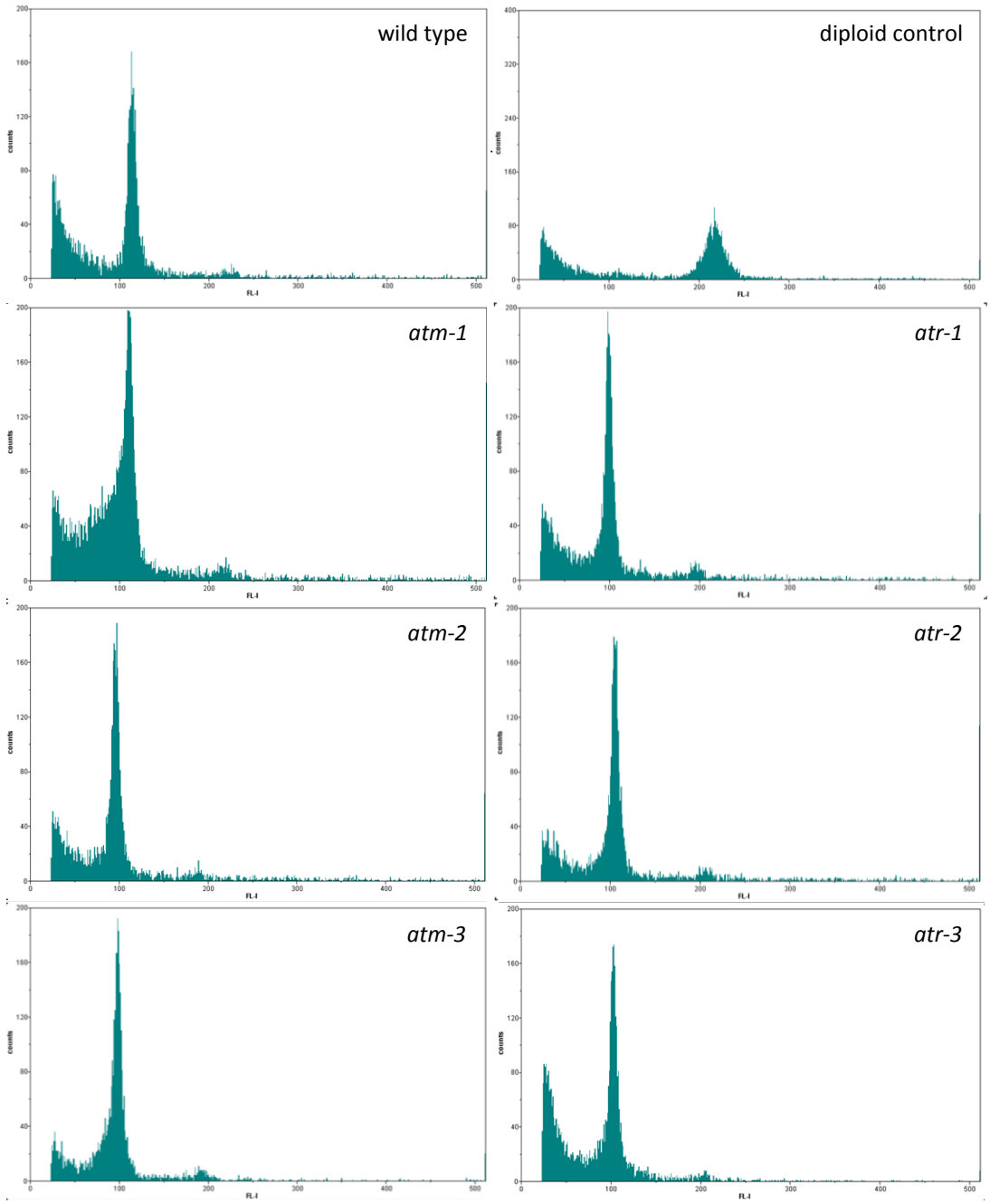

Figure 3.2-9: Flow cytometric analysis of select ATM and ATR mutant lines. To analyze ploidy levels in the *atm-1, atm-2, atm-3, atr-1, atr-2, atr-3* transformants as well as wild type and a known diploid line, nuclei were extracted from 7 day old protonema filaments. Crude nuclei extracts were then stained with DAPI, a DNA specific fluorescent stain. Fluorescence signals were subsequently quantified via flow cytometry. Starting from the left the first peak visible is very likely suspected to originate from cell wall fragments or other unspecific background signals from the DAPI staining. The second and largest peak resembles that of 1C G2 stage nuclei. The 1C G1 peak is not visible in the measurement as the majority of cells in *P. patens* are supposed to be arrested in G2 and the low number of 1C G1 nuclei are insufficient to generate a signal above the level of background noise. The third, usually small peak resembles 2C G2 nuclei and is only really prominent in the diploid control line.

3.2.2.5 Obtaining a double mutant by crossing of single mutant lines

Having obtained a number of verified ATM and ATR single mutant lines they were sexually crossed in an attempt to generate a double mutant line. The lines chosen for this were those previously characterized as the most suitable for further phenotypic studies, *atm-1*, *atm-2*, *atm-3*, *atr-1*, *atr-2* and *atr-3*. Crossings were performed between each of the individual *ATM* and *ATR* mutant lines. For this purpose plant material of each pairing was grown under conditions favoring cross fertilization until the emergence of mature spores marked completion of the lifecycle. To screen for successful crossing events the obtained spores were then germinated on media with double antibiotic selection. Since the single mutants carried a resistance marker for only one of the two antibiotics used, only a double mutant should be capable of surviving this selection regime. Out of all of the conducted crosses only one produced doubly resistant plantlets, the one between *atm-1* and *atr-1*. To verify the successful crossing event the line was PCR screened for the presence of the targeted *ATM* and *ATR* locus, utilizing the set of PCR reactions which were previously used in the analysis of single mutants. The PCR results confirmed the presence of altered *ATM* and *ATR* loci (data not shown). Therefore the obtained double mutant line was designated *atmatr-1* and implemented in the following phenotypic analysis of *ATM* and *ATR* mutants.

3.2.3 The function of *P. patens* ATM and ATR in the genotoxic stress response

ATM and ATR are known for their important role in the repair of DNA DSBs, a potentially lethal lesion. To evaluate the relevance of the *P. patens* ATM and ATR homologues in the repair of DNA DSBs, the wild type as well as mutant lines were assayed in regard to their sensitivity towards the DNA DSB inducing agents bleomycin and mitomycin c. The difference between these two agents is that they generate two different types of DNA DSBs. The radiomimetic bleomycin is presumed to directly generate random DNA DSBs independent of the cell cycle stage (Dresp et al. 1978). Mitomycin c on the other hand is known for indirectly generating DNA DSBs, initially inducing DNA interstrand crosslinks (Iyer and Szybalski 1964). If these DNA crosslinks are not resolved prior to the onset of DNA replication, they may trigger a replication fork collapse, resulting in a DSB (Niedernhofer et al. 2004). In addition to the ATM and ATR mutant lines generated in the course of this work a *rad51AB* mutant line was also included into the experimental setup. Rad51 is a presumably essential downstream component of the ATM and ATR controlled pathway for DSB repair and as the *P. patens Rad51AB* double mutant had been found highly sensitive to DSB inducing agents in a previous study (Markmann-Mulisch et al. 2007) it was to serve as a positive control.

3.2.3.1 ATR is much more important than ATM for the repair of DNA DSBs in *P. patens*

To quantitatively assess the sensitivity of our lines towards these two genotoxic agents, a protoplast based assay system was employed. In short, Protoplasts of wild type and mutant lines were isolated from actively growing protonema subcultures. After being treated with increasing concentrations of either bleomycin or mitomycin, protoplasts were regenerated on media without genotoxic agents. After 10 days of regeneration, the percentage of surviving protoplasts, which by that time had grown into small plantlets, was scored in relation to the untreated controls.

Subjecting wild type and mutant lines to treatment with the DNA DSB inducing genotoxic agent bleomycin it was found that *atm-1* displayed a similar sensitivity as did the wild type. The lines *atr-1*, *atmatr-1* and *rad51AB* however each displayed a significant increase in sensitivity (**Figure 3.2-10a)**. Our results implied that ATR as well as Rad51AB have an important function in the repair of bleomycin mediated DNA DSBs in *P. patens* whereas ATM does not.

Testing the susceptibility of the mutant lines to mitomycin C treatment no heightened sensitivity was observed for *atm-1* when compared to the wild type **(Figure 3.2-10b)**. The *rad51AB* mutant displayed an intermediate degree of sensitivity while *atr-1* and *atmatr-1* both displayed a high level of sensitivity.

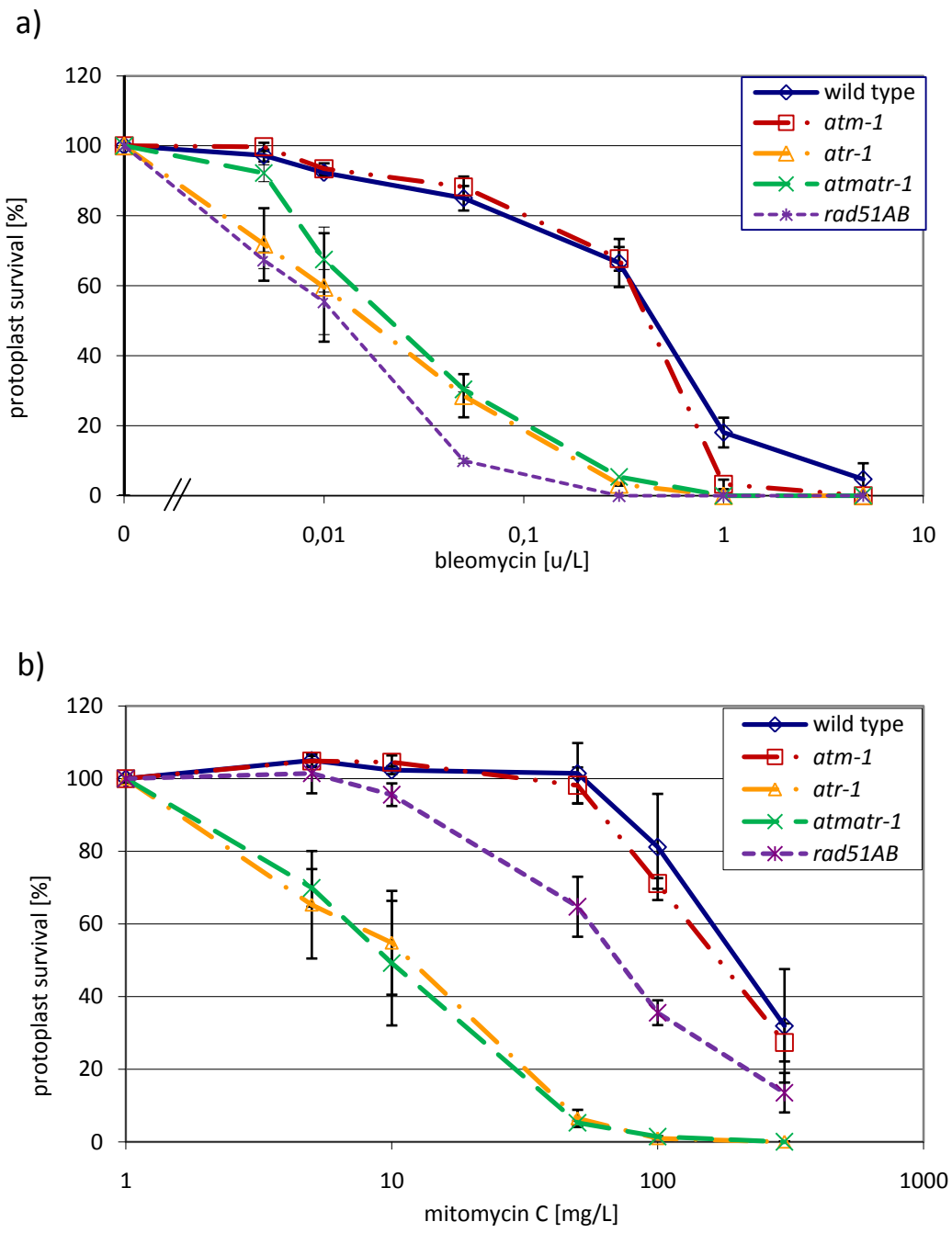

Figure 3.2-10: Sensitivity of wild type and mutant lines to DNA DSB inducing agents bleomycin and mitomycin C. a) Survival curves of *P. patens* wt, *atm, atm/atr* and *rad51AB* protoplasts treated with 0, 0.005, 0.01, 0.05, 0.5, 1 and 5 U/L of bleomycin. Error bars denote standard error (n=2). The observed decrease in protoplast survival of *atr-1*, *atmatr-1* and *rad51AB* as compared to wild type and *atm-1* was found to be significant (p=0.05) according to ANOVA and post hoc Tukey's test for treatment with 0.01 and 0.05 U/L of bleomycin. **b)** Survival curves of *P. patens* wild type, *atm-1, atr-1, atmatr-1* and *rad51AB* protoplasts treated with 0, 5, 10, 50, 100 and 300 mg/L of mitomycin. Error bars denote standard error (n=2). The observed differences in protoplast survival were found to be significant according to ANOVA and post hoc Tukey's test for treatment with 50 mg/L mitomycin C.

To verify the findings of our protoplast based DNA damage assay and to also evaluate the effect of constant genotoxic stress on growth, an additional DNA damage assay was performed. In this assay, small pre-grown plantlets of wild type and mutant lines were selected for similar size and transferred to media containing increasing concentrations of again bleomycin or mitomycin. Plantlets were then grown for two weeks on these plates.

It was found that *atm-1* plantlets displayed a slight hypersensitivity phenotype in response to bleomycin, plantlets being markedly smaller than those of the wild type after two weeks of growth (**Figure 3.2-11a)**. Judging from their size after 2 weeks of growth *atr-1* plantlets were found to be of intermediate sensitivity and *atmatr-1* and *rad51AB* plantlets highly sensitive. At higher concentrations plantlets of the latter three mutant lines appeared to hardly grow at all, instead turning brown quickly and presumably dying. Compared to the previous protoplast based bleomycin sensitivity assay the results obtained in this approach differed in two major aspects. The first was that the *atm-1* line displayed a distinct sensitivity to bleomycin in this assay but not the protoplast one. Thus the *P. patens* ATM protein does have a function in the response to random DSBs after all, albeit it seems to be minor to ATR as its effects appear to only be apparent after long term exposure. Second, contrary to the protoplast assay *atmatr-1* and *rad51AB* lines were shown to be even more sensitive than *atr-1*. The fact that the *atmatr-1* double mutant displayed an increased sensitivity compared to either single mutant furthermore suggested that both *P. patens* ATM and ATR contribute independently to the repair of random bleomycin mediated DNA damage. Finally, the fact that the *atmatr-1* double mutant displayed the same sensitivity as the r*ad51AB* mutant, which is likely completely deficient in HR based DSB repair (Schaefer et al. 2010), suggested that in *P. patens* loss of ATM and ATR both may have a similar effect.

Repeating this type of assay with mitomycin C, *atm-1* was found to display no increased sensitivity compared to the wild type (**Figure 3.2-11b)**. *rad51AB* displayed an intermediate sensitivity while *atr-1* and *atmatr-1* were found to be highly sensitivity. Thus contrary to bleomycin the mitomycin protoplast and plantlet based assays gave the same results regarding the sensitivity of mutant lines.

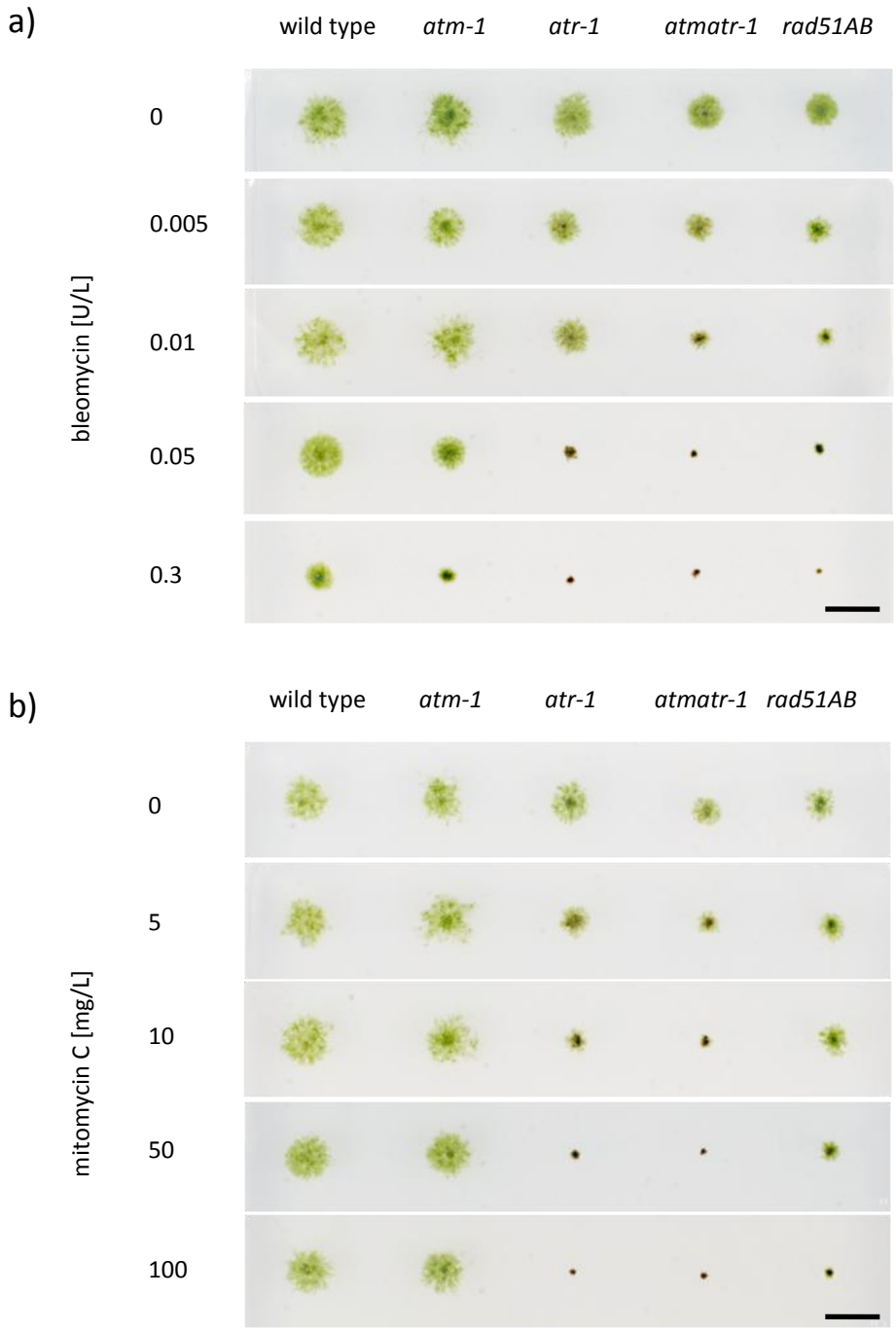

Figure 3.2-11: Sensitivity of wild type and mutant lines to DNA DSB inducing agents bleomycin and mitomycin C. a) Plantlets of wild type, *atm-1*, *atr-1*, *atmatr-1* and *rad51AB* were grown for two weeks on standard PPNH4 media supplemented with 0, 0.005, 0.01, 0.05 or 0.3 U/L bleomycin. Pictures were taken on day 14. **b)** Plantlets of wild type, *atm-1*, *atr-1*, *atmatr-1* and *rad51AB* were grown for two weeks on standard PPNH4 media supplemented with 0, 5, 10, 50 or 100 mg/L mitomycin. Pictures were taken on day 14. Scale bar = 1 cm.

Observing the effect of bleomycin and mitomycin on the level of individual filaments and cells two additional interesting observations were made. First, with increasing bleomycin or mitomycin concentrations the growth of plantlets of all the lines became more compact, less and less caulonema filaments protruding outwards from the central part **(Figure 3.2-12a)**. This suggested that caulonema type filaments may be more susceptible to this type of genotoxic stress than chloronema. Considering one of the major differences between caulonema and chloronema cells is that the former proliferate at a much faster rate there may be a connection between that trait of caulonema and the apparent increase in sensitivity. Second, concerning individual cells, the plantlets of wild type and *atm-1*, despite being markedly growth impaired at high bleomycin concentrations, hardly displayed any visibly dead cells as a result of prolonged growth in presence of bleomycin **(Figure 3.2-12b)**. Contrary to that, *atr-1* and *atmatr-1* plantlets displayed plenty of aberrant and dead looking cells soon after being transferred to media containing bleomycin. Interestingly, the *rad51AB* mutant, although similarly impaired in growth as *atr-1* and *atmatr-1* at high bleomycin concentrations, did also not display a marked increase in dead cells. As the cell death phenotype was prominent in only lines which lacked ATR this finding suggests *P. patens* ATR might have a vital function in preventing/delaying death of cells exposed to genotoxic stress.

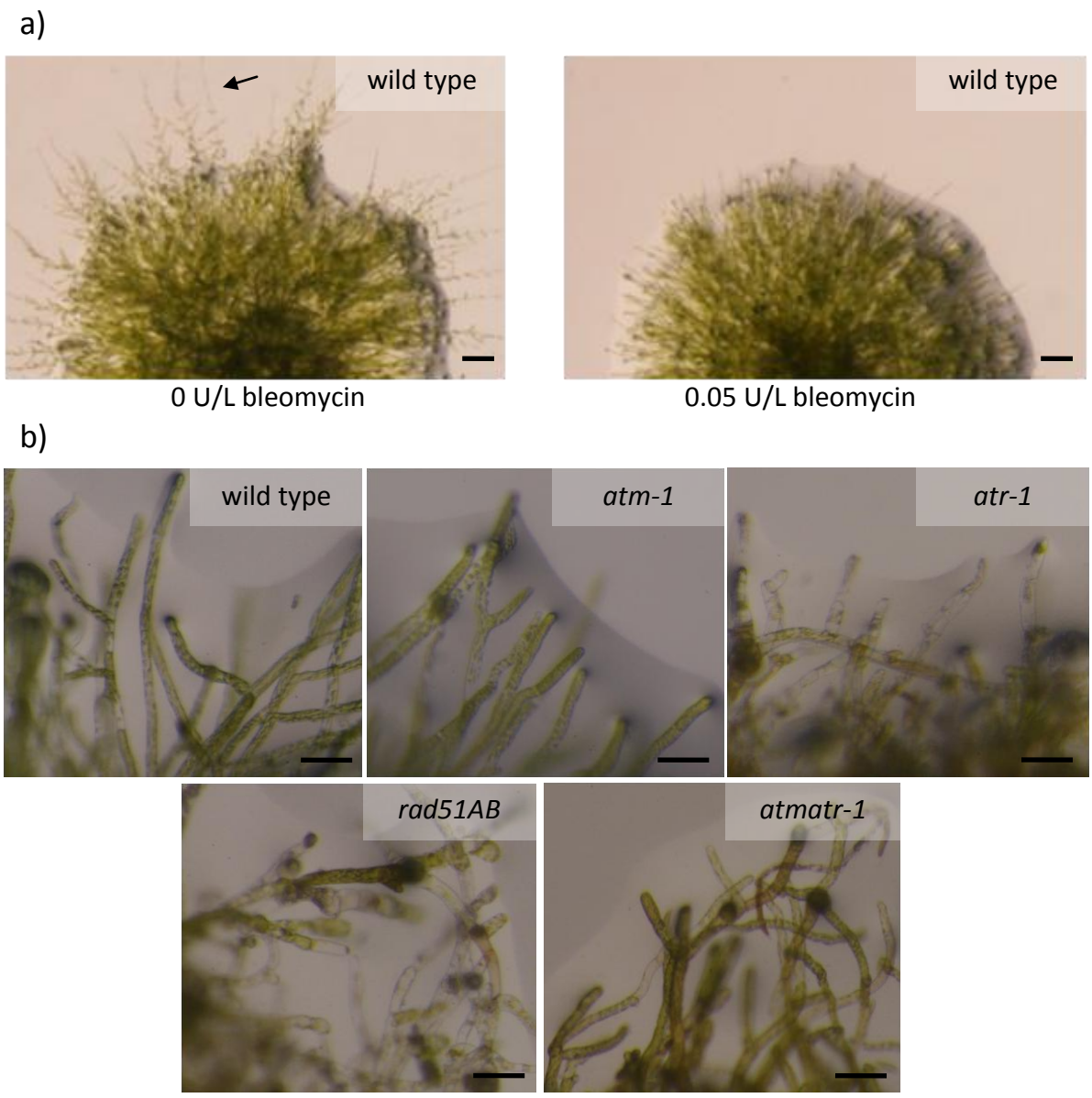

Figure 3.2-12: Effect of genotoxic stress on filamentous growth and individual cell survival. (a) Morphology of wild type plantlets grown for 5 days on media with or without 0.05 U/L of bleomycin. Long protruding caulonema type filaments, indicated by arrow, as seen on media without bleomycin, are absent in plantlets grown in the presence of 0.05 U/L bleomycin. Scale bars indicate 200 µm. **(b)** Close up of individual filaments of wild type and mutant lines five days after transfer to standard PPNH4 media supplemented with 1 U/L of bleomycin. Wild type and *atm-1* display no marked increase in dead cells *atr-1* and *atmatr-1* display a steep increase in seemingly dead or dying cells while *rad51AB* display a minor effect if at all. Scale bars indicate 100 µm.

3.2.3.2 *P. patens* ATR is likely involved in the repair of DNA damage other than DSBs

In the previous DNA damage assays, in which mitomycin was used to induce DNA DSBs, the rad*51AB* mutant was found to be less sensitive than *atr-1* and *atmatr-1*. This was unexpected as RAD51 is presumably an essential downstream component of ATR in homologous recombination mediated DSB repair (Shinohara and Ogawa 1995, Sørensen et al. 2005, Schaefer et al. 2010). Therefore, the fact that *rad51AB* displayed a reduced sensitivity to mitomycin as compared to *atr-1* and *atmatr-1* suggested that ATR may have an additional function besides in HR mediated repair of DSBs which is important in the resistance to the genotoxic agent mitomycin. At this point it is important to note that DSBs generated via mitomycin are a secondary effect of unresolved interstrand crosslinks. It was therefore a distinct possibility that ATR may also play a role in nucleotide excision repair (NER), the pathway responsible for resolving interstrand crosslinks. To test whether this possible increase in function of *P. patens* ATR was restricted to NER an additional DNA damage assay, using the genotoxic substance MNU, was performed. MNU is an alkylating agent which is known to methylate DNA bases (Beranek 1990), a lesion, which if left unrepaired, will also induce DSBs during replication (Groth et al. 2010). Instead of the crosslinks which are repaired via NER, alkylated bases are repaired by the base excision repair pathway (BER) (Krokan and Bjørås 2013). Testing the sensitivity of mutant lines to MNU was thus intended to reveal whether the hypothesized function of ATR in other DNA repair pathways than HR would be restricted to only NER or if there was more to it.

Performing the previously described plantlet growth assay with increasing concentrations of MNU, the pattern of sensitivity was similar to that obtained with mitomycin **(Figure 3.2-13)**. Wild type and *atm-1* were found least sensitive, r*ad51AB* displayed an intermediate sensitivity and *atr-1* and *atmatr-1* displayed the highest sensitivity. This result suggested that ATR in addition to NER may also be involved in BER. As an alternative to the suggested role of ATR in these individual repair pathways the results could also be interpreted in the sense that ATR may provide a more general function of which repair processes as a whole may benefit or rely on.

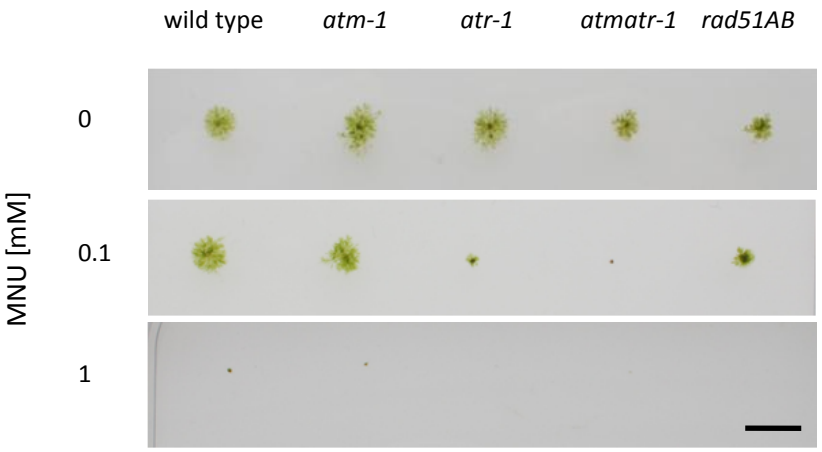

Figure 3.2-13: Sensitivity of wild type and mutant lines to DNA DSB inducing agent MNU. Plantlets of wild type, *atm-1*, *atr-1*, *atmatr-1* and *rad51AB* were grown for two weeks on standard PPNH4 media supplemented with 0, 0.1 mM and 1 mM of MNU. Pictures were taken on day 14. Scale bar indicates 1 cm.

3.2.3.3 *P. patens* ATR plays a major role in DNA damage checkpoint control

The previous DNA damage assays provided information on whether or not ATM and ATR are required in the repair of DNA DSB lesions in *P. patens*. They did not provide any insight into whether or not specific functions of ATM and ATR in the DNA damage response, as known from other organisms, might be conserved in the *P. patens* homologs. While both proteins are known for their capacity to activate a large variety of downstream effectors in the DNA damage response (Cimprich and Cortez 2008, Shiloh and Ziv 2013), one of their key responsibilities is to arrest the cell cycle in the event of DNA double strand breaks (Wright et al. 1998, Liu et al. 2000, Abraham 2001). This function is of vital importance as progression of cells into mitosis with unrepaired DNA DSBs will lead to loss of genomic information and very likely cell death.

To test whether *P. patens* ATM and ATR homologs are also involved in initiating cell cycle arrest in response to DNA DSBs an assay was devised. In short, actively growing protonema filaments were first subjected to bleomycin treatment to induce DNA DSBs and thus activation of cell cycle checkpoints. Subsequently these filaments were monitored for progression through S-phase by growing them for a limited time in the presence of EDU, a chemically modified base analog. All cells replicating their DNA during that growth period would incorporate EDU into their genome which could then be detected by chemically coupling the EDU with an alexa488 fluorophore. After counterstaining the samples with dapi the percentage of alexa488 positive nuclei in individual filaments was manually assessed using a fluorescence microscope and used as a measure for proliferation.

First, comparing the mock treated samples from wild type, *atm-1*, *atr-1* and *atmatr-1* no difference in the abundance/percentage of alexa488 positive nuclei was observed **(Figure 3.2-14, Figure 3.2-15)**. This suggested that there is no difference in basic proliferation speed between any of the lines. Comparing the percentage of alexa488 positive nuclei between mock and bleomycin treated wild type samples it was found that the latter displayed hardly any alexa488 positive nuclei. This implied that the cell cycle was efficiently arrested in the wild type upon exposure to the genotoxic agent bleomycin. While *atm-1* appeared to display a slight increase in the number of alexa488 signals it was not significant implying that also in this line the DNA damage checkpoints are functional still. *atr-1* and *atmatr-1* on the other hand displayed a similar percentage of alexa488 positive nuclei in mock as well as bleomycin treated samples. This suggested that the DNA damage checkpoints are completely deficient in these two lines. At the same time it also suggested that in *P. patens* ATR but not ATM plays the major role in the control of DNA damage checkpoints.

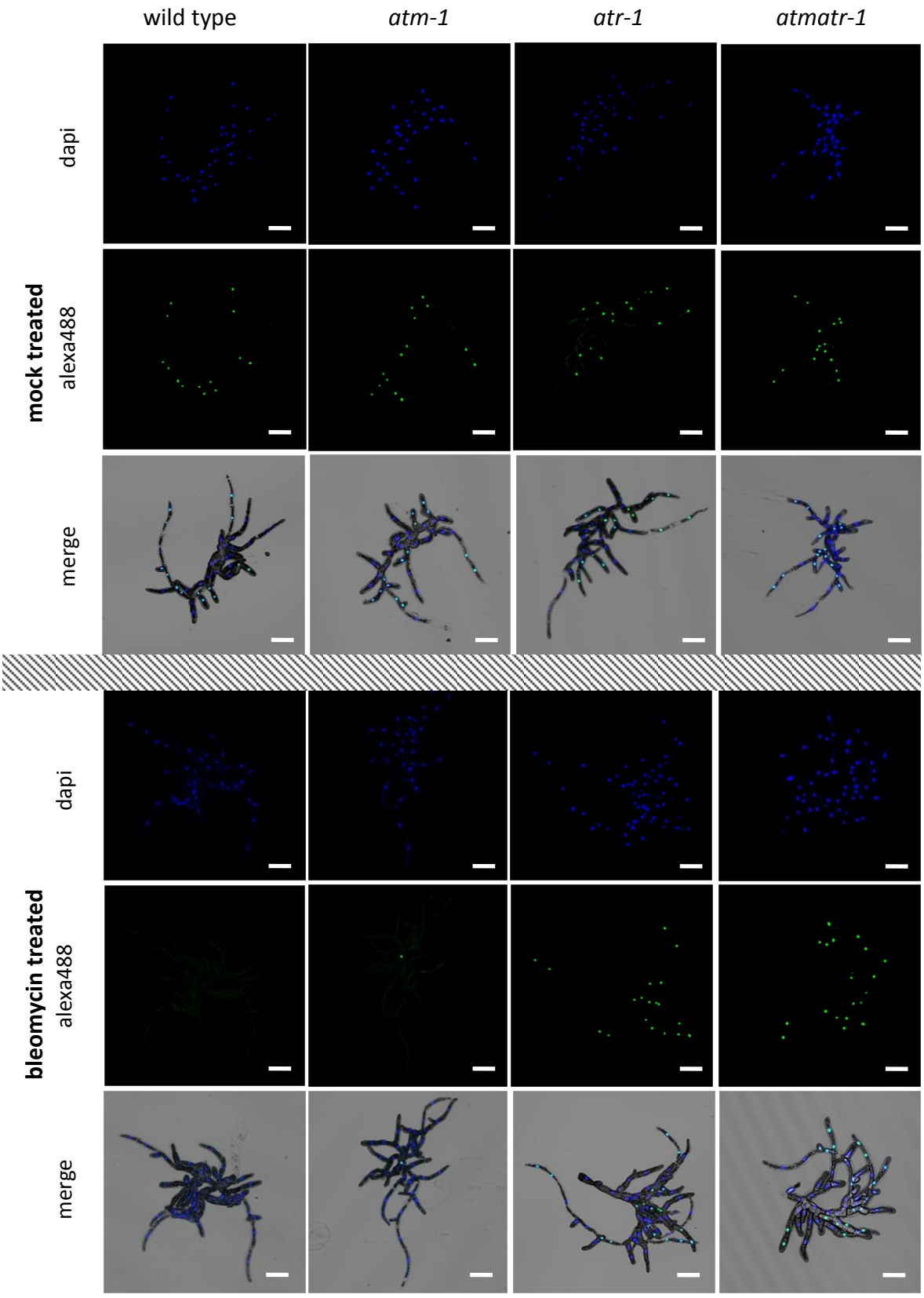

Figure 3.2-14: Proliferation appears not to be arrested in response to treatment with the genotoxic agent bleomycin in *atr-1* and *atmatr-1*. Bleomycin and mock treated protonema of wild type, *atm-1*, *atr-1* and *atmatr-1* were grown for 6 hours in liquid PPNH4 media supplemented with 20µM of the base analog EDU. Proliferating cells were visualized by coupling of genome incorporated EDU with an alexa488 fluorophore. Scale bar = 100µm

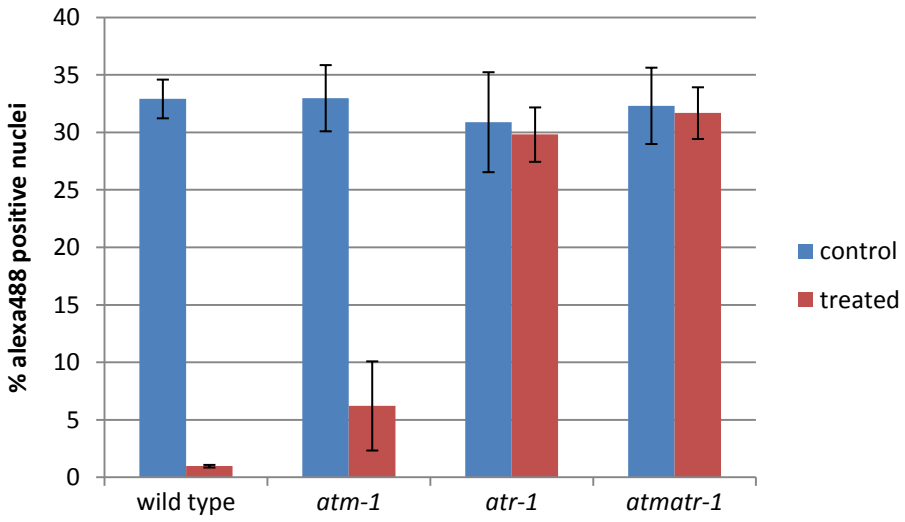

Figure 3.2-15: Proliferation is not arrested in response to treatment with the genotoxic agent bleomycin in *atr-1* and *atmatr-1*. Quantitative evaluation of proliferation in bleomycin and mock treated plantlets of wild type and mutant lines subjected to the fluorescence based proliferation assay. Ratio of alexa488 positive nuclei, indicating DNA replication, was determined for 20 individual plantlets per line and treatment. Error bars denote standard deviation in three biological replicates. Testing for difference of means between mock treated samples of wild type, *atm-1*, *atr-1* and *atmatr-1* via one-way ANOVA, no significant differences were found ($F(3,8) = 0.27$, $P = 0.84$). Testing for difference of means between treated samples of wild type, *atm-1*, *atr-1* and *atmatr-1* via one-way ANOVA significant differences were found ($F(3,8) = 117.19$, $P = 5.98E-07$). Tukey's post hoc test confirmed that differences in the percentage of alexa488 positive nuclei were significant between wild type and *atr-1*, wild type and *atmatr-1*, *atm-1* and *atr-1* as well as *atm-1* and *atmatr-1* ($P<0.05$).

To verify the previous results obtained by manually counting alexa488 positive nuclei in an intermediate number of filaments, a large scale assay was performed. While the experimental setup remained largely similar to before, the evaluation differed in that a large number of nuclei from mock and bleomycin treated protonema was extracted and quantified via flow cytometric analysis. The flow cytometric analysis was conducted by our collaborator Jörg Fuchs (IPK Gatersleben). As can be observed, the trends found in the previous experimental approach were confirmed with this setup also (**Figure 3.2-16**). The only notable difference was that the percentage of alexa488 positive nuclei in bleomycin treated samples of wild type and *atm-1* was notably increased compared to the previous approach. As this phenomenon was not observed during the previous experiments it may have resulted from the small alterations to the protocol necessary for the later flow cytometric evaluation.

Nevertheless this set of experiments showcased that in *P. patens* ATR and not ATM is the major component required to arrest the cell cyle in response to bleomycin induced DNA damage.

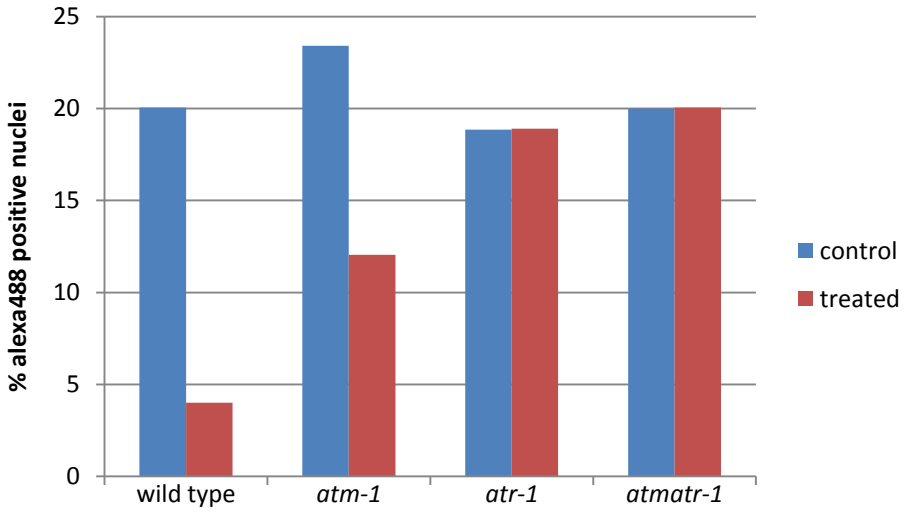

Figure 3.2-16: Proliferation is not slowed down in response to treatment with the genotoxic agent bleomycin in *atr-1* and *atmatr-1*. Quantitative evaluation of proliferation in bleomycin and mock treated plantlets of wild type and mutant lines subjected to fluorescence based proliferation assay. Ratio of alexa488 positive nuclei, indicating DNA replication, was determined via flow cytometric measurement of a large number of nuclei.

3.2.4 Assessing the function of *P. patens* ATM and ATR in recombination

ATM and ATR are known to be key regulative components of the HR based response to DNA DSBs (Shiotani and Zou 2009). Therefore DNA damage assays such as those discussed in the previous chapter are often used to assess their importance in the HR mediated repair of these lesions. However one has to consider that the observed sensitivity phenotypes are likely not only related to the role ATM and ATR play in facilitating the HR process itself. Instead the sensitivity phenotypes observed in these assays could also be related to their role in DNA damage checkpoints or even other DNA repair pathways. Bleomycin induced DNA DSBs for example could, alternatively to HR, also be repaired via NHEJ.

To gain insight into whether ATM and ATR actually affect the efficiency of the homologous recombination process *P. patens* offers an interesting opportunity. This is because gene targeting, the precise modification of genomic loci via a homologous recombination based process, is highly efficient in *P. patens*. Using gene targeting as an assay system in ATM and ATR mutants of *P. patens* would therefore allow one to directly observe whether these two proteins are required for efficient HR or not.

3.2.4.1 Transformation efficiency is increased in *atm-1*

Wild type and mutant lines were transformed with the pKoCol2 targeting construct in a total of seven experiments, obtaining altogether 193, 1078, 434 and 264 stable transformants for the wild type, *atm-1*, *atr-1* and *atmatr-1* respectively. The reason why so many replicates were necessary becomes evident when observing the absolute numbers of stable transformants obtained in the initial three experiments (**Table 3.2-6**). First, the numbers of stable transformants obtained especially in *atmatr-1* were very low. This would have been a problem in the later PCR based analysis as the results would be based on only a small sample size. Second, there are considerable variations in the relative numbers of transformants obtained. This is true when comparing the individual lines amongst themselves and with each other.

Since the number of stable transformants obtained depended on protoplast regeneration efficiency, a factor which may vary in each experiment, the data were normalized to compensate for this effect. However, as could be observed, calculating the number of stable transformants per 10000 regenerated protoplasts did not really alleviate the large variation (**Table 3.2-7**).

	I	II	III	IV	V	VI	VII
wild type	21	27	13	10	25	31	66
atm-1	62	210	117	80	211	146	252
atr-1	17	50	40	55	102	105	65
atmatr-1	9	15	12	37	61	65	65

Table 3.2-6: Absolute number of stable transformants obtained per line in the individual gene targeting experiments.

	I	II	III	IV	V	VI	VII
wild type	6,95	10,67	5,14	1,61	20,82	22,06	15,26
atm-1	19,03	65,26	36,36	19,41	87,77	67,66	82,08
atr-1	6,08	15,63	12,51	8,66	42,38	41,94	11,15
atmatr-1	2,78	5,09	4,07	6,65	10,48	13,45	18,06

Table 3.2-7: Number of stable transformants obtained in the individual gene targeting experiments normalized per 10000 regenerating protoplasts.

Therefore it appeared that additional unknown parameters affected the transformation efficiencies in each individual experiment. These might have been related to differing qualities of either DNA or protoplasts between individual transformation experiments, not noticeable during routine observation of both these parameters. Therefore, to obtain reliable data, we tried to counterbalance the high degree of variation observed in this type of experiment by an increased number of replicates.

Calculating the average number of transformants obtained per line across the seven targeting experiments **(Figure 3.2-17)**, only *atm-1* displayed a significant increase in the number of stable transformants.

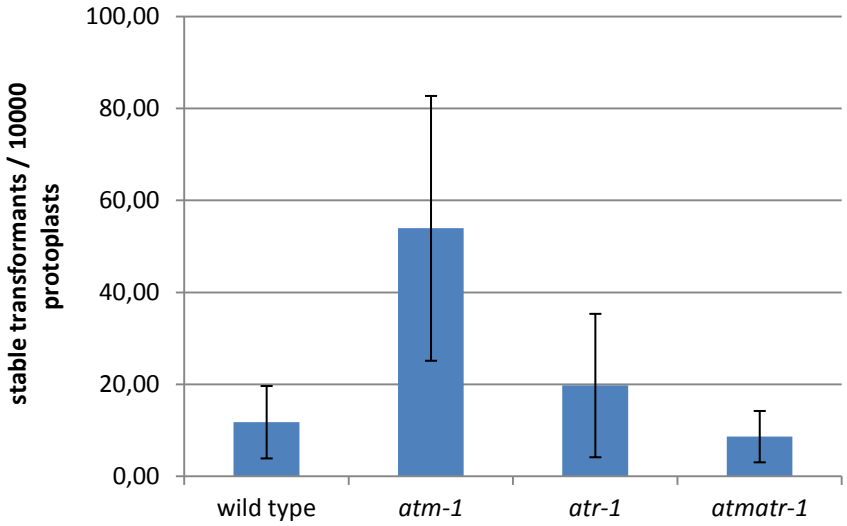

Figure 3.2-17: The absolute number of stable transformants obtained in gene targeting experiments is increased in *atm-1*.Testing for significant differences in the mean number of transformants obtained per 10000 protoplasts in wild type, *atm-1*, *atr-1* and *atmatr-1* via one-way ANOVA was successful ($F(3,24) = 10.4$, $P = 0.00014$). Tukey's post hoc test confirmed that the increase in the number of stable transformants obtained in *atm-1* was significant compared to the wild type ($P<0.05$).

3.2.4.2 Random integrations are increased in *atm-1* and targeted integrations decreased in *atmatr-1*

After evaluating the overall transformation efficiency of mutant lines, the next step was to determine whether there would also be any effects on the mode of targeting construct integration. Since ATM and ATR are components of the homologous recombination pathway, which is required for targeted integration of transgenes, a shift towards random integrations was expected. To identify the mode of integration in the stable transformants they were analyzed via PCR. While transformations I and II out of the total of 7 transformations were fully analysed via PCR, only an aliquot of at least 13 transformants was analysed for the remaining 5 experiments, if available. An exception was made for *atmatr-1* due to the fact that transformant numbers obtained in the first two experiments were comparably low. To increase the total number of PCR analyzed transformants all of the *atmatr-1* transformants obtained in experiment VII were analyzed. At first the PCR results were divided into two categories. Transformants whose PCR results indicated that the targeting vector had not integrated or interacted with the targeted locus were categorized as random. Any transformants whose PCR data indicated integration or interaction of the targeting construct at the target locus were summarized as targeted (**Table 3.2-8**). In order to compare the absolute numbers of random or

targeted integrations between the lines it was necessary to extrapolate the PCR results for the partially analyzed experiments. This was deemed to be a valid procedure as the ratios of targeted to random integrations was similar in the fully and partially analyzed experiments (**5.3**).

Table 3.2-8: PCR analysis of stable transformants obtained in the 7 individual gene targeting experiments. PCR assayed transformants were categorized as targeted if either the 5' or 3' PCR produced the band expected from a successful targeting event at that respective locus. If the PCR only produced wild type bands, the transformant was classified as random. While only in Experiment I and II all of the transformants were PCR analyzed, aliquots of at least 13 transformants, if available, were analyzed in the remainder of experiments. Transformants whose PCR results were inconclusive and suggested PCR failure were excluded from the dataset.

	wild type		***atm-1***		***atr-1***		***atmatr-1***	
	targeted	**random**	**targeted**	**random**	**targeted**	**random**	**targeted**	**random**
I	14	5	2	38	6	8	0	6
II	23	1	36	145	35	13	1	13
III	9	1	4	12	25	11	0	12
IV	7	2	1	12	10	2	2	11
V	10	0	0	12	7	3	1	11
VI	10	1	1	12	5	6	0	13
VII	9	0	1	12	5	7	4	57

Calculating the average number of targeted and random transformants across the seven experiments, *atm-1* was found to display a significant increase in the number of random integration (**Figure 3.2-18**). While a similar trend was observed in *atr-1* and *atmatr-1* the differences were found to be insignificant. Regarding the number of targeted integrations only *atmatr-1* displayed a significant decrease, with *atm-1* and *atr-1* being similar to the wild type. These results also implied that the increased transformation efficieny reported for *atm-1* earlier was related to only or at least largely an increase in random integrations.

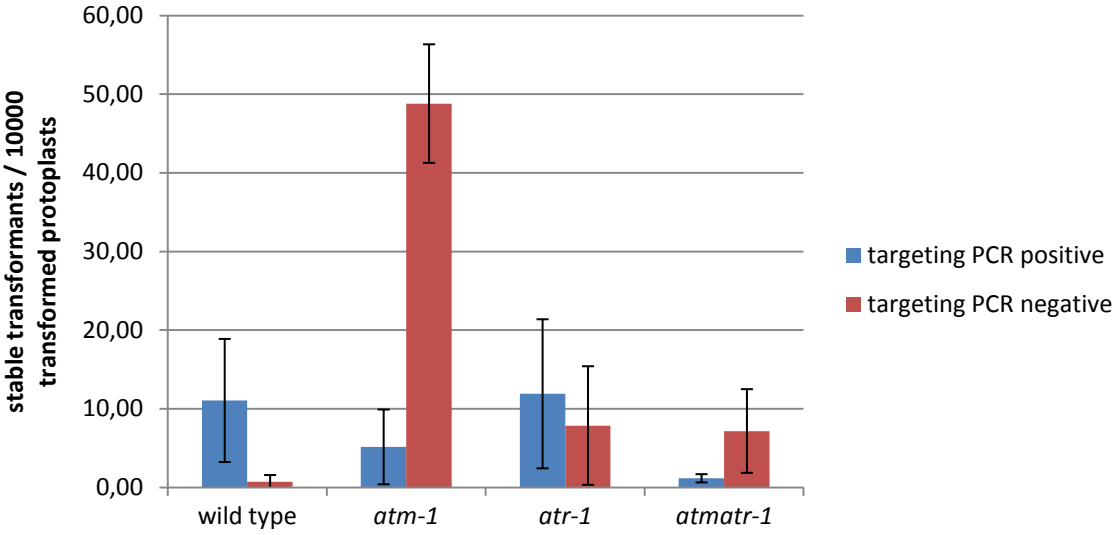

Figure 3.2-18: Random integrations are increased in *atm-1* and targeted integrations are decreased in *atmatr-1*. Protoplasts of wild type, *atm-1*, *atr-1* and *atmatr-1* were transformed with the pKoCol2 targeting construct and analyzed for successful targeting events via PCR. Numbers of targeted and random transformants were averaged across all 7 experiments and normalized per 10000 regenerated protoplasts. Error bars denote the standard deviation in the 7 replicate experiments. Testing for significant differences in the mean number of targeting PCR positive transformants via one-way ANOVA was successful ($F(3,24) = 4.62$, $P = 0.01$). Tukey's post hoc test revealed that the decrease of targeting PCR positive transformants observed in *atmatr-1* compared to the wild type was significant ($P<0.05$). Conducting one-way ANOVA on the means of targeting PCR negative transformants significant differences were detected ($F(3,24) = 15.1$, $P = 9.85E-06$) Post hoc Tukey's test showed increase in targeting PCR negative transformants in *atm-1* to be significant compared to the wild type ($P<0.05$).

3.2.5 Analysis of the transcriptional DNA damage response in wild type and mutant lines

One of the functions of ATM and ATR is to serve as transducers in the DNA damage response. Studies in yeast, Arabidopsis as well as mammalian cell lines have shown that these genes are involved in orchestrating the transcriptional response to genotoxic stress (Culligan et al. 2006, Matsuoka et al. 2007, Jaehnig et al. 2013). The transcriptome changes reported in these studies were assessed utilizing microarray based experimental setups. While sufficient for many applications, microarrays provide analog datasets only and low abundant transcripts are difficult to examine at all (Matsumura et al. 2008). The latter may be especially problematic regarding the analysis of the DNA damage response as transcripts of DNA repair factors are presumed to be low abundant (Bachant and Elledge 1999, Watson et al. 2004).

More recently the sequencing based SuperSAGE technique has become a feasible alternative to microarrays in expression profiling (Matsumura et al. 2008). This technique is based on sequencing a large number of defined fragments of individual mRNAs (tags), the obtained sequence being subsequently used to identify the related gene via BLAST search. This method provides a digital dataset and has also been suggested suitable in detecting low abundant transcripts (Matsumura et al. 2008). Therefore, utilizing SuperSAGE as a means to study the transcriptional response of *P. patens* wild type, *atm-1*, *atr-1* and *atmatr-1* mutant lines had the added benefit of possibly obtaining a more complete picture of the transcriptional DNA damage response compared to previous studies.

3.2.5.1 Bleomycin treatment elicits a diverse transcriptional response

5 day old actively growing protonema subcultures from wild type and mutant lines were utilized for the experiment. To initiate the transcriptional DNA damage response, plant material was incubated with 0.3 U/L of the DNA DSB inducing agent bleomycin. After a period of 3 more hours, without the genotoxic agent, RNA was extracted and sent for SuperSAGE analysis to our collaborators at GenXPro (Frankfurt am Main, germany). The timepoint for RNA extraction was chosen due to previous RT-PCR based experiments having suggested the expression of RAD51A being greatest at 3 hours post DNA damage induction. Considering RAD51A is presumed to be an essential component of the homologous recombination repair process in *P. patens* (Schaefer et al. 2010), this timepoint was deemed most suitable for detecting other components involved in the repair process.

The total number of transcripts identified in the SuperSAGE analysis was 15561. To improve the quality of the subsequent analysis out of the total of 15561 transcripts only those were considered which totaled 10 tags in untreated and bleomycin treated wild type samples combined. This reduced the number of transcripts available for evaluation to 14332. To obtain an overview of the transcriptional damage response to bleomycin mediated genotoxic stress the share of transcripts

induced/repressed at least 2 fold was assessed in wild type, *atm-1*, *atr-1* and *atmatr-1*. The top 50 induced/repressed genes, according to fold change, in the wild type in response to bleomycin treatment can be found in the Appendix (**5.4**). Comparing the effect of bleomycin treatment on wild type and mutant lines it is obvious that mutant lines deviate in variying degrees regarding the total number of transcripts induced/repressed in response to bleomycin treatment (**Figure 3.2-19**). While annotation for most of the transcripts is severely lacking, assessing the abundance of GO-terms in differentially expressed transcripts provides an overview of the scope of the transcriptional response to bleomycin treatment (**Table 3.2-9**). It is interesting to note that albeit the experiment was intended to induce the transcriptional DNA damage response, the GO-term DNA repair is strongly underrepresented. Instead the majority of differentially regulated transcripts according to GO annotation are involved in more general cellular functions such as metabolism or development.

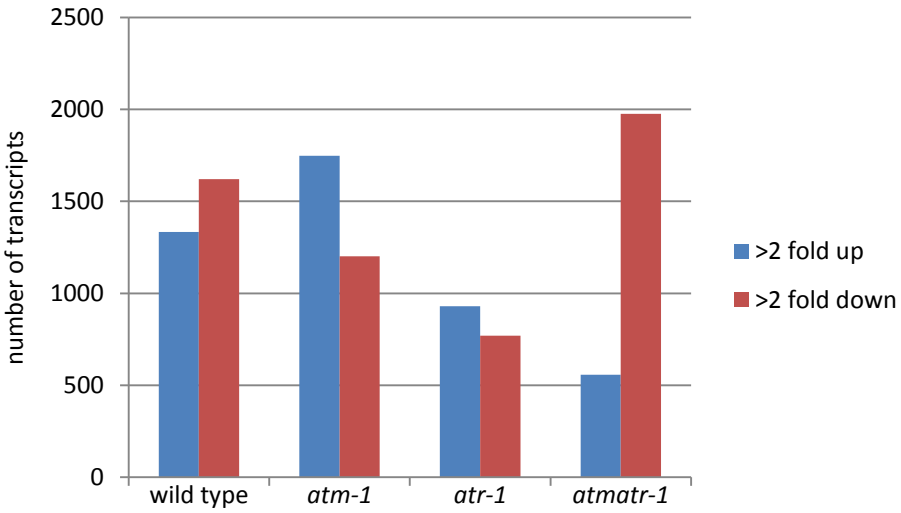

Figure 3.2-19: Tanscriptional response to the genotoxic agent bleomycin in wild type and mutant lines. Shown is the absolute number of transcripts induced/repressed at least 2-fold after treatment with 0.3 U of bleomycin.

Table 3.2-9: Incidence of GO terms amongst transcripts induced or repressed equal to or more than 2 fold in wild type and mutant lines.

	wild type		*atm-1*		*atr-1*		*atmatr-1*	
GO-term	>= 2	<= 2	>= 2	<= 2	>= 2	<= 2	>= 2	<= 2
developmental process	52	61	61	61	31	31	18	89
DNA repair	5	5	7	5	4	3	1	8
growth	12	11	12	11	4	2	4	27
metabolic process	349	314	429	312	180	177	145	482
regulation of biological process	91	95	132	94	50	56	27	158
regulation of cell cycle	0	2	3	2	0	1	0	2
response to stress	79	72	90	72	51	39	31	146

The previous evaluation had showcased that the transcriptional DNA damage response in mutant lines is affected in terms of the numbers of induced/repressed transcripts. However another important piece of information would be whether those genes which are differentially regulated upon bleomycin induced genotoxic stress in the mutants are actually the same as those in the wild type. To address this question it was determined how many of the specific transcripts induced/repressed in the wild type were similarly regulated in the mutant lines As is depicted in **Figure 3.2-20** the majority of the transcripts induced/repressed in response to bleomycin in the mutant lines are actually different from those in the wild type. It is also evident that loss of ATR function has a greater negative effect on maintaining a similar DNA damage response as observed in the wild type.

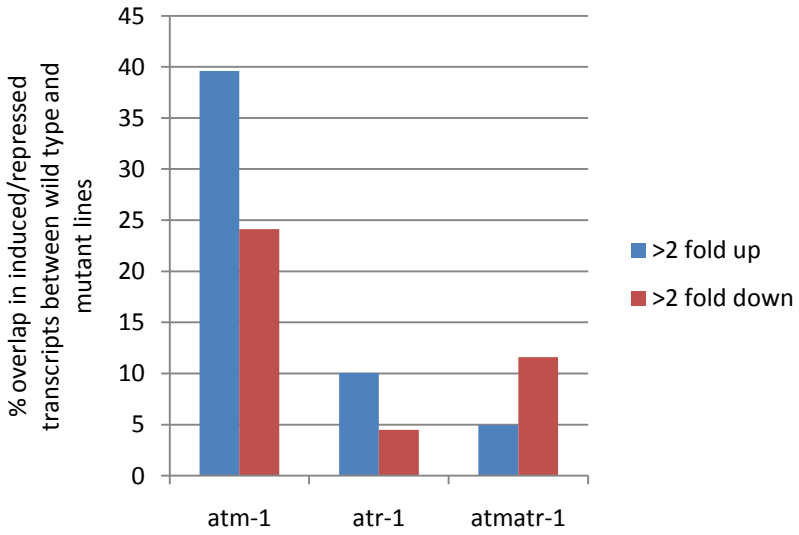

Figure 3.2-20: The identity of genes induced/repressed in response to bleomycin in mutant lines differs to a great extent from those induced/repressed in the wild type. In order to determine the similarity in the transcriptional response to bleomycin between wild type and mutant lines it was assessed how many of the genes induced/repressed in *atm-1*, *atr-1* and *atmatr-1* in response to bleomycin are the same as in the wild type

Basically there are two possible explanations for the apparent lack of induction/repression in the transcriptional DNA damage response of the mutant lines. The most obvious being that the respective genes are simply not induced/repressed upon the genotoxic stimulus due to lack of ATM/ATR functionality. However, another possible explanation would be that the respective genes are constitutively induced/repressed in the mutant lines. To check for the latter phenomenon it was assessed whether in the untreated mutant lines the transcripts differentially regulated in the wild type upon bleomycin treatment were already increased/decreased in abundance. As is evident from **Figure 3.2-21** a share of transcripts induced upon genotoxic stress treatment in the wild type is indeed constitutively induced/repressed in the mutant lines. This effect is markedly more

pronounced in lines lacking functional ATR. Thus the DNA damage response of *atm-1*, *atr-1* and *atmatr-1* lines is affected in the total number of differentially regulated transcripts as well as their identity.

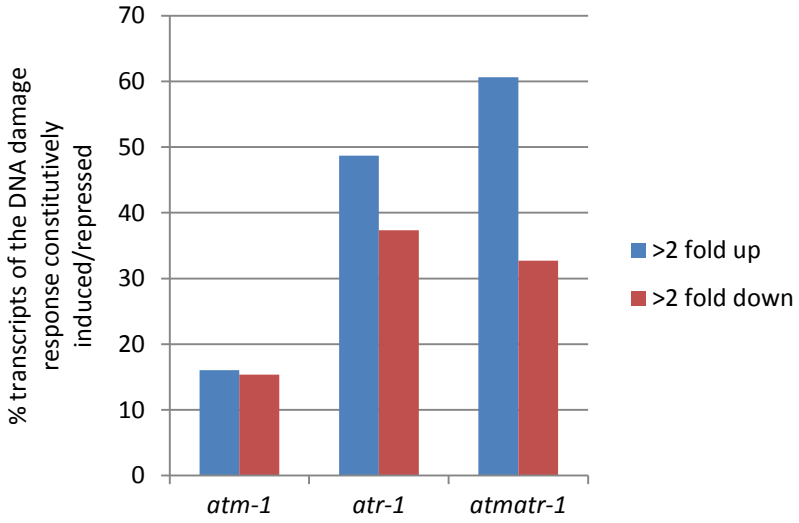

Figure 3.2-21: A percentage of genes differentially regulated upon bleomycin treatment in the wild type are constitutively induced/repressed in mutant lines. To assess whether any of the 1333 and 1621 genes, found to be induced and repressed respectively in response to bleomycin treatment in the wild type, might be constitutively induced/repressed in mutant lines, their transcript count in untreated *atm-1*, *atr-1* and *atmatr-1* was related to the untreated wild type.

3.2.5.2 Transcriptional response of the subset of DNA repair/recombination genes

As mentioned in the previous paragraph, the annotation of the majority of genes found to be transcriptionally induced/repressed in the SuperSAGE analysis was lacking. Especially the fact that only so few transcripts carrying the GO-term DNA repair were detected was inconvenient as it limited the scope of the evaluation. However as was presented in a previous chapter one of the components of this work was to assess the inventory of DNA recombination/repair genes available in *P. patens*. Using this list the SuperSAGE data were evaluated once more, this time only in respect to the subset of genes confirmed to be involved in DNA repair/recombination.

From the total set of 183 distinct genes compiled in the list, including paralogs, 171 genes were represented in the SuperSAGE data. Filtering out any transcripts for which there were less than 10 tags detected in the untreated and treated wild type library combined, 119 remained. **Table 3.2-10**

displays fold changes between treated and untreated as well as mutant and wild type lines. In the wild type 26 genes were found to be induced and 17 repressed in response to bleomycin treatment. Compared to the previous GO-term based analysis which only found 5 DNA repair annotated genes being induced and repressed this represented a substantial improvement. Looking at the identity of individual genes induced upon bleomycin treatment and the repair pathways associated with them some interesting observations were made. First off, bleomycin is a genotoxic agent which is generally used to induce DNA DSBs. DNA DSBs are known to require either the HR or NHEJ pathways for repair. In accordance with that the genes induced upon bleomycin treatment included central HR factors such as Rad51, Rad54 and WRIP as well as key NHEJ components KU70, Ku80 and XRCC4. However even genes generally unrelated to DSB repair such as components of base excision repair (PARP2), nucleotide excision repair (ERCC2, ERCC6), crosslink repair (FACD2), UV repair (FEN1, DDB2) and mismatch repair (MLH2) were found to be induced. Even SPO11, known for its central role in meiotic recombination but generally not thought involved in somatic recombination, was found induced. In addition, the fact that components of HR such as TOP3B or MUS81 were actually repressed upon bleomycin treatment was surprising as well. It is also worth noting that out of the 29 HR components in the compiled list of DNA repair genes only 6 were found to be transcriptionally induced in the SuperSAGE data. This suggested that either the bleomycin induced DNA damage response may to some degree occur on a post translational level instead.

Table 3.2-10: bleomycin induced/repressed DNA repair/recombination genes in wild type as well as mutant lines. Numbers represent fold change between either bleomycin untreated (0) and treated (0.3) samples of individual lines of untreated *atm-1*, *atr-1* and *atmatr-1* in respect to untreated wild type (wt). A light green backdrop indicates a 2-fold or higher increase in transcript level whereas a light red backdrop indicates a 2-fold or higher decrease.

Gene locus	Gene name	wt (0.3) vs wt (0)	*atm-1* (0.3) vs *atm-1* (0)	*atr-1* (0.3) vs *atr-1* (0)	*atmatr-1* (0.3) *vs* *atmatr-1* (0)	*atm-1* (0) vs wt (0)	*atr-1* (0) vs wt (0)	*atmatr-1* (0) vs wt (0)
checkpoint								
Pp1s135_65V6.1	ATM	1.46	-1.22	-1.32	-6.33	-3.43	1.24	-1.77
Pp1s60_281V6.1	ATRIP	-1.20	-1.77	1.01	-1.22	-1.05	-1.21	1.04
Pp1s34_370V6.1	ATRX	1.38	2.25	1.10	1.01	1.33	1.23	2.17
Pp1s59_63V6.1	HUS1	2.60	5.14	-1.47	-1.16	-1.35	1.77	2.04
Pp1s207_63V6.1	MAPK1	-∞	+∞	-6.62	-∞	-∞	3.49	1.10
Pp1s138_117V6.1	MAPK1	1.52	-1.46	1.13	1.34	-1.12	1.03	1.19
Pp1s207_100V6.1	PMYT1	-1.62	-1.20	1.08	-1.33	1.04	-1.04	1.20
Pp1s184_70V6.1	RAD17	2.18	1.07	1.01	1.09	1.37	1.72	1.56
Pp1s130_202V6.1	RAD9A	20.98	27.56	-1.10	-2.04	-2.65	2.63	4.73
Pp1s251_11V6.1	SOG1	-1.63	1.10	1.33	1.33	-1.99	-1.89	-1.99

Pp1s197_56V6.1	WEE1	-1.09	1.43	1.51	1.66	-1.52	1.13	-1.54
homologous recombination								
Pp1s54_11V6.2	BRCC36	-∞	2.50	-2.21	-3.57	-2.40	-1.20	1.31
Pp1s242_87V6.1	COM1	1.27	1.25	-2.21	-∞	1.30	1.29	2.04
Pp1s304_7V6.1	INO80	-∞	-1.60	-1.77	-1.07	1.75	2.33	1.10
Pp1s15_297V6.1	MUS81	-3.74	-2.99	-1.66	-1.28	-1.46	-3.67	-1.29
Pp1s219_52V6.1	NBN	-1.24	-1.56	1.00	-1.15	1.04	-1.42	-1.38
Pp1s97_25V6.1	PAXI1	-1.27	-1.27	-1.13	-2.29	-1.19	-1.05	1.81
Pp1s160_107V6.1	PAXI1	3.11	-2.40	4.53	-6.42	1.59	-1.89	3.00
Pp1s35_92V6.1	PAXI1	2.62	3.03	-1.23	-1.82	-1.56	2.03	3.77
Pp1s232_74V6.1	PAXI1	-1.82	1.00	-1.61	-1.16	-1.42	-1.12	1.15
Pp1s129_197V6.1	RA51B	-2.54	1.00	1.04	1.60	1.01	1.40	-1.13
Pp1s236_47V6.1	RA51C	1.76	1.00	1.21	1.12	-1.34	1.34	1.18
Pp1s212_41V6.1	RA54B	2.82	4.18	1.88	-1.12	-1.44	-1.24	1.20
Pp1s51_220V6.1	RAD50	-1.38	-1.04	1.59	-1.31	1.58	-1.35	1.28
Pp1s31_236V6.1	RAD51A	5.23	6.01	2.69	1.09	-1.10	-1.28	1.54
Pp1s42_140V6.1	RAD51B	1.01	3.76	-1.38	-1.43	-1.94	1.71	1.73
Pp1s341_67V6.1	RAD54	2.79	4.87	1.16	1.03	-1.40	1.42	2.15
Pp1s236_78V6.1	RAD54	-2.10	-1.76	1.36	1.03	1.34	-2.06	1.45
Pp1s93_66V6.1	RecQ1	-2.39	1.70	1.64	1.40	-1.33	-1.17	-1.29
Pp1s201_114V6.1	RMI1	-1.92	-1.16	1.21	-1.17	-1.88	-1.62	-1.44
Pp1s159_85V6.1	RMI2	1.36	2.45	1.16	1.27	-1.09	1.39	1.42
Pp1s152_88V6.1	RQSIM	1.44	1.79	1.98	1.00	-1.26	-1.11	1.50
Pp1s4_73V6.1	RUVB1	-2.28	-1.12	-2.21	-1.07	1.57	-1.12	1.27
Pp1s255_64V6.1	RUVB2	-2.45	-1.69	1.39	1.56	-1.01	-1.48	-1.70
Pp1s474_3V6.1	TOP3B	-5.87	1.25	1.40	1.82	-1.64	-1.05	-1.83
Pp1s16_272V6.1	WRIP1	2.09	2.98	-3.24	-1.16	-1.78	2.27	1.92
Pp1s455_6V6.1	WRIP1	-2.09	1.05	1.29	-1.46	-3.28	-1.74	-1.41
Pp1s135_47V6.1	wrx	1.32	1.42	-1.58	-1.79	1.07	3.30	2.97
Pp1s4_355V6.1	wrx	1.18	1.52	-2.22	-1.57	1.06	2.19	1.85
Pp1s45_271V6.1	XRCC2	-1.49	1.63	1.18	-1.05	-1.75	-1.35	1.58
Non homologous end joining								
Pp1s299_4V6.1	KU70	7.77	4.46	2.59	-2.46	-1.24	-1.11	2.78
Pp1s121_27V6.1	KU80	5.35	4.79	-1.06	-1.15	-1.12	2.46	3.77
Pp1s78_226V6.1	PRKDC	-1.17	-1.03	1.06	-1.19	1.31	-1.15	-2.18
Pp1s147_88V6.1	XRCC4	5.14	11.08	4.83	1.64	-4.51	-3.92	-2.27
Pp1s224_52V6.1	XRCC1	-1.23	-1.33	-1.47	-3.92	1.04	-1.20	1.44
meiotic recombination								
Pp1s335_13V6.1	HOP2	-1.13	-1.05	1.18	1.09	-1.22	-1.36	-1.17
Pp1s41_172V6.1	MND1	1.02	1.42	1.88	1.15	-1.92	-2.22	-1.39
Pp1s62_130V6.1	SPO11	2.20	1.79	-1.46	1.82	1.48	2.87	1.94
UV repair								
Pp1s458_4V6.1	DDB1	1.07	-1.11	-1.19	1.30	-1.04	-1.22	-1.32
Pp1s203_55V6.1	DDB1	-1.20	1.49	-1.10	-1.02	1.00	1.37	1.10
Pp1s114_132V6.1	DDB2	3.57	2.81	-1.06	-3.02	-1.13	1.61	2.94
Pp1s456_8V6.1	FEN1	3.51	1.25	2.26	1.40	1.35	-1.12	1.98
Pp1s39_160V6.1	FEN1	2.06	1.67	4.35	-1.43	-1.18	-2.85	1.41
Pp1s58_148V6.1	RAD23	-1.87	-1.33	1.27	-1.05	-1.06	-1.53	-1.23
Pp1s286_52V6.1	RAD23	-2.28	-1.06	-1.44	1.50	1.20	1.12	1.01
Pp1s3_105V6.1	RAD23	1.89	1.14	-2.34	-2.10	1.08	1.62	2.38
Pp1s91_87V6.1	UBC2	1.03	1.09	1.05	-1.44	1.05	1.10	1.52
Pp1s219_106V6.1	UBC2	1.04	1.07	1.11	1.03	-1.65	-1.22	-1.27
crosslink repair								
Pp1s120_12V6.1	DCR1A	-1.05	-∞	-1.10	-1.43	1.95	1.94	-1.64
Pp1s68_3V6.1	DCR1B	-∞	-1.20	-2.21	7.01	1.09	-1.38	-4.36

Pp1s204_101V6.1	FACD2	2.75	1.33	1.21	-1.36	1.71	1.20	2.53
Pp1s156_74V6.1	FANCL	-1.04	-1.85	-1.30	-1.04	-1.05	-1.44	-1.28
nucleotide excision repair								
Pp1s117_170V6.1	ERCC1	-1.33	1.05	1.11	-1.07	-1.09	-1.23	1.17
Pp1s117_181V6.1	ERCC1	1.08	1.25	-6.62	-17.12	-3.03	1.32	4.98
Pp1s145_26V6.3	ERCC2	2.42	3.63	-1.22	1.10	-2.00	1.13	1.64
Pp1s177_124V6.1	ERCC3	-1.02	1.70	-1.10	1.45	-1.46	1.01	-1.27
Pp1s3_646V6.1	ERCC4	1.46	3.01	-1.02	1.33	-2.14	1.16	1.09
Pp1s31_24V6.1	ERCC5	1.03	-1.09	1.10	1.13	1.06	-1.01	-1.09
Pp1s66_144V6.1	ERCC6	4.79	3.48	1.16	-1.76	1.30	2.30	3.12
Pp1s155_61V6.3	ERCC6	-1.23	-1.06	1.51	1.00	-1.20	-1.60	-1.09
Pp1s67_171V6.1	RAD1	-1.64	-1.53	1.60	-1.10	-1.61	-2.47	-2.59
Pp1s3_639V6.1	RAD16	1.29	3.07	-1.03	-1.17	-1.38	1.84	2.22
Pp1s132_19V6.1	RAD16	-1.71	1.34	-1.22	-1.02	1.40	1.54	1.47
Pp1s139_28V6.1	SYF1	1.48	-1.47	1.32	1.21	1.11	1.06	1.30
Pp1s12_235V6.1	XPC	1.14	10.02	1.09	4.21	-3.42	1.45	-5.45
base excision repair								
Pp1s324_39V6.1	PARP2	6.01	3.46	1.22	-3.94	-1.31	1.00	2.96
mismatch repair								
Pp1s72_302V6.1	EME1	-1.78	1.16	1.32	2.10	1.34	2.29	1.44
Pp1s10_231V6.2	EXO1	1.20	1.36	1.15	1.20	-1.04	1.36	1.26
Pp1s58_199V6.1	MLH1	1.66	1.41	1.15	-1.51	-1.18	1.04	1.39
Pp1s5_400V6.1	MLH3	2.48	1.17	1.62	-1.39	-1.21	-1.30	1.42
Pp1s251_77V6.1	MSH2	1.29	3.90	-1.01	-1.17	-2.53	1.36	1.77
Pp1s30_339V6.1	MSH3	1.43	1.21	-1.48	-2.17	-1.04	1.29	1.52
Pp1s84_88V6.1	MSH5	-1.10	1.33	1.36	-1.05	-1.17	1.14	1.31
Pp1s90_86V6.1	MSH6	-2.41	2.19	2.49	-1.32	-2.35	-2.36	-1.15
Pp1s3_417V6.1	Muts	-1.75	1.00	-1.10	1.05	1.46	1.45	2.20
Pp1s474_7V6.1	PMS1/PMS2	-1.54	-1.40	1.58	1.10	-1.03	-1.91	-1.50
general functions								
Pp1s370_33V6.1	DDX11	1.63	1.25	3.02	2.80	1.67	-1.20	-1.91
Pp1s223_54V6.2	DNL1	1.47	-1.18	1.42	-1.08	1.03	-1.72	-1.22
Pp1s300_57V6.1	PIF1	2.79	1.13	-1.81	1.08	1.30	1.91	2.40
Pp1s240_31V6.1	PNKP	4.56	10.65	3.32	1.06	-1.93	-2.58	2.69
Pp1s77_195V6.2	RAD21	-1.90	1.50	1.49	-1.39	-1.34	-1.97	-1.36
Pp1s491_21V6.1	RNF4	-1.05	-1.08	-1.10	-1.07	-1.42	-2.34	-1.34
Pp1s121_28V6.2	RNF4	1.08	1.09	-1.28	-1.84	1.41	1.35	2.90
Pp1s3_270V6.1	RNF4	-1.21	-1.26	1.08	1.23	-1.18	-1.58	-1.14
Pp1s104_102V6.1	SCC2	-1.62	-1.22	1.32	-1.23	1.37	-1.47	1.02
Pp1s68_122V6.2	SIR2	-1.51	1.17	-1.35	-1.53	1.54	1.30	1.76
Pp1s272_33V6.1	SIR2	2.33	1.16	1.47	1.09	1.22	2.11	2.25
Pp1s15_90V6.2	SIR2	-1.75	-1.09	-1.08	-1.13	-1.21	-1.12	-1.41
Pp1s91_43V6.1	SMC1A	1.27	1.79	-1.55	-1.25	-1.10	2.11	1.52
Pp1s410_17V6.1	SMC3	1.14	1.25	-1.10	-1.57	-1.03	1.75	1.34
Pp1s274_85V6.1	SMC5	-1.71	-1.15	1.19	-1.08	-1.45	-1.34	-1.39
Pp1s61_278V6.1	SMC6	3.51	8.77	-1.66	-4.28	-2.23	1.34	1.69
Pp1s199_157V6.1	STAG1	1.30	2.51	2.42	-2.00	-1.88	-2.52	1.17
ATM/ATR interactors								
Pp1s641_1V6.1	CDC5L	1.39	2.86	-1.51	1.58	-1.81	2.04	1.14
Pp1s33_329V6.1	CHD4	-1.87	1.31	-1.31	-1.21	-1.22	-1.02	-1.04
Pp1s235_76V6.1	CHD4	-2.02	-1.25	1.28	1.13	1.02	-1.28	-1.17
Pp1s22_60V6.1	E2F1	-1.01	-1.40	-1.31	-1.05	-1.20	-1.05	1.21
Pp1s55_112V6.1	H2AX	1.12	1.37	-1.24	-1.08	1.42	1.36	1.48
Pp1s452_4V6.1	H2AX	-1.22	1.42	1.03	1.05	1.02	-1.27	-1.06
Pp1s188_35V6.1	H2AX	1.49	1.25	+∞	4.21	-3.94	-∞	-6.27

Pp1s223_52V6.1	HDAC1	-1.91	2.16	1.32	-1.03	-1.70	-1.44	-1.29
Pp1s351_29V6.1	HDAC1	1.33	-1.22	1.09	1.30	1.09	-1.11	1.35
Pp1s180_68V6.1	HDAC1	1.22	1.86	-1.23	-2.19	-1.29	1.30	2.73
Pp1s9_156V6.1	MCM3	-1.05	-∞	-1.10	4.91	-2.06	-1.03	-1.64
Pp1s31_86V6.1	MCM7	-1.90	-3.19	-1.10	2.45	1.44	-1.40	-2.21
Pp1s226_56V6.1	PTPA	1.04	1.65	1.05	1.02	-1.44	-1.33	-1.06
Pp1s111_153V6.1	PTPA	-7.19	-1.40	-2.21	-1.27	-2.01	-3.52	-1.40
Pp1s10_103V6.1	RENT1	-2.54	1.67	1.93	2.65	-1.66	-1.25	-1.76
Pp1s44_135V6.1	RENT1	1.09	-1.60	-1.10	4.21	1.11	1.11	-2.86
Pp1s222_133V6.1	RFA1	1.16	2.76	1.41	-1.30	-1.18	-1.32	1.06
Pp1s357_53V6.1	RFA2	1.06	-1.56	-1.01	1.17	1.32	1.44	1.20
Pp1s112_133V6.1	SOSB1	-1.65	-1.13	1.65	1.25	1.24	-1.18	-1.62
Pp1s217_52V6.1	STRAP	-1.62	-2.79	-1.77	-1.28	1.10	1.26	-1.12
Pp1s25_22V6.1	STRAP	-1.41	-1.12	1.09	-1.25	-1.35	-1.30	-1.08
Pp1s114_137V6.1	TERF1/2	-1.57	-1.74	1.19	1.24	-1.36	-1.91	-2.09
Pp1s176_113V6.2	TERF1/2	1.83	1.71	-1.37	-2.23	1.09	1.86	1.87
Pp1s1_349V6.1	TERF1/2	-1.21	-1.62	-1.10	1.13	-1.07	-1.04	-1.01
Pp1s49_258V6.1	TERF1/2	2.17	1.67	1.29	1.05	-1.20	1.94	2.10
Pp1s152_10V6.1	TERF1/2	1.95	1.25	1.04	-4.99	-1.40	-1.01	1.25

Looking at the overall effect of ATM and ATR loss of function on the induction/repression of DNA repair transcripts the observed effects were similar to the global transcript analysis reported previously. First, the total number of DNA repair genes induced/repressed varied in all of the mutant lines (**Figure 3.2-22**).

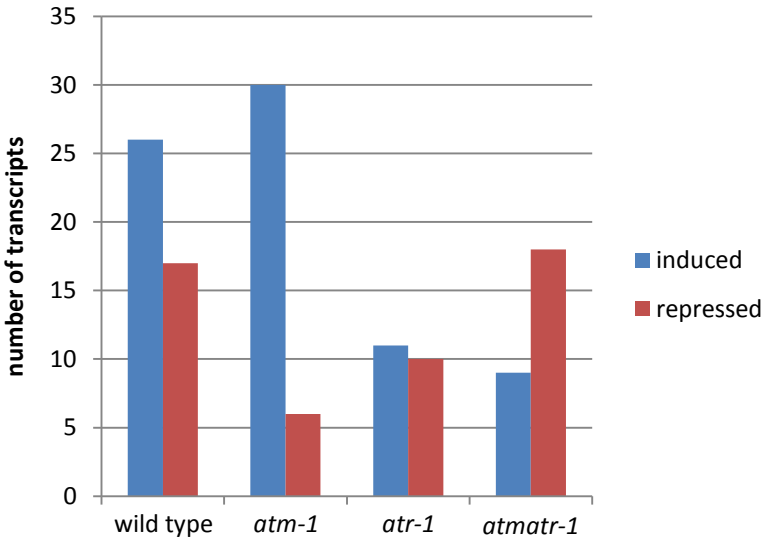

Figure 3.2-22: The transcriptional response of the subset of DNA repair/recombination genes of wild type and mutant lines. Shown is the number of DNA repair/recombination genes in wild type, *atm-1*, *atr-1* and *atmatr-1* lines which is up- or downregulated at least 2-fold after bleomycin treatment.

Second, the identity of genes induced/repressed in the mutant lines following bleomycin treatment was to a large degree different to the wild type, especially in lines lacking functional ATR (**Figure 3.2-23**). Third, a share of DNA damage responsive transcripts observed in the wild type were found constitutively induced/repressed in the mutant lines. The latter effect was once more most prominent in the ATR mutant lines (**Figure 3.2-24**). What is worth noting is that *ATM* and *ATR* mutants are not only deficient in upregulation of HR components such as Rad51 and Rad9 but also key components of the NHEJ pathway such as Ku70 and XRCC4. This is rather unexpected as ATM and ATR are presumed to be central components of the HR but not the NHEJ pathway.

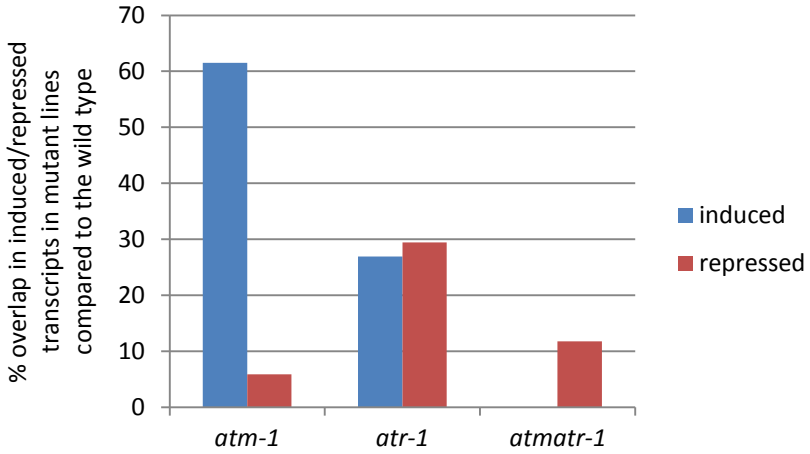

Figure 3.2-23: Genes induced/repressed in the mutant lines upon bleomycin are only partially identical to those induced/repressed in the wild type. Shown is the percentage of more than 2-fold induced/repressed DNA repair/recombination genes in *atm-1*, *atr-1* and *atmatr-1,* in response to bleomycin treatment, which are similarly regulated in the wild type.

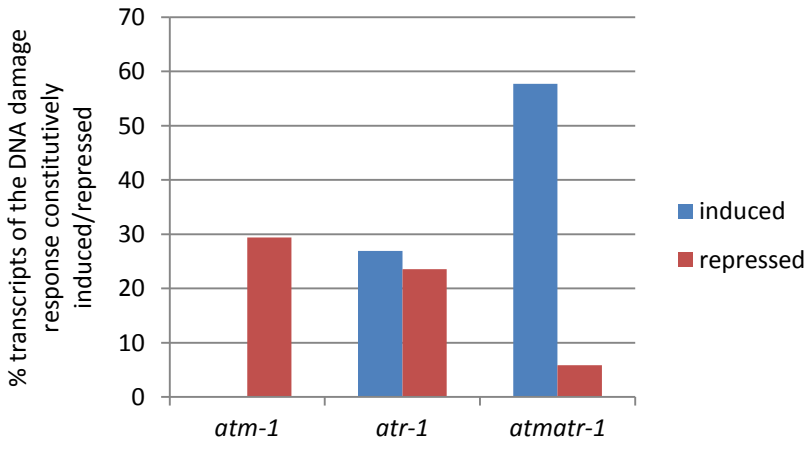

Figure 3.2-24: A share of DNA damage responsive genes, as observed in the wild type are up- downregulated in mutant lines already. Shown is the number of genes which is up- or downregulated in untreated mutant lines in relation to the untreated wild type, which had previously been found differentially regulated upon bleomycin treatment in the wild type.

To showcase the previously found effect of ATM and ATR loss on the transcriptional DNA damage response the expression profile of a number of key HR and NHEJ components of the DSB repair is shown in **Figure 3.2-25**. Observing at the *atm-1* line it is evident that all of the selected genes are still induced upon DNA damage. However, the absolute transcript levels after induction are in some cases lower than those in the wild type. Both *atr-1* and *atmatr-1* meanwhile display a much more severe effect, the transcript levels of genes often being increased in the untreated samples and the increase in transcript levels upon genotoxic stress being either absent or weaker than in the other lines.

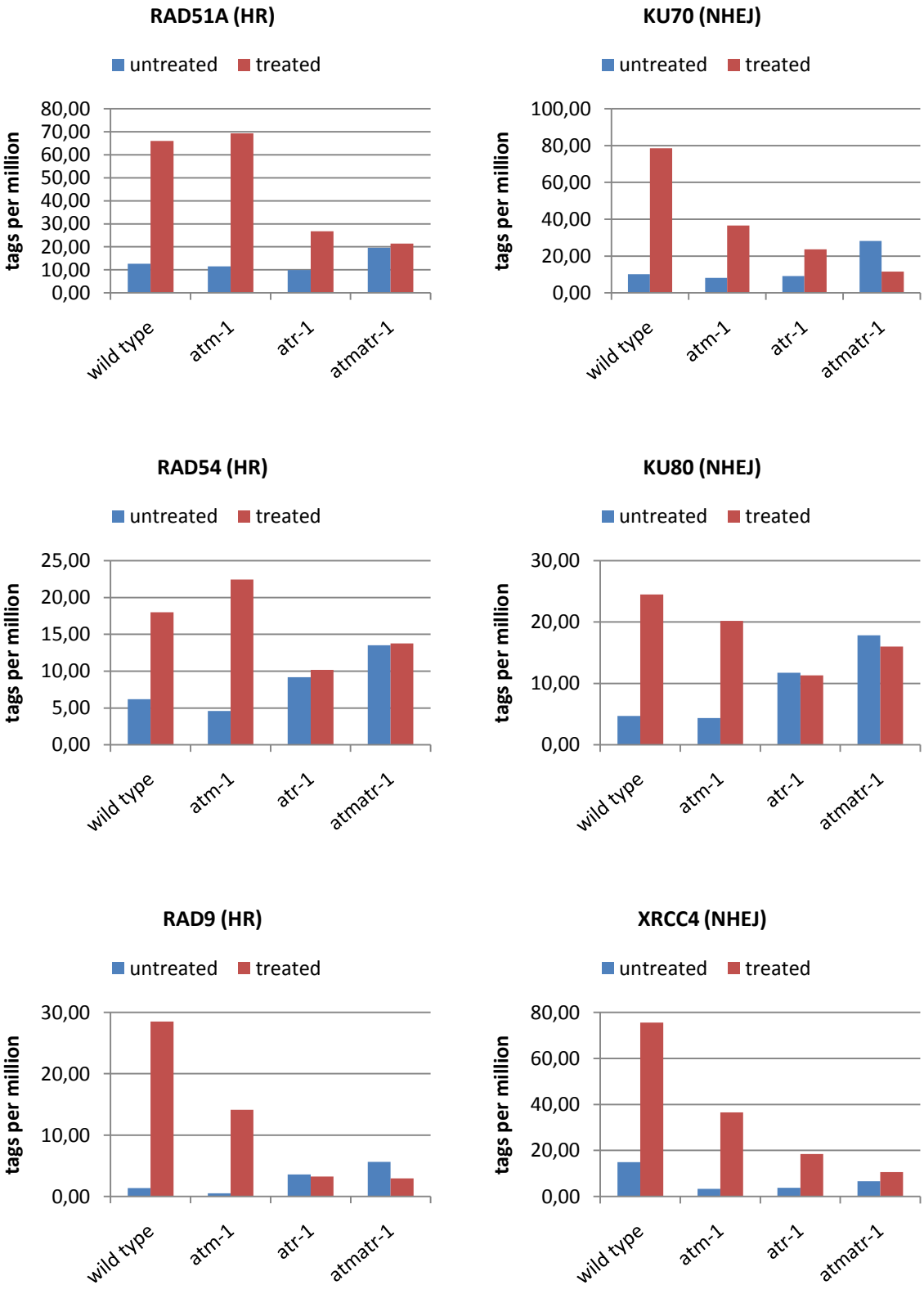

Figure 3.2-25: Transcriptional response of key HR and NHEJ genes is affected in mutant lines. Tags per million count of select HR and NHEJ transcripts in untreated and treated (0.3 U/L bleomycin) samples of wild type and mutant lines.

3.2.6 Proteome analysis of wild type and mutant lines

In the previous chapter the transcriptional response of wild type and mutant lines to the genotoxic agent bleomycin was assessed. However, since there is active discussion to which degree transcriptional changes may relate to actual changes in protein abundance (Muers 2011) it was decided to supplement the transcriptome data with a proteome analysis.
The starting plant material for the proteome analysis was prepared using the same experimental protocol as used for the transcriptome approach. 5 day old actively growing protonema subcultures of wild type*, atm-1, atr-1 and atmatr-1* lines were subjected to either mock or bleomycin (0.3 U/L) treatment for 1 hour. Afterwards the material was washed and incubated for 3 more hours in fresh growth media to provide time for the physiological response to the stress treatment. Subsequently Proteins were extracted and separated via 2D gel electrophoresis. Protein spots were evaluated via 2D image analysis software and spots which were found to be differentially regulated at least 1.5 fold ($P < 0.05$) were isolated from the gel. The identity of proteins in each of these spots was determined via mass spectrometric analysis followed by a BLAST search utilizing the identified peptide.

3.2.6.1 A low number of proteins is found differentially expressed in mutant lines

In the previous transcriptome analysis the base expression level of a large number of genes was found to be different in all of the mutant lines. Thus the first thing to be evaluated was whether this phenomenon would also be observable on the protein level. Comparing untreated wild type with untreated *atm-1, atr-1 and atmatr-1* only 1, 3 and 6 proteins respectively were found to be differentially regulated in the mutant lines (**Table 3.2-11**). As the transcriptome dataset showed 1000's of genes to be constitutively induced/repressed in the mutant lines compared to the wild type these low numbers of differentially expressed proteins are surprising. Comparing the expression data for these 10 genes in the transcript and protein dataset it is also worth mentioning that only 7 out of 10 display the same trend regarding induction/repression (data not shown).
Looking at the individual proteins which are differentially expressed it is worth noting that among them are key metabolic components such as RuBisCO activase and small subunit. This in addition to the vast transcriptome changes reported earlier further supports the notion that basic cellular homeostasis is likely disturbed in mutant lines. Among the set of 10 differentially expressed proteins in the mutant lines two more are worth mentioning in this regard. One is the MLP-like protein 423 which is increased in abundance in *atr-1* as well as *atmatr-1*. While the exact function of this protein has not yet been discovered, research conducted in Arabidopsis has revealed it to be differentially expressed in response to biotic (Ascencio-Ibáñez et al. 2008) and abiotic (Abercrombie et al. 2008) stress. The second protein of note is STR1, which is upregulated in only *atmatr-1*. The Arabidopsis

homologue of this protein has been found to be important in developmental processes, a T-DNA insertion line displaying arrested/delayed embryo development (Mao et al. 2011).

Table 3.2-11: Differentially expressed proteins in ATM and ATR mutant lines. Fold change calculated in relation to the wild type. Only proteins are listed whose fold change exceeded 1.5 (P<0.05)

mutant	*Physcomitrella* accession	Fold change	Arabidopsis homolog	Full Name	Main function
atm-1	Pp1s339_15V6.1	10,37	RBS1A_ARATH	RuBisCO small subunit 1A	metabolic
atr-1	Pp1s5_83V6.2	-2.33	RCA_ARATH	RuBisCO activase	metabolic
	Pp1s360_13V6.1	-2	DIR21_ARATH	Dirigent protein 21	Secondary metabolism
	Pp1s22_322V6.1	1,89	ML423_ARATH	MLP-like protein 423	Stress response
atmatr-1	Pp1s276_75V6.1	1,67	CPNB1_ARATH	Chaperonin 60 subunit beta 1	chaperone
	Pp1s17_355V6.1	1,94	GDL53_ARATH	GDSL esterase/lipase	metabolic
	Pp1s22_322V6.1	2,32	ML423_ARATH	MLP-like protein 423	Stress response
	Pp1s248_84V6.1	2,38	Y5224_ARATH	Uncharacterized protein	unknown
	Pp1s78_4V6.2	3,64	STR1_ARATH	Thiosulfate/3-mercaptopyruvate sulfurtransferase 1	development
	Pp1s339_15V6.1	6,39	RBS1A_ARATH	RuBisCO small subunit 1A	metabolic

3.2.6.2 Hardly any proteome changes were observed in response to genotoxic stress

Having assessed the differences in base protein levels between wild type and mutant lines the next step was to evaluate the effect of bleomycin treatment. Comparing the abundance of proteins between treated and untreated samples of each of the individual lines only a single protein was found to be significantly altered in its expression level, in only the *atr-1* line (**Table 3.2-12**). This protein, "Dirigent protein 21", is not known to have any known function in regard to DNA damage repair. Instead the sparse information available suggests this protein to be involved in a defense response against vermin (Ralph et al. 2007). The fact that it was only differentially expressed in the *atr-1* line but not the wild type further suggested that it may not even be part of the regular DNA damage response. Thus there was practically no DNA damage response detected in the proteome dataset of wild type and mutant lines.

Table 3.2-12: Differentially expressed proteins in response to bleomycin treatment in wild type and mutant lines. Fold change was calculated between untreated and treated samples of each individual line. Only proteins with significant fold change according to t-test (P<0.05) are listed.

line	*Physcomitrella* accession	Fold change	Arabidopsis homolog	Full Name	Main function
wild type	-	-	-	-	-
atm-1	-	-	-	-	-
atr-1	Pp1s360_13V6.1	-2	DIR21_ARATH	Dirigent protein 21	Secondary metabolism
atmatr-1	-	-	-	-	-

3.2.7 The vegetative and generative phenotype of ATM and ATR mutant lines

In the previous chapters the *atm-1*, *atr-1* and *atmatr-1* mutant lines were found to be hypersensitive to genotoxic stress, deficient in checkpoint control and the base expression level of a large number of genes involved in a variety of processes such as DNA repair, metabolism and stress response were found to be increased/decreased. It is worth noting that these deficiencies/irregularities were generally most prominent in the *atr-1* and *atmatr-1* mutant lines. Taking this into consideration it was expected that the mutations of ATM but even more so ATR in *P. patens* would also produce growth and/or developmental phenotypes.

3.2.7.1 Loss of ATM and ATR affects gametophytic growth

To obtain an overview regarding possible growth and developmental phenotypes of the *atm-1*, *atr-1*, and *atmatr-1* lines they were at first observed during the gametophytic stage of growth. Starting material for this assay were small protonema colonies pregrown on standard PPNH4 medium overlaid with cellophane. The semipermeable cellophane layer used in growth of the precultures simplified the later transfer of plantlets due to preventing growth of filaments into the agar, whilst still allowing for nutrient uptake. From these pregrown plantlets, similar sized ones were selected and transferred onto fresh plates for the actual growth assay. Growth of plantlets was observed for 5 weeks. Replicating this type of experiment multiple times, a reproducible set of observations was obtained.

The juvenile stage of gametophytic development of *P. patens* is characterized by the two dimensional growth of two distinct types of filaments, chloronema and caulonema. Chloronema filaments proliferate comparably slow, branch irregularly and contain a large number of chloroplasts in accordance with their mainly assimilatory role (Reski 1998, Cove 2005). Caulonema filaments on the other hand proliferate faster, branch regularly and contain only few chloroplasts, befitting to their assumed role of colonizing the growth substrate and nutrient uptake (Cove 2005, Menand et al. 2007). Under our experimental conditions, the body of the plantlets built up by these two filament types forms a round shape, dominated by chloronema filaments, interspersed with a lower number of caulonema. Comparing mutant and wild type plantlets at this stage, we found that plantlets of *atm-1* and *atr-1* displayed a less dense outer region **(Figure 3.2-26a)**. Furthermore plantlets of these two lines had spread faster on the growth media, their diameter being noticeably increased compared to the wild type. In contrast, the double mutant *atmatr-1* appeared to be similar in growth to the wild type, being equal in size and displaying densely grown fringe regions.

The next phase of *P. patens* development, the adult gametophytic stage, is marked by the emergence of leafy structures, the gametophores. These are formed out of so called buds, three faced apical cells which develop out of side branch initials of mainly caulonema type filaments (Reski 1998).

Observing plantlets in this phase of growth, the plantlet surface of wild type and *atm-1* plantlets was quickly saturated with large numbers of gametophores **(Figure 3.2-26b)**. *Atr-1* and *atmatr-1* plantlets on the other hand produced few or hardly any gametophores respectively. In addition *atr-1* and *atmatr-1* plantlets appeared to be brownishly discoloured in their central area. However the same discoloration is present in the wild type albeit hidden under the densely grown gametophores. These results implied that the loss of ATM and especially ATR affected different stages of gametophytic development to varying degrees.

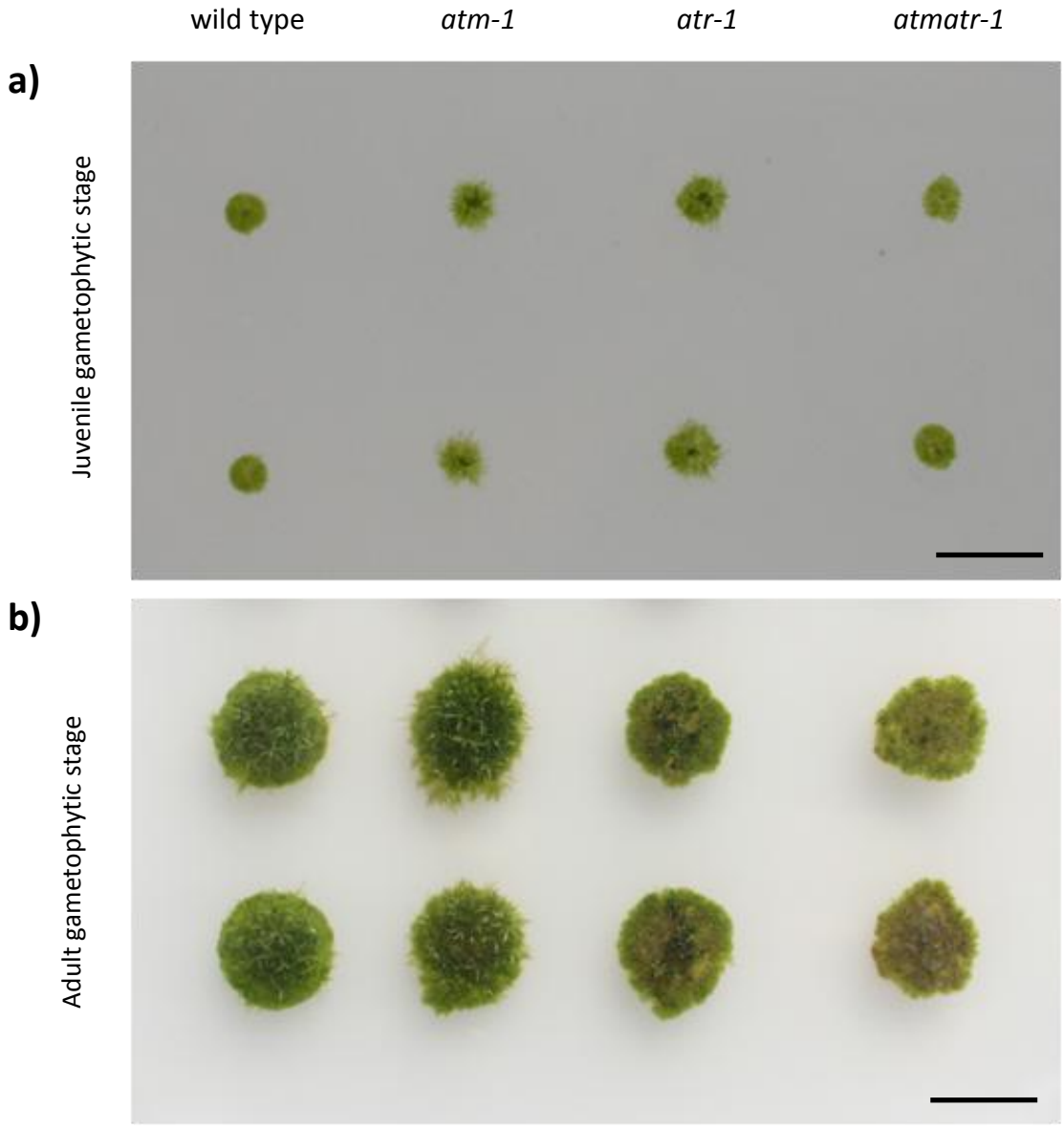

Figure 3.2-26: Overview of the two main stages in *P. patens* gametophytic growth in wild type and mutant lines. Starting material for the growth assays were similar sized 1 week old plantlets pregrown from small

protonema fragments. Wild type, *atm-1*, *atr-1* and *atmatr-1* plantlets were grown for a total of 5 weeks and pictures were taken after 2 (**a**) and 5 (**b**) weeks of growth. Scale bar = 1 cm.

3.2.7.2 Mutant lines are affected to varying degrees in filament differentiation and regeneration

In the initial set of growth experiments protonema colonies of *atm-1* and *atr-1* were found to be growing faster in diameter, as well as displaying a less dense filament structure in the outer regions. Since these differences were likely to manifest in the early stages of filament development an additional growth assay was performed, focusing on the early stages of filament development. This time, instead of pregrown and similar sized protonema colonies, a large number of random filaments, only a few cells in size were utilized as starting material. Filaments were grown on standard medium and their growth observed till the formation of gametophores marked the beginning of the adult gametophytic stage.

The early stage of filamentous development appeared to be similar in wild type and mutant lines regarding the growth pattern of filaments. Differences were observed once plantlets attained their characteristic rounded shape (**Figure 3.2-27**). At this stage, wild type plantlets were dominated by a densely grown central region with few and comparably short protruding caulonema type filaments. *atm-1* and *atr-1* plantlets on the other hand displayed a less dense central part coupled with a much more pronounced caulonemal outgrowth. Caulonema filaments protruded farther from the central region and were more numerous in general. The filament morphology of *atmatr-1* plantlets meanwhile seemed to be similar to the wild type again. Therefore the increased size gain and less dense growth of *atm-1* and *atr-1* plantlets observed in the previous experiment seemed to stem from an increased ratio of caulonema to chloronema. In support of this hypothesis is the fact that *P. patens* caulonema type filaments have been found to grow faster than chloronema (Cove 2005, Menand et al. 2007) and are generally attributed with substrate colonization (Menand et al. 2007).

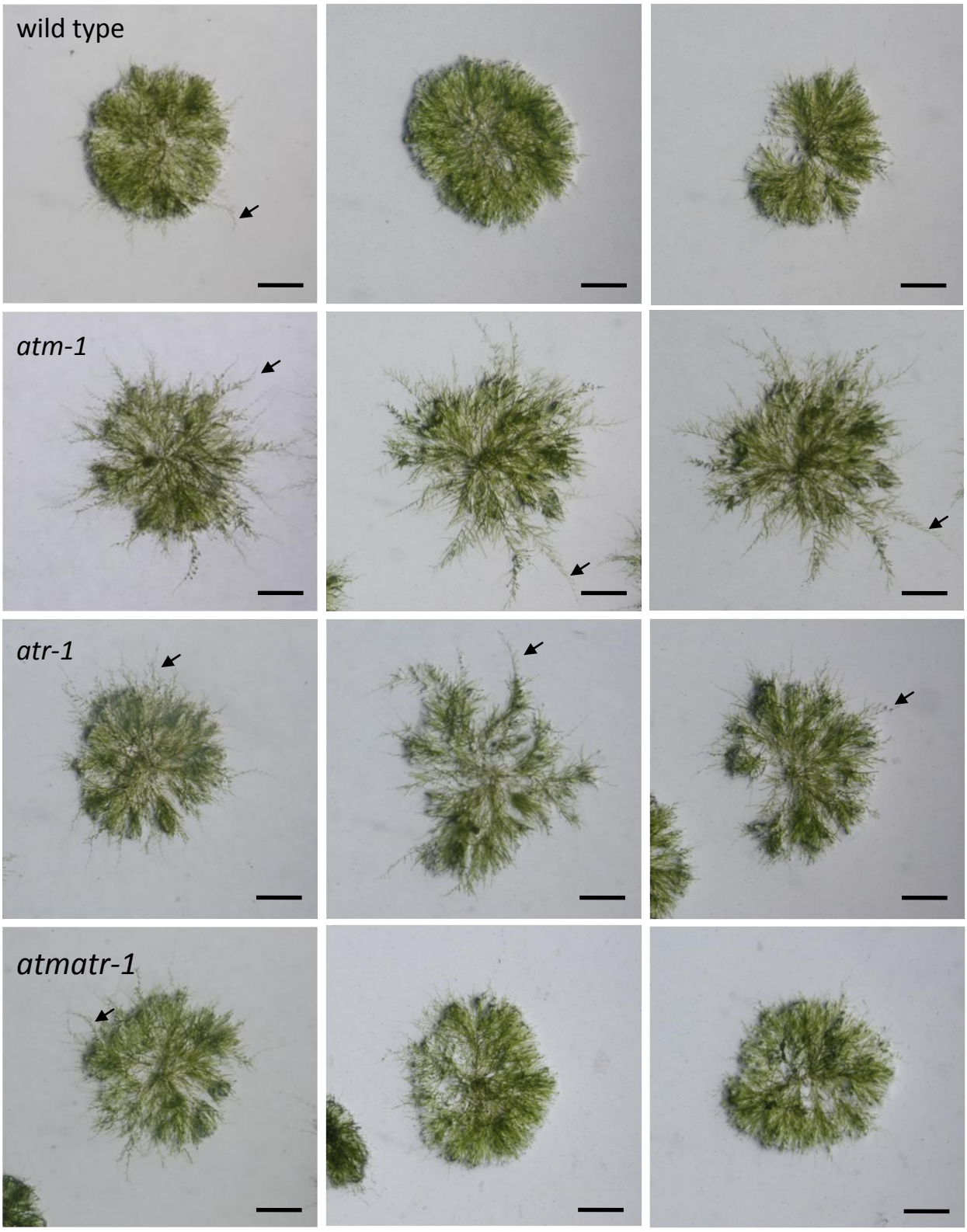

Figure 3.2-27: Plantlet morphology is markedly different in *atm-1* and *atr-1*, especially in regard to caulonemal growth. Depicted plantlets were regenerated from similar sized small protonema fragments obtained from homogenization and filtration of 7 day old subcultures. Plantlets were grown for a total of 14 days in permanent light. Arrows indicate a representative of a caulonema type filament, if at all observed in the respective plantlet. Scale bar = 1 mm

Another observation which was made during early regeneration was that a number of filaments appeared to be growing much more slowly than the rest **(indicated by arrows, Figure 3.2-28a)**. Whereas this appeared to occur in wild type as well as mutant lines it was more common in especially *atr-1* and *atmatr-1*. To verify this observation we quantified the incidence of small plantlets by measuring the surface area of a large number of individual plantlets and sorting them into size categories for comparison **(Figure 3.2-28b)**. As is evident, the *atr-1* and *atmatr-1* lines display a significant increase in small plantlets who were likely suffering from reduced growth.

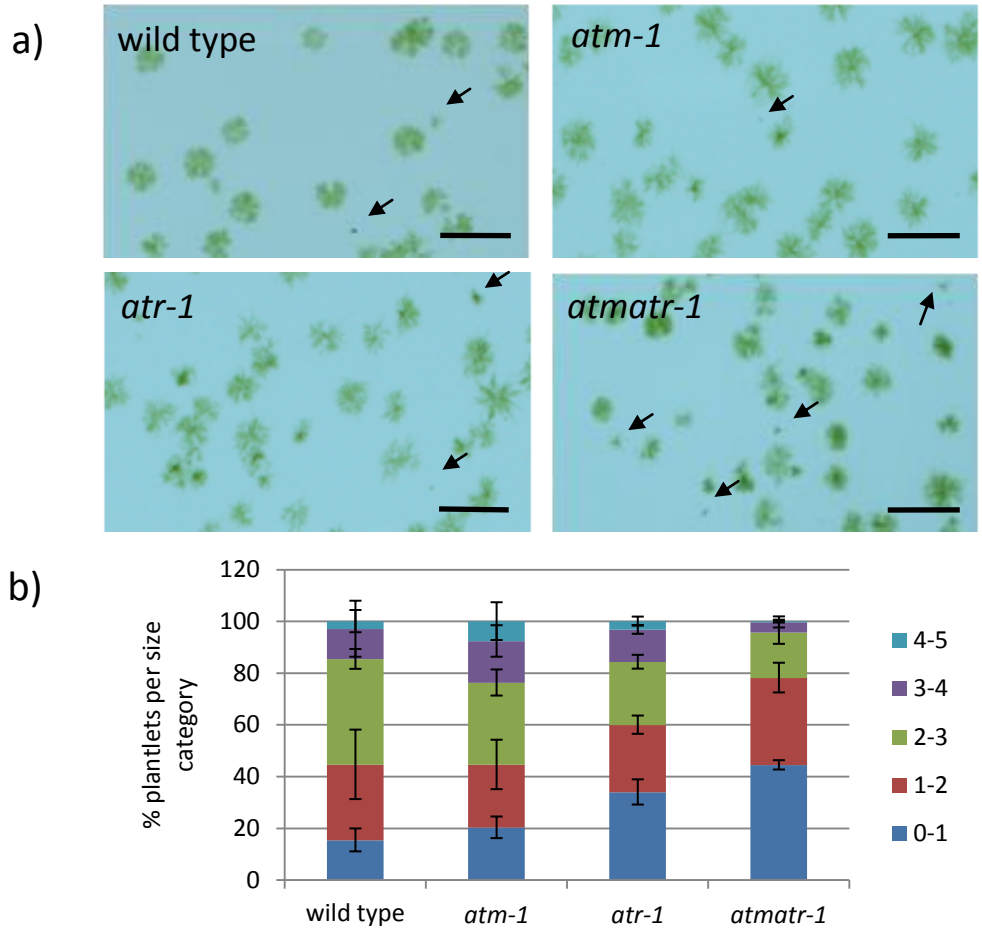

Figure 3.2-28: Incidence of small sized plantlets is increased in *atr-1* and *atmatr-1* after 10 days of growth. (**a**) 7 day old subcultures of wild type and mutant lines were homogenized and regenerated for 10 days in permanent light on standard medium. Black arrows indicate filaments which appeared to have grown back very slowly in wild type as well as mutant lines. Scale bar = 0.5 cm (**b**) Individual surface areas of large number of 10 day old regenerated plantlets were measured via 2D image analysis software. Error bars indicate standard deviation per size category for three independent experiments. Testing for difference of means in 0-1 mm^2 size group via one-way ANOVA revealed significant differences ($F(3,8) = 32.57$, P = 7.81E-06). Tukey's post hoc test confirmed significant difference in percentage of plantlets belonging to 0-1mm^2 size group between all lines but wt and *atm-1* (P<0.05).

3.2.7.3 Enhanced caulonemal growth in *atm-1* and *atr-1* may be a stress response

One of the two main phenotypes of mutant lines we observed during the juvenile stage was that the growth of caulonema type filaments was markedly increased in the *atm-1* and *atr-1* lines but not *atmatr-1.* Moreover, while the role of ATM and ATR is well documented in cell cycle control and DNA damage repair, no function has been attributed to them in the control of cellular differentiation. Therefore the increased caulonemal growth was likely to be an indirect effect of reduced ATM or ATR function.

In wild type plantlets, the formation of caulonema type filaments can be enhanced by adjusting growth conditions. For instance, we observed that limited availability of nitrogen lead to a much decreased growth rate while at the same time increasing caulonema formation. Since caulonema are believed to be important for nutrient uptake (Menand et al. 2007) the increased caulonemal growth could likely be a response to nutrient stress. In order to provide additional evidence to the correlation of increased caulonemal growth and stress in general, we tested the effect of several presumed stress conditions on growth of our wild type as well as mutant plantlets.

In this assay, individual small protonema colonies were pregrown under standard growth conditions for a number of days before similar sized plantlets were then transferred to the specific growth conditions aimed at stimulating caulonemal growth. Three conditions were tested, which were overlaying the media with a sheet of cellophane, omission of ammonium tartrate from the media or subjecting plantlets to a low dosage of UV-B radiation during growth. Withholding additional ammonium tartrate as well as overlaying media with cellophane were supposed to impose nutrient stress. While omission of ammonium tartrate limited nitrogen abundance directly, the cellophane overlay prohibited growth of filaments into the agar, restricting nutrient accessibility in general. The low dosage of UV-B light on the other hand was to impose a measure of genotoxic stress. It may not be ruled out however that UV might also function as a developmental cue as it has been reported in Arabidopsis (Favory et al. 2009, Rizzini et al. 2011).

Evaluating the outcome of this comparative experiment, three distinct observations could be made. First, wild type caulonema growth was strongly induced in all three of the altered growth conditions (**Figure 3.2-29**).

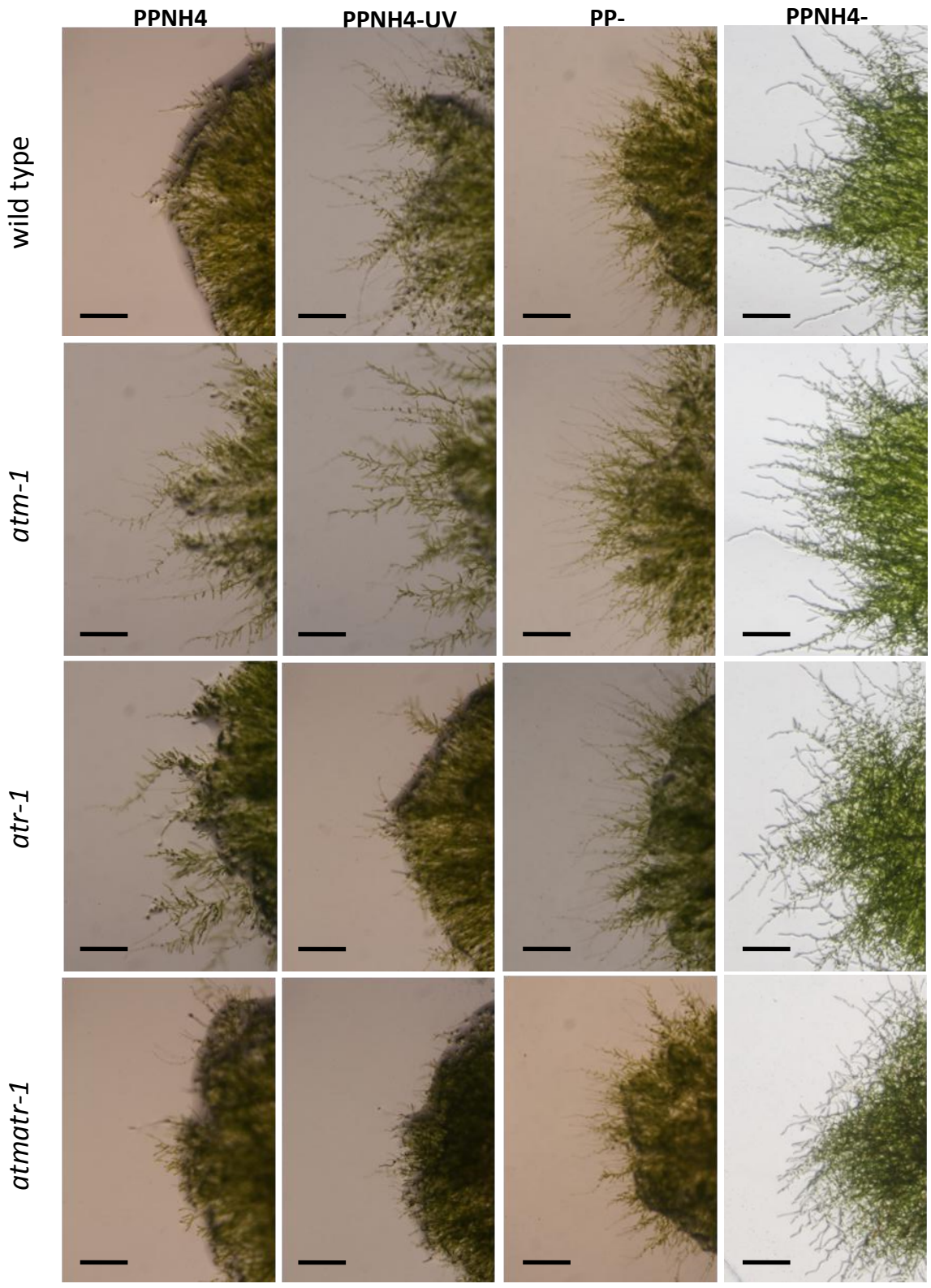

Figure 3.2-29: Caulonemal growth similar to that of *atm-1* and *atr-1* can be induced in wild type by variation of growth conditions. Pictures show fringe regions of wild type, *atm-1*, *atr-1* and *atmatr-1* plantlets after a total of 14 days of growth in permanent light. Protonema filaments protruding outwards from center mass are of the caulonema type. In addition to the standard growth condition used in the majority of experiments plantlet growth was observed on different media or with low intensity UV light. PPNH4, standard medium; PP-, standard medium minus ammonium tartrate as nitrogen source; PPNH4-UV, standard medium with a low dose (0.05-0.06 mW/cm^2) of UV, PPNH4 + cellophane, standard medium overlaid with cellophane as a diffusion barrier. Scale bar = 0.5 mm

This lead to a much more similar or even equal caulonema configuration in wild type plantlets compared to *atm-1* and *atr-1* than on the standard media. Second, *atr-1* and *atmatr-1* displayed a clearly reduced caulonemal growth to the other two lines when subjected to UV-B light. Third, albeit caulonemal growth was also enhanced in *atmatr-1* plantlets when grown without ammonium tartrate or with cellophane overlay, the increase in caulonemal growth was distinctly less than in the wild type.

As was found, all of the tested stress conditions increased caulonemal growth in the wild type, suggesting that enhanced caulonemal growth may indeed be an indicator of cellular stress. Considering the important roles of ATM and ATR in cell cycle control and DNA damage repair it therefore appeared likely that the increased caulonemal growth growth of the single mutant lines might be due to impairment of these central cellular processes and the resulting stress. The lack of caulonemal growth in *atr-1* and *atmatr-1* in the presence of UV light meanwhile is likely related to an increased sensitivity to genotoxic stress in these lines, an aspect which will be discussed in detail in a later chapter.

The fact that caulonemal growth was less in the double mutant as compared to the wild type when grown under caulonema inducing conditions, suggested a possible growth defect of this type of filament in this line. To address this matter further we conducted an additional growth experiment, utilizing a special growth regime.

3.2.7.4 Caulonemal growth is markedly impaired in *atr-1* and especially *atmatr-1*

It was previously shown that the double mutant line did not share the prominent increase in caulonemal growth as was evident in the single mutants under standard growth conditions. Moreover, when growth conditions were adjusted to promote caulonemal growth, the increase was markedly less than in the wild type. This suggested that the growth of caulonemal filaments may be somehow affected in the double mutant. The two most likely explanations for this phenomenon were that either individual Caulonema cells were shorter or their proliferation markedly impaired. To discern between these two possibilities, an additional growth experiment was performed to measure the length of individual caulonema cells. To obtain a large number of caulonemal cells and avoid interference by chloronema type cells we employed a special growth condition, reported to restrict filamentous growth to only caulonema type filaments (Jenkins et al. 1986).

In this assay, protonema filaments were grown in darkness, on standard PPNH4 media supplied with additional glucose. Moreover, vertical placement of agar plates lead to unidirectional growth of

caulonema filaments due to their negative gravitropism, simplifying length comparison. Plates were incubated for a total of 3 weeks in constant darkness before growth was evaluated. It should be stated that filaments grown under these conditions were different from the usual caulonema in that they were brownish and completely devoid of chloroplasts. However the hallmarks of caulonema filaments which are fast growth speed and oblique cell walls were observed. Comparing the length of filaments between the lines, wild type and *atm-1* appeared to be similar (**Figure 3.2-30a**). *Atr-1* meanwhile displayed a clear reduction in the abundance of longest caulonema filaments (**Figure 3.2-30b**) while *atmatr-1* filaments were altogether strongly reduced in length **(Figure 3.2-30a)**. Measuring the length of individual cells in these caulonema filaments, we found that there were no significant deviations in cell length among wild type and mutant lines (**Figure 3.2-31**).

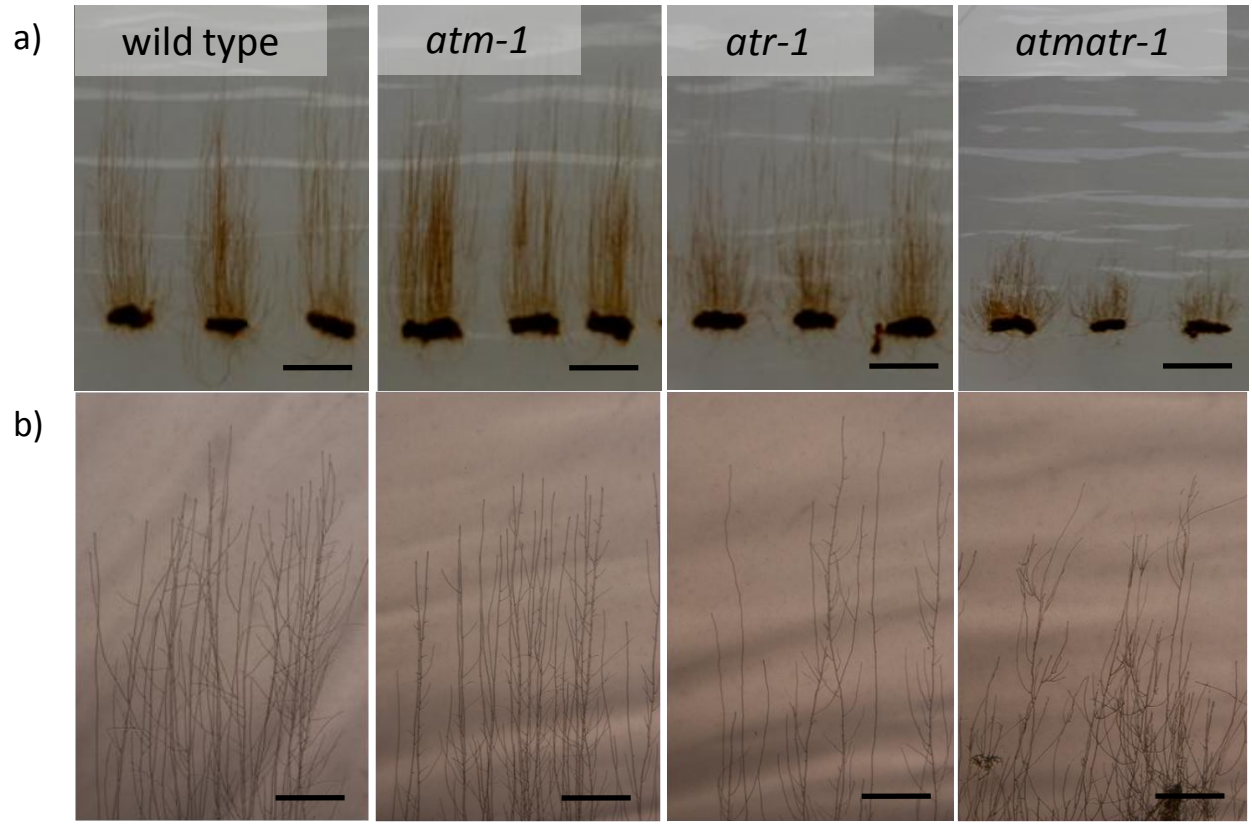

Figure 3.2-30: Restricting growth to caulonema type filaments reveals caulonemal growth deficiency in *atr-1* and *atmatr-1*. (**a**) Overview of caulonema filaments regenerated from small pieces of protonema tissue in darkness, on PPNH4 media supplemented with glucose, for three weeks. Notice the reduced length in *atr-1* and *atmatr-1*. Scale bar = 0.5 cm. (**b**) Closer view of the apical filament region showing the density and morphology of the longest caulonema filaments in each line.

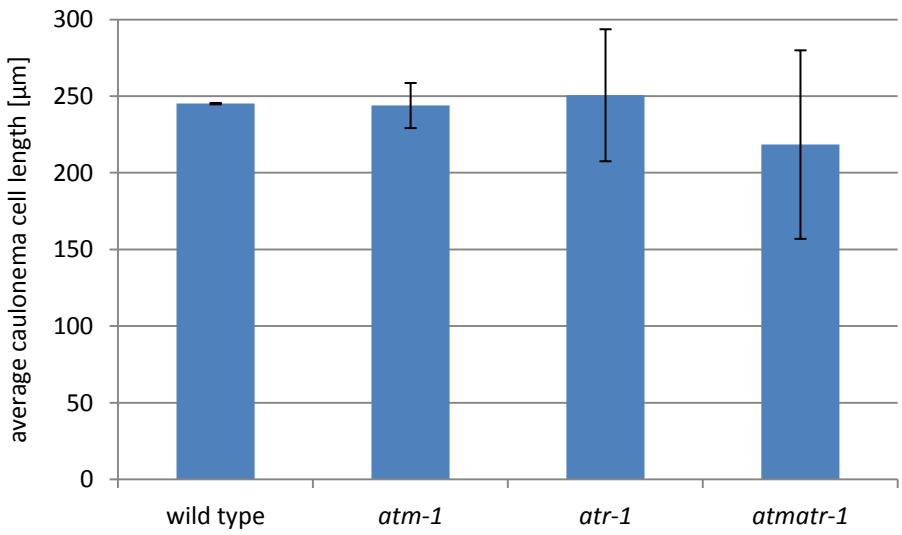

Figure 3.2-31: Caulonema cells show no significant decrease in length in any of the mutant lines. The average length of cells in protonema filaments grown for 3 weeks in darkness on PPNH4 media supplemented with glucose is shown for wild type and mutant lines. Error bars show standard deviation in between two replicate experiments. Statistical analysis via one-way ANOVA confirmed that no significant differences in cell length were observed between the lines *(F*(3,4) = 0.28, P = 0.83).

Considering that caulonema filament length but not the length of individual cells was affected in *atr-1* and much more prominently *atmatr-1* it can be assumed that proliferation of caulonemal cells is impaired slightly in *atr-1* and but much more severely in *atmatr-1*. This finding also helps in understanding the lack of an increased caulonemal growth phenotype of the double mutant in the initial growth assays. There it was presumed that lack of fully functional ATM or ATR had lead to cellular stress, which plantlets responded to with increased caulonemal growth as was observed in the single mutants. If this hypothesis was true the double mutant should have by any means be subjected to even more stress, suggesting an at least equally strong increase in caulonemal growth. With the knowledge that caulonemal proliferation in especially the double mutant is strongly impaired however the increased caulonemal growth could simply be counterbalanced by this. The findings also suggest that ATM and ATR function in a partially redundant manner in assuring proper caulonema proliferation, as only the double knockout displayed severe caulonemal growth impairment.

3.2.7.5 Growth retardation observed during early filament regeneration of *atr-1* and *atmatr-1* mutants appears to be largely temporary.

Besides the aforementioned differences in caulonemal growth, another phenotype of early filament growth was that specifically *atr-1* and *atmatr-1* displayed an increased number of slowly growing filaments. ATM and ATR are known for their important role in DNA repair and cell cycle control. It therefore appeared possible that the increased numbers of growth retarded filaments in *atr-1* and *atmatr-1* lines may stem from detrimental mutations occurring at a higher rate than in the wild type. To determine whether a genomic defect was indeed the cause of this phenotype it was observed whether or not the slower growth speed in the affected filaments was a permanent effect. For this purpose, filaments which were markedly impaired in regeneration at day 10 of culture were marked and observed again after 8 more days of growth. It was found that the vast majority of initially slow growing filaments increased their growth speed considerably in wild type as well as mutant lines (**Figure 3.2-32**). This implies that that the increased number of initially growth deficient filaments in *atr-1* and *atmatr-1* are likely not due to mutational effects. However, there were still about 20 % of *atr-1* and *atmatr-1* filaments which did not increase their growth speed during the observed time frame. To test whether the cells of these filaments were indeed permanently growth impaired small filament pieces were extracted from a number of them and transferred onto fresh plates for an additional cycle of regeneration. Regenerating them once more, it was observed that indeed the majority of these filaments were again impaired in growth (data not shown). Thus while the majority of growth retarded filaments, which occurred at an increased rate in *atr-1* and *atmatr-1*, were temporary and likely not caused by genome aberrations it cannot be ruled out that the aforementioned lines may suffer from increased mutational rates.

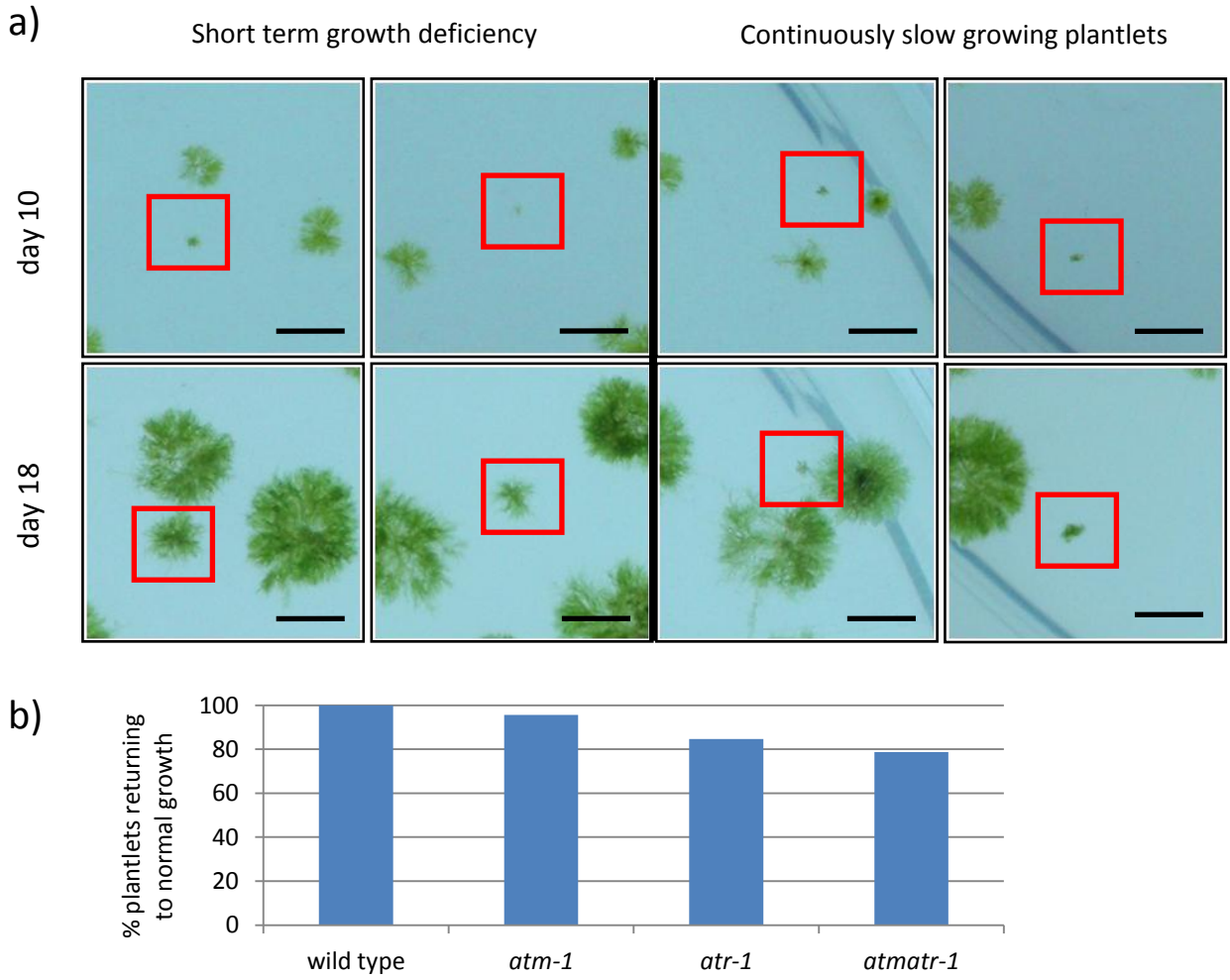

Figure 3.2-32: Growth retardations observed during early stage of filament regeneration are mostly temporary. (**a**) Selection of *atmatr-1* plantlets which had grown very slowly during the initial 10 days after plating and either continued to do so for at least eight more days (right) or increased their growth speed considerably (left). (**b**) Percentage of initially slow growing plantlets of wild type, *atm-1*, *atr-1* and *atmatr-1* plantlets which appeared to increase their growth speed considerably from day 10 to 18. The numbers in brackets refer to the total amount of initially small plantlets which were evaluated in this regard. Size bar indicates 0.5 cm.

3.2.7.6 Temporary growth deficiencies observed during early regeneration of *atr-1* and *atmatr-1* filaments are likely related to the method of sample preparation.

The fact that the growth retardation observed in *atr-1* and *atmatr-1* was a largely temporary effect suggested that it may be related to sample preparation. This suspicion was supported by the fact that sample preparation involved shredding of large filaments into smaller ones by using a tissue homogenizer. This meant that while some cells were broken apart outright others were likely to have suffered damage to their cell wall, membrane and maybe organelles. Considering mutant lines are already disturbed in cellular homeostasis, as is evident from the large amount of transcriptome changes reported in a previous chapter, the additional stress from the homogenization process may affect them to a greater degree than the wild type.

Thus it was decided to test whether the process of sample preparation, as used for the growth assays, may indeed have had an effect on the subsequent regeneration process. For this purpose the regeneration efficiency of small filament fragments, as obtained via mechanical disruption, was compared to that of individual protoplasts, which were isolated by enzymatic cell wall digestion. Regeneration efficiency was measured as percentage of filaments/protoplasts which survived the initial stage of regeneration and regenerated into small plantlets.

Comparing the result of the two different experiments it was found that while regeneration efficiencies of *atr-1* and *atmatr-1* were reduced utilizing mechanically fragmented filaments as starting material this was not the case using the protoplast based approach (**Figure 3.2-33**). Thus it seems that the deficiencies observed during early regeneration in *atr-1* and *atmatr-1* in the previous growth assays was indeed related to the method of sample preparation. This suggested that loss of especially ATR functionality may sensitize cells to mechanical stress and its consequences as imposed by high shearing forces.

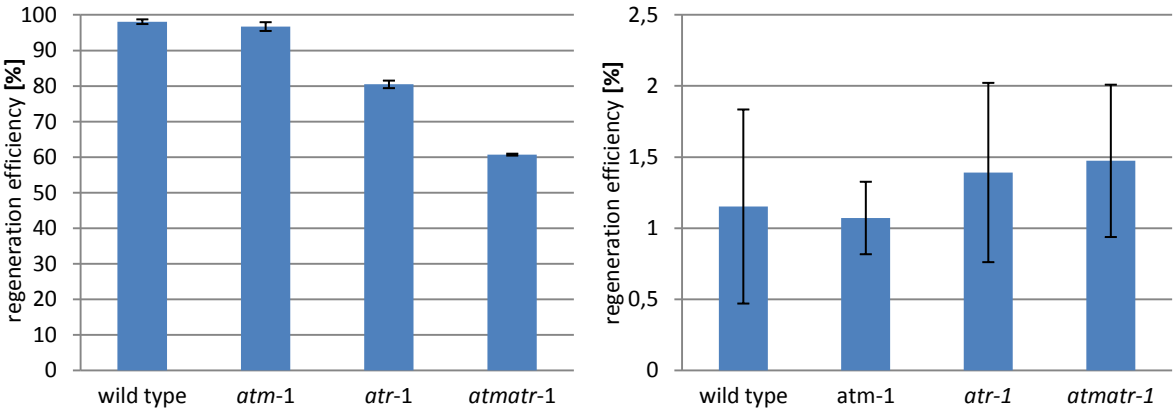

Figure 3.2-33: Regeneration efficiency of *atr-1* and *atmatr-1* mutants is dependent on the method of sample preparation. (**a**) 7 day old subcultures of wild type and mutant lines were shredded via tissue homogenizer. Obtained filament fragments of approximately 1-5 cells in size were regenerated on standard media for 6 days. Regeneration efficiencies were calculated from regenerated plantlets assessed at day 6 of growth and number of initially plated filaments. According to one-way ANOVA regeneration efficiencies were found to differ significantly in between the lines ($F(3,8) = 1165.13$, $P = 6.68E-11$). Tukey's post hoc test confirmed significant differences in regeneration efficiency between all lines but wt and *atm-1* ($P<0.05$). (**b**) Protoplasts of wild type and mutant lines were isolated from 7 day old subcultures and regenerated on standard media supplied with an osmoticum. Regeneration efficiency was calculated from regenerated plantlets counted after 10 days of growth and initial number of visually healthy protoplasts. Error bars indicate standard deviation in between 6 independent experiments. As determined by one way ANOVA there were no statistically significant differences between group means ($F(3,24) = 0.84$, $P = 0.48$).

3.2.7.7 Prolonged vegetative propagation did not markedly deteriorate growth capacities of mutant lines.

As was previously assessed, a number of filaments of especially *atr-1* and *atmatr-1* appeared to display permanent growth retardation. It was therefore speculated that the aforementioned lines may suffer from a hypermutation phenotype, the accumulation of detrimental mutations permanently impairing growth of affected cells. While the main functionality ascribed to ATM and ATR is indeed to maintain genome integrity, they are mainly thought to do so by providing checkpoint control and facilitate the repair of DNA DSBs. While a lack of these functions would not necessarily lead to an increased mutational rate, recent studies have found evidence supporting an involvement of ATM and ATR in nucleotide excision repair (NER) (Colton et al. 2006, Wu et al. 2006, Shell et al. 2009)**.** As deficiencies in the NER pathway are indeed known to promote incidence of mutations (Sancar 1996, Scott et al. 1999, Friedberg et al. 2000), a possible involvement of ATM and ATR in this process may indeed have produced the permanent growth retardation observed in a number of *atr-1* and *atmatr-1* filaments. To address the question of whether or not mutations may accumulate in the mutant lines, it was opted to perform a growth assay in which old and young cultures were compared regarding their growth capacity.

In this assay, small filament fragments were prepared from cultures which had undergone differing numbers of vegetative propagation cycles and plated on standard media. After 10 days of growth, the average surface area of plantlets was measured and the percentaged change in surface area from younger to older cultures was calculated. This type of assay was performed twice, comparing plant material which had undergone 6 and 18 propagation cycles as well as 8 and 41 cycles. Observing the changes in average surface area from younger to older cultures, no distinctly negative tendency was observed in any of the mutant lines **(Figure 3.2-34)**. This implied that there was no marked increase in cells harboring growth impairing mutations in any of the lines as a result of prolonged vegetative propagation. This result suggested an either weak or no mutator phenotype in any of the mutant lines.

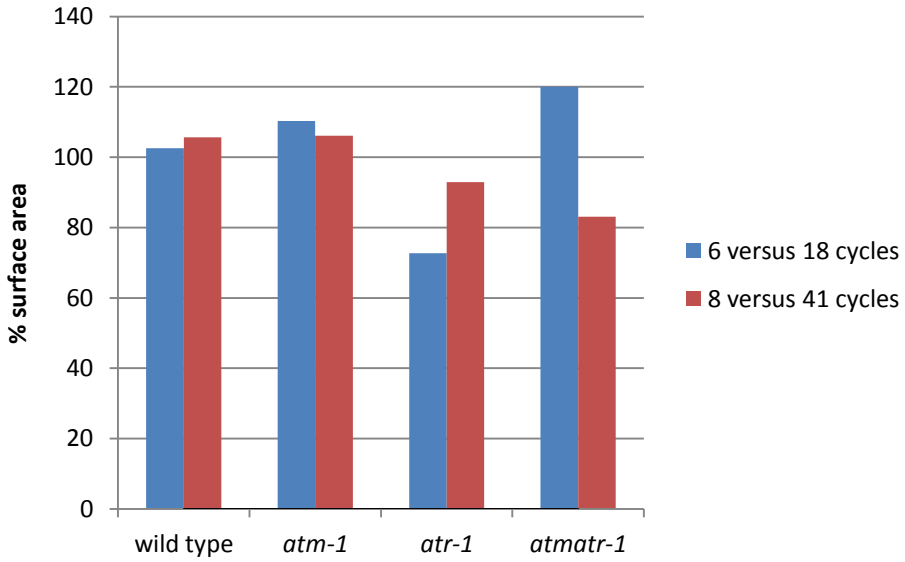

Figure 3.2-34: Average growth speed of plantlets is not affected by number of vegetative propagation cycles. Percentage change in average plantlet surface area when comparing young cultures which were vegetatively propagated 6 or 8 times versus cultures propagated 18 and 41 times respectively.

3.2.7.8 Gametophore development is impaired in *atr-1* and *atmatr-1*

The beginning of the adult gametophytic stage of *P. patens* is marked by the switch from purely filamentous growth to the formation of more complex leafy structures, the gametophores. The initial stage of gametophore development starts with the formation of a bud, a meristematic tissue which forms out of a caulonema side branch initial cell. This meristem then proceeds to form a simple leafy shoot, consisting of a stem, leaflets and rhizoids.

During the initial observation of this lifecycle stage it was found that the number of developing gametophores was much reduced in *atr-1* and *atmatr-1*. To examine this phenomenon in greater detail, gametophore development was closely monitored in individual plantlets.

In this assay similarly sized small plantlets pregrown on standard PPNH4 medium overlaid with cellophane were once more used as starting material. These plantlets were then grown for up to 8 weeks to observe gametophore development over an extended period of time.
It was observed that in wild type and *atm-1* plantlets a large number of big and healthy looking gametophores had developed after a total of about 4 weeks of growth **(Figure 3.2-35)**. In *atr-1* and *atmatr-1* plantlets on the other hand only few or close to no gametophores were observed respectively. Isolating gametophores and observing them under the microscope it was furthermore found that *atr-1* and *atmatr-1* gametophores displayed severe deformities. The outgrowth of leaflets was often asymmetrical, with leaflets themselves regularly appearing twisted in shape. Continued culture of these plantlets for 4 additional weeks did not yield any significant improvement on that

phenotype, the number of visible gametophores remaining low and their morphology aberrant. This implied that ATR as well as ATM play a role in gametophore development albeit the functionality of ATM appears to be redundant to ATR in this regard. This still left the question of what may have been the immediate cause for the highly reduced number of bigger gametophores in *atr-1* and *atmatr-1* plantlets. Two explanations seemed possible. Either the growth of the majority of gametophores in these lines was impaired so severely or even arrested that they never got big, or the developmental switch from caulonema to buds, the precursor structures of gametophores, could have been affected. Observing 4 week old plantlets under transmissive light (**Figure 3.2-36**) it appeared as if a similar number of gametophores had actually formed in *atr-1* and *atmatr-1*. This implied that the reduced number of larger gametophores was in fact largely related to a severe growth impairment of these structures.

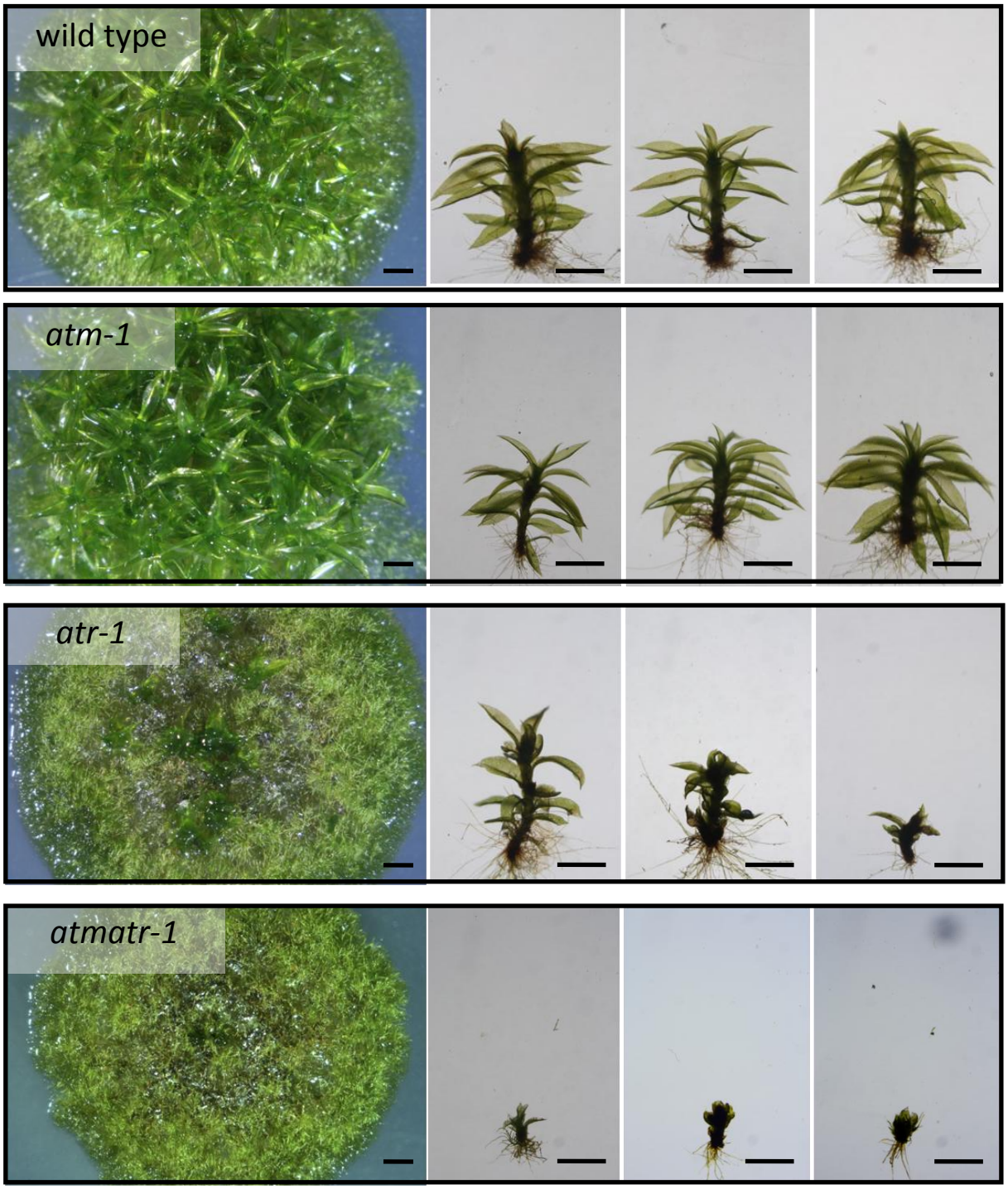

Figure 3.2-35: Gametophore growth appears to be drastically reduced as well as aberrant in *atr-1* and even more so *atmatr-1*. Plantlets were grown on standard PPNH4 media in constant light for a total of 4 weeks. Isolated gametophores are representative for the majority gametophores as observed in wild type and mutant lines respectively. Scale bars = 1 mm.

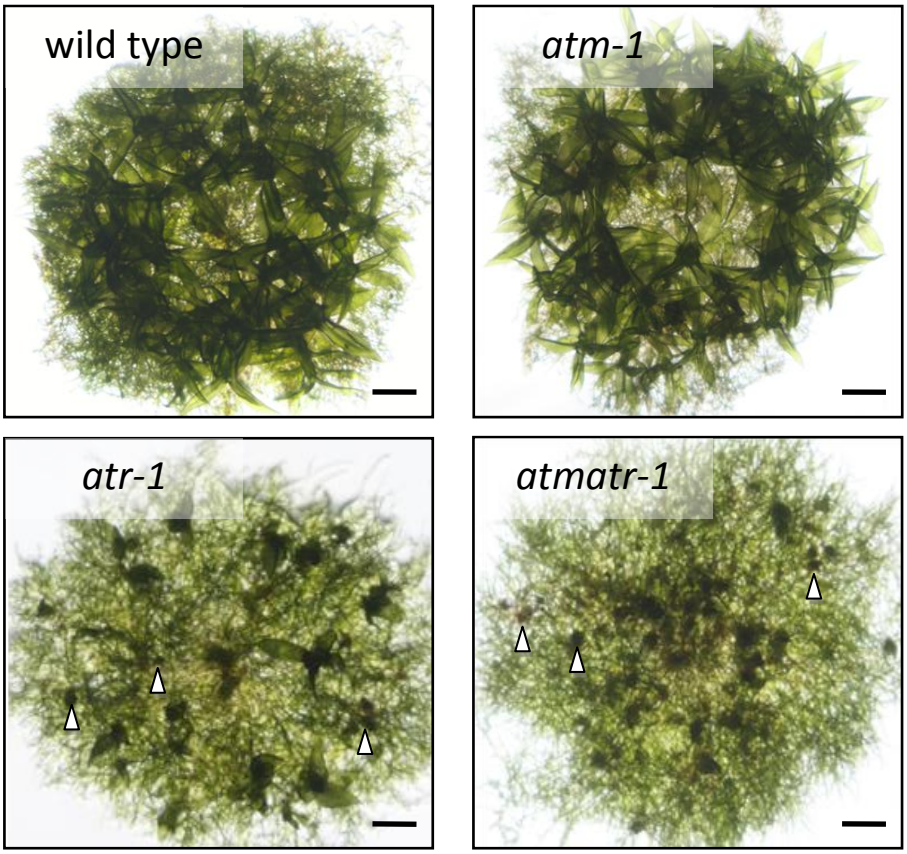

Figure 3.2-36: Developmental switch from protonemal to gametophore growth appears not to be markedly impaired in *atr-1* and *atmatr-1*. Four week old plantlets of wild type and mutant lines are shown as they appear under intense transmissive light. White arrows point out dense clumps of cells which indicate young gametophore structures as they are readily formed in *atr-1* and *atmatr-1*. Scale bar = 1 mm.

3.2.7.9 Severity of *atr-1* and *atmatr-1* Gametophore phenotype is affected by growth conditions

In order to try and obtain spores of our mutant lines plantlets had to be grown on PP- medium to promote sexual propagation. This medium differs from the standard medium in that it lacks the ammonium tartrate as an added nitrogen source. Growing plantlets on this type of media, a distinct effect on the previously reported gametophore phenotype of the *ATR* mutants was observed. To elaborate on this observation an additional study of gametophore development on this media type was performed. In this assay, analogous to the previous one, small pregrown protonema colonies of similar size were used as starting material. This time however they were transferred onto PP- instead of PPNH4 media for further growth.

The first general thing to be noticed was that the gametophores as well as plantlets as a whole were considerably smaller after the same time of growth on PP- than on PPNH4 (compare **Figure 3.2-35** and **Figure 3.2-37**). Second, the gametophores of *atr-1* and to a lesser degree *atmatr-1* grew markedly better on PP- than they did on PPNH4. While on the PPNH4 media the differences between

number and morphology of gametophores compared to the wild type were considerable, these differences were markedly less pronounced on PP-.

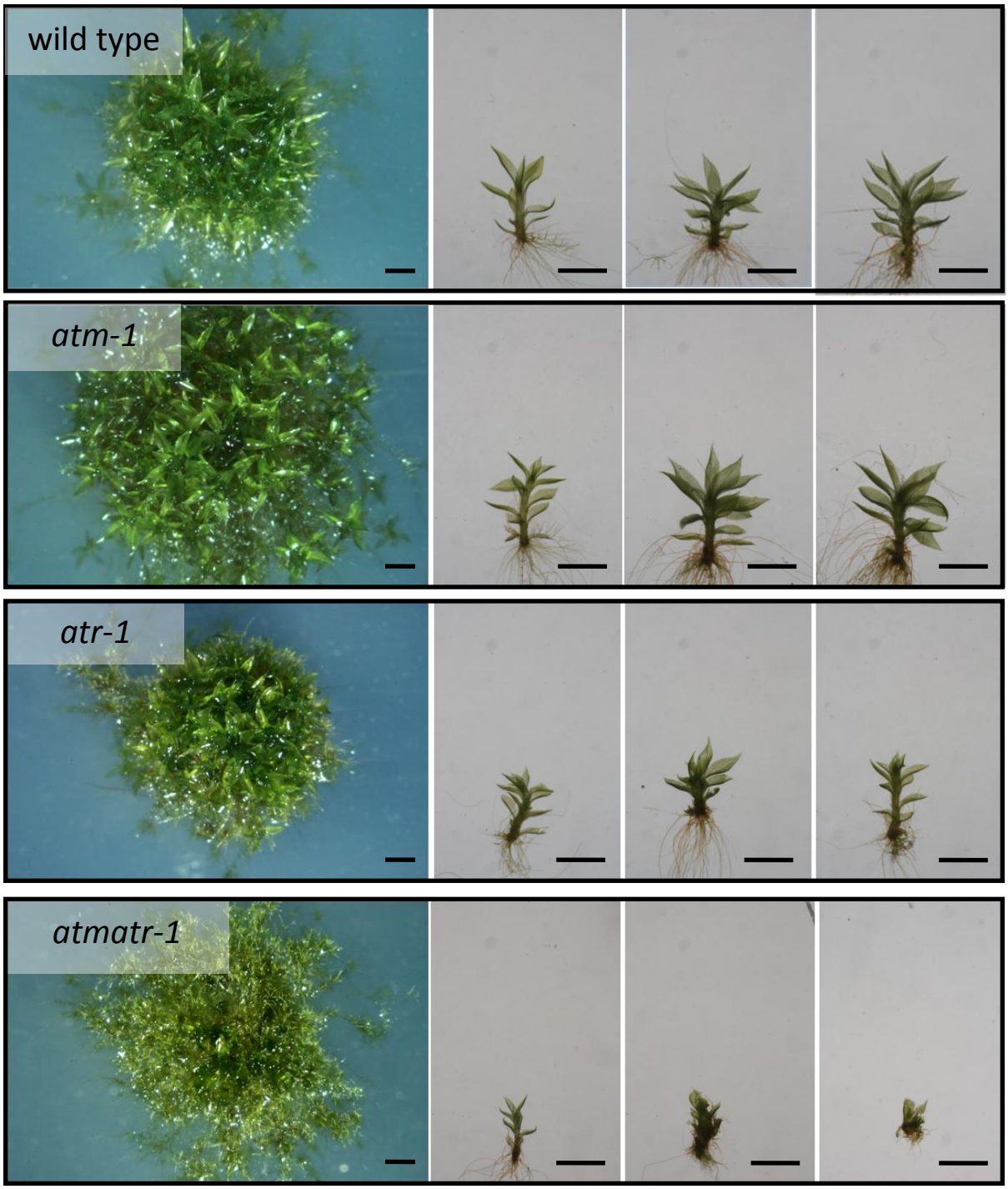

Figure 3.2-37: Gametophore formation is improved in *atr-1* and *atmatr-1* mutant plantlets if grown on less nutrient rich media. Plantlets were grown on standard PPNH4 media in constant light for a total of 4 weeks. Isolated gametophores are representative for the majority gametophores as observed in wild type and mutant lines respectively. Scale bar = 1 mm.

Thus it appeared that the previously reported impaired gametophore growth in *atr-1* and *atmatr-1* could be alleviated by growing plantlets on less nutrient rich media. As mentioned before the omission of nitrogen also led to an overall reduced size of plantlets as well as gametophores. Since starting material was similar sized and gametophore development commenced after a similar number of days on both media, this finding suggested that lack of nitrogen may have restricted and also slowed down growth on the PP- media. This proposed that a reduction in proliferation speed may have alleviated the severity of the gametophore phenotypes of *atr-1* and *atmatr-1*.

3.2.7.10 *Atr-1* and *atmatr-1* plantlets are sterile

The reproductive stage in *P. patens* development begins with the formation of the male and female gametangia, designated as antheridia and archegonia respectively. These form at the apex of mature gametophores and in due time the antheridia will release the spermatozoids which may subsequently fertilize the egg cells in the archegonia. This will give rise to the diploid sporophyte in which the spores for the following generation will be generated via meiosis.

To evaluate the capacity for spore formation in our mutant lines, the reproductive development was observed from the development of gametangia to the formation of spores.
In this assay, plantlets were grown on PP- medium, and subjected to a specific light and temperature regime to promote sexual propagation. To compensate for any delays in sporophyte formation in our mutant lines, plantlets were incubated under these conditions for up to two years.

In wild type as well as *atm-1* plantlets the first gametangia were observed after about 2 months. In *atr-1* and *atmatr-1* however the formation of gametangia was found to be delayed about 4 and 10 weeks respectively. Moreover, comparing the morphology of individual gametangia under the microscope, it was noticed that *atr-1* and *atmatr-1* archegonia, but not antheridia, were largely aberrant (**Figure 3.2-38**).
Monitoring the further development of plantlets, mature sporophytes were observed after about a total of 4 months in wild type as well as *atm-1* but not *atr-1* and *atmatr-1* (**Figure 3.2-39**). Although cultures were maintained for up to two years, to compensate for any delay in sporophyte formation, *atr-1* and *atmatr-1* were found to be practically sterile. To determine whether the observed sterility was due to defective male or female gametes, a set of gametophores which had produced sporophytes in a successful cross of *ATM* and *ATR* mutant lines was genotyped (data not shown). As it turned out, all of the sporophytes originated from *ATM* gametophores. This implied that in this analyzed subset all of the crosses originated from a fusion of male gametes from an *ATR* mutant with female gametes from an *ATM* mutant. This suggested a male sterility phenotype of *ATR* mutants.

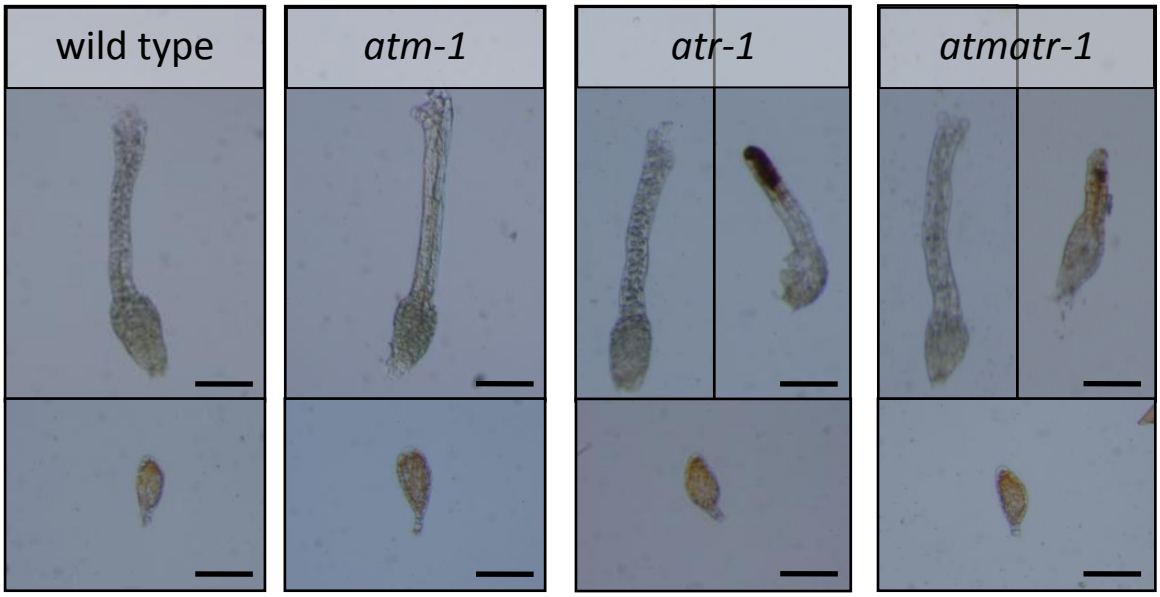

Figure 3.2-38: Female gametangia (archegonia) are *atr-1* and *atmatr-1* display aberrant archegonia. Pictures show female (archegonia, top) and male (antheridia, bottom) gametangia as they are regularly found in the respective lines. Scale bar = 100 µm

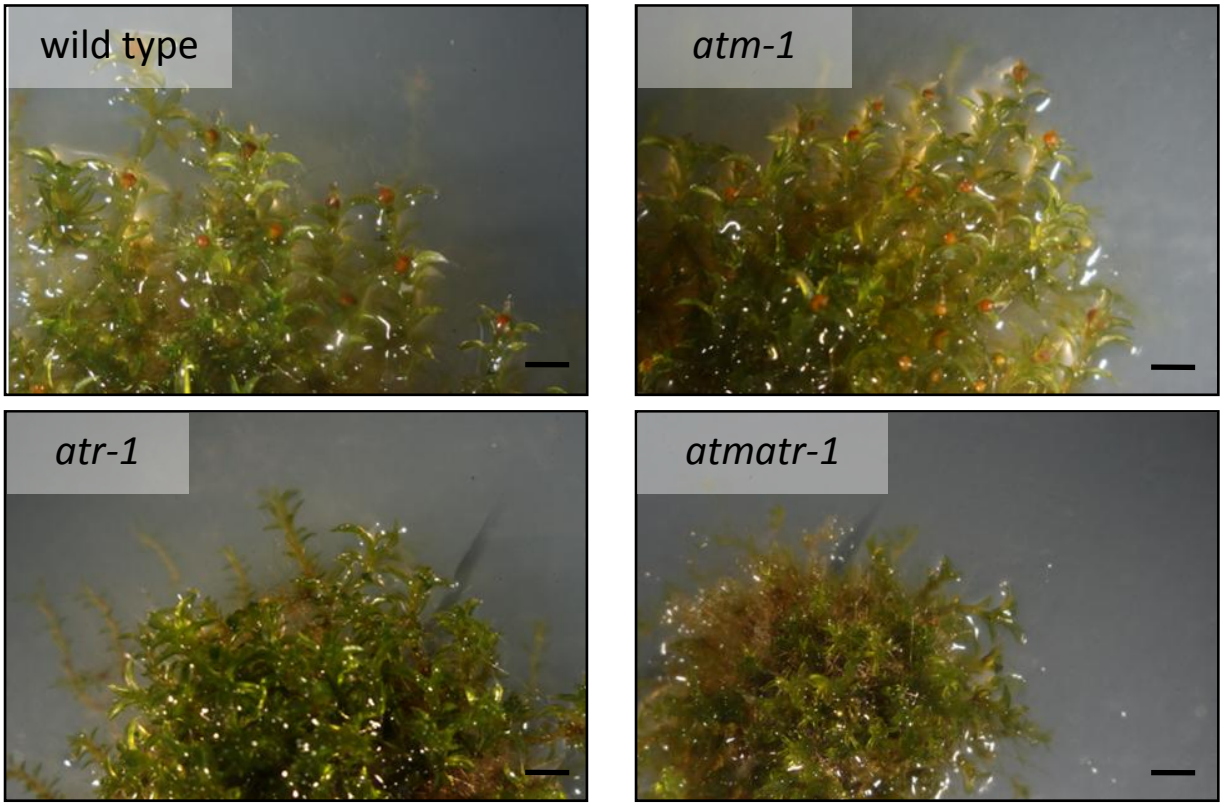

Figure 3.2-39: Plantlets of *atr-1* and *atmatr-1* are practically sterile. The plantlets shown were grown for a total of 4 months on PP- media. Growth conditions were switched from the standard 24h light, 26°C growth conditions to 16h light and 15°C after the first two months to induce sexual propagation. Scale bar = 100 µm.

3.2.7.11 Observed growth phenotypes are unlikely to be a cause of secondary mutations

The central function of ATM and ATR is to maintain genome integrity. In addition ATR mutants were shown to regularly produce growth retarded plantlets whose origin was hypothesized to stem from an increased mutation rate in these lines. Therefore, to support the claim that the previously reported phenotypes were indeed due to loss of ATM and ATR function and not some secondary mutation, key phenotypic observations were checked again by assaying additional independent ATM and ATR single mutant lines. First the enhanced caulonemal growth phenotype as reported in **3.2.7.2**. It was found that the additional *ATM* mutant lines, *atm-2* and *atm-3* displayed a similarly increased caulonemal growth as previously shown for *atm-1* (**Appendix**, **Figure 5.5-1**). In case of the ATR mutants the result was not as clear, while *atr*-2 displayed the same increase in caulonemal growth as shown for *atr-1, atr*-3 did not. Including another *ATR* mutant into the analysis, *atr-4*, that line was again found to show the increased caulonemal growth phenotype. Considering 3 out of 4 independent ATR mutant lines displayed the caulonema phenotype it appears reasonable to assume that it is related to the *ATR* mutation still. The second aspect which was checked was the regeneration efficiency of homogenized filament fragments as tested for the standard lines in **3.2.7.6.** It was found that the additionally tested lines *atm*-2, *atm*-3, *atr*-2 and *atr*-3 all behaved similar to *atm-1* and *atr-1* respectively (**Appendix**, **Figure 5.5-2**). During the adult gametophytic stage *atr-1* was shown to be impaired in gametophore growth, a phenotype which could be remedied by growing plantlets on media with reduced nitrogen availability. Reproducing the experiment as described in **3.2.7.8** and **3.2.7.9**. It was found that gametophore growth displayed the same deficiencies in *atr*-2 and *atr*-3 as were observed in *atr-1*, including the reduction in phenotype severity when grown on PP- instead of PPNH4 medium (**Appendix**, **Figure 5.5-3**). Finally, the sterility phenotype of *atr-1* was also observed in *atr*-2 and *atr*-3 (data not shown).

It therefore seems very likely that the previously described growth and developmental phenotypes described for *atm-1* and *atr-1* as well were indeed specific to the loss of ATM and ATR function and not due to secondary mutations. Additional double mutant lines were not tested since these were generated from the previously tested single mutants via crossing.

4 Discussion

4.1 Introduction

Gene targeting (GT) is a currently developing technique with important implications to agricultural research. GT designates the site-specific integration of an external DNA fragment into the genome of a host organism based on sequence homology and using the host's endogenous homologous recombination (HR) mechanism. In principle, GT enables the creation of arbitrary, designed modifications in the genome therefore it is also referred to as precise genome engineering, a powerful tool for "next generation" plant breeding.

While most crop plants are very inefficient in regards to GT, the moss *P. patens* exhibits very good GT properties. Higher plant species tested so far (both monocot and dicot) typically integrate transgenes to random genomic positions via the non homologous end joining (NHEJ) pathway, targeted transgene integrations occurvery rarely (Halfter et al. 1992, Zhu et al. 1999, Hanin et al. 2001, Iida and Terada 2004). In contrast, targeted integration events based on homologous recombination are frequent in *P. patens* (Schaefer and Zryd 1997, Kamisugi et al. 2006). Therefore *P. patens* quickly became a model organism of HR and gene targeting. One of the long term goals of *P. patens* research is to reveal the molecular mechanisms behind its exceptional recombination and therefore gene targeting properties and to transfer them to other plant species. However, to date there has been no conclusive answer to the question what makes *P. patens* so proficient in homologous recombination and thus gene targeting (Puchta and Fauser 2013). There are two main possibilities to explain this phenomenon. Firstly, while higher plants are also able to incorporate DNA constructs via HR, it happens rarely and integration occurs preferably at random, via the non homologous end joining (NHEJ) pathway. This implies that higher plants actually have all the genes necessary for HR. Thus the key difference to *P. patens* might lie with the regulation of the genes that decide which pathway should be used to integrate the linear DNA fragments either randomly via NHEJ or targeted via HR. Second, it also has to be considered that *P. patens* may have evolved a different set of recombination genes to achieve more efficient HR, which may include different gene variants of the known recombination genes, or the existence of specific new genes as well as the absence of components of the NHEJ pathway. Even if this second option may first sound unlikely, it has been revealed that in comparison to crop plants and Arabidopsis*, P. patens* in fact contains an additional gene encoding for a RAD51 recombinase, one of the key components of the recombination machinery (Ayora et al. 2002, Markmann-Mulisch et al. 2002, Markmann-Mulisch et al. 2007). At the time of these publications resources for *P. patens* were not so developed as today and genes had to be isolated and studied individually. With time and the advance of technology the genome of *P. patens* was sequenced in 2008 (Rensing et al. 2008), but a systematic comparison of

recombination genes between yeast, mammals, higher plants and *P. patens* was yet to be done. Intriguingly, merely expressing any of the two *P. patens Rad51* genes, or even both of them simultaneously in Arabidopsis did not dramatically increase GT efficiency (Bernd Reiss, unpublished) which suggests that the true explanation of *Physcomitrella*'s recombination properties may involve both hypothesis.

The goal of this PhD thesis is to extend the knowledge regarding both of these hypotheses. The first part of the present thesis is the compilation of a comparative recombination gene list from human, yeast, Arabidopsis and *P. patens*, by extracting information of present day databases with the help of bioinformatics. This list was expected to reveal differences on the level of presence or absence of genes based on sequence homologies. This catalogue of *P. patens* recombination genes may be of additional use as a basis for more detailed bioinformatic investigations, which may involve sequence comparisons, domain analysis, or the study of evolutionary relations.

The second part of this thesis focuses on the functional analysis of ATM and ATR, two central components of homologous recombination (Cimprich and Cortez 2008, Shiloh and Ziv 2013). *P. patens* is an exceptionally suitable model organism to study the function of these two genes due to its previously mentioned bias towards HR as compared to Arabidopsis which mainly utilizes NHEJ. In addition, the proficiency of *P. patens* in gene targeting simplifies the mutation of its *ATM* and *ATR* genes. ATM and ATR are two centrally important kinases in the signal transduction of DNA double strand breaks (DSB), a lesion which may be repaired via HR or NHEJ. These two kinases are located at upstream positions in the signaling cascade, possibly being involved in determining which one of these two options for DSB repair to use. Building on this property, the second chapter of the present thesis involves the preparation and detailed phenotypic analysis on *ATM* and *ATR* mutants. Here in the discussion the results are evaluated in a broader context, especially in comparison to mutations in the homolog genes in Arabidopsis published earlier (Garcia et al. 2003, Culligan et al. 2004, Culligan et al. 2006), but also phylogenetically more distant organisms like yeast and mammals.

4.2 The DNA repair and recombination gene subset of *P. patens*

Sequencing the *P. patens* genome in 2008 and opening the data to the public in the COSMOSS database made it possible to systematically compare the DNA repair and recombination gene set of *P. patens* with those of other organisms. Maintaining genome integrity is a very important function, therefore genes involved in DNA damage repair and HR seem well conserved from bacteria to eukaryotes (Cromie et al. 2001). However, at the same time there are also prominent differences, some organisms lacking genes which in others have been deemed highly important or even essential. For instance the cell cycle checkpoint kinases Chk1 and Chk2, which are responsible to halt the cell

cycle in response to DNA DSB damage in mammals and yeast (Shieh et al. 2000, Sørensen et al. 2005, Huang et al. 2008) have not been found in plants (De Veylder et al. 2007, Cools et al. 2011). Another example is the Rad52 gene, which is essential in yeast but a sequence homolog is missing from plants, although recently a potential functional ortholog was described in Arabidopsis. The systematic comparison of such evolutionary divergences may reveal key points in the specific life strategies of these organisms.

From the point of view of this thesis the main question was, whether the identified differences can suggest candidates responsible for the more efficient gene targeting properties in *P. patens*. It should be noted that this topic has also been discussed in the 2008 release paper for the *P. patens* genome draft (Rensing et al. 2008). However, this dataset of genome annotation was based on an automated BLAST procedure, without additional verification of BLAST hits via backBLAST or further in depth manual curation. This suggested that the annotation and thus the discussion of presence/absence of recombination genes contained inaccuracies. Indeed, when comparing the results of our largely manual and more elaborate effort of annotating the set of recombination genes in *P. patens* a number of inaccuracies were found in the previously published dataset. In support of our findings a recent revision of the *P. patens* genome annotation has fixed many if not all of the identified inaccuracies (Zimmer et al. 2013). To assess the available recombination genes in *P. patens* a BLAST search using protein sequences of known recombination genes from human, yeast and Arabidopsis was conducted. The identified putative recombination gene homologs were verified via reciprocal BLAST and in some cases the domain structure was also analyzed and phylogenetic trees were constructed. As an overall evaluation, it was found that from the known recombination gene sets of human, yeast and Arabidopsis 72 %, 72 % and 94 % respectively could be identified in *P. patens*. This is well reflected in the closer phylogenetic distance between Arabidopsis and *P. patens*. However it is also obvious that the overlap between *P. patens* and yeast as well as human is still quite high. Having obtained the list of available recombination genes the first question was whether the dominance of HR over NHEJ in *P. patens* might be due to missing NHEJ genes, as was speculated earlier. In agreement with the findings of Rensing and coworkers (Rensing et al. 2008) it was observed that all the central NHEJ components such as Ku70, Ku80, DNA ligase 4, XRCC4 and NHEJ1 (Hefferin and Tomkinson 2005), are present in *P. patens*. Therefore it seems that the bias of *P.patens* towards HR is not because it is missing certain NHEJ genes. In fact, there is one central NHEJ component which was found present in *P. patens* but is not available in Arabidopsis. The gene in question is the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Studies conducted in mammalian model systems have shown that DNA-PKcs forms a heterotrimer with two other important NHEJ components, Ku70 and Ku80 to constitute the DNA-PK holoenzyme (Spagnolo et al. 2006). DNA-PK is a pivotal component of NHEJ-based DSB repair as it facilitates the early stage of DSB

rejoining and is also known to phosphorylate additional downstream repair components (Smith and Jackson 1999, Hill and Lee 2010). The necessity of DNA-PKcs for DSB repair has been demonstrated in the mouse model system as well as in human cell culture, as mutations in the *DNA-PKcs* gene caused increased sensitivity to DNA DSB inducing agents (Houghtaling et al. 2005). It is therefore interesting to point out that an organism such as Arabidopsis, which has been found to mainly utilize NHEJ for DSB repair as well as transgene integration (Reiss 2003, Markmann-Mulisch et al. 2007), would be lacking a prominent NHEJ component while *P. patens*, which is supposed to rely largely on HR (Schaefer 2001, Markmann-Mulisch et al. 2007) does not. Finally, considering that all of the central NHEJ genes are available in *P. patens* there seems to be no grounds to claim that its bias towards HR is related to an incomplete/lacking assortment of known NHEJ components.

Next it was investigated whether *Physcomitrella* has some additional, species-specific genes to enhance HR and thereby gene targeting. Out of the total of 119 DNA repair gene homologues which were found in *P. patens*, 7 are not found in Arabidopsis. These are *PKMYT1, WRN, Rad54B, DDX11, MutS, SIR2* and the previously mentioned PRKDC. Out of these 7 genes *WRN* and *Rad54B* are known to be involved in homologous recombination. However, it is assumed that the function of WRN is actually represented in Arabidopsis, but split between two other genes, *WRX* and RecQ2 (Hartung et al. 2000, Plchova et al. 2003, Kobbe et al. 2008). This leaves Rad54B as the remaining candidate. Evidence suggesting an involvement of Rad54B in homologous recombination has been established in mammals. First, Rad54B has been found to colocalize with Rad51, a key component of homologous recombination (Tanaka et al. 2000). Second, it was shown that the HR based process of gene targeting was less efficient in a human *Rad54B* mutant cell line (Miyagawa et al. 2002). It is interesting to note that in the same mutant the measured overall resistance against DSB inducing agents was not compromised. This is unexpected as HR is supposed to be utilized for the artificial gene targeting process as well as somatic DSB repair. Indeed, another study conducted in mouse Rad54B deficient embryonal stem cells showed that in fact Rad54B was also important for the resistance to treatment with DSB inducing agents (Wesoly et al. 2006). Therefore, while there is some ambiguity regarding the HR function of Rad54B, this may be an interesting candidate gene to explore in regards to its capacity of influencing gene targeting efficiency.

Just like some known recombination genes present in *P. patens* were not found in Arabidopsis, there are also 10 genes in our list which are present in Arabidopsis but missing from *P. patens*. These include BRCA1, BRCA2, BARD1, RQL3, SYCP1, SYCP2, HDAC2 and XRCC3, which are also found in human but not in yeast. The remaining two, RAD7 and MEK1, meanwhile are available in yeast but not human. Among these genes there are four, which are known to be involved in homologous recombination, namely *BRCA1, BRCA2, BARD1 and XRCC3*. BRCA1 and BARD1 function together as a

heterodimer (Brzovic et al. 2001, Xia et al. 2003) and provide a number of functions including HR mediated DSB repair (Moynahan et al. 1999, Moynahan et al. 2001) but also act in regulation of cell cycle checkpoints in response to DNA damage (Xu et al. 2001, Yarden et al. 2002). BRCA2 was found to directly interact with the essential HR component Rad51 in mammals (Chen et al. 1998, Esashi et al. 2007) and has also been proven important in HR mediated DSB repair (Moynahan et al. 2001, Xia et al. 2001). Similarly, XRCC3 has also been shown to function in HR mediated DSB repair (Johnson et al. 1999) and it also interacts with Rad51, playing a role in the accumulation of the latter at sites of DNA breaks (Tambini et al. 2010). The fact that these four important components of the HR mediated DSB repair pathway are present in Arabidopsis but not *P. patens* may appear to contradict the hypothesis that the presence of specific HR genes are the reason of *P. patens* outstanding gene targeting ability. However the lack of these genes is also intriguing as yeast, another organism known for its bias towards HR, also does not contain any of these genes. It should be mentioned however that at least the functionality of BRCA2 is thought to be provided by another gene in yeast, *Rad52* (Sung 1997). Still, the absence of these four genes displays an interesting parallel in between the HR focused recombination apparatus in yeast and *P. patens* on the one hand and NHEJ biased mammals and Arabidopsis. The absence of these four factors implies a different setup of the HR pathways in these organisms which might very well be related to the differences in HR fidelity.

Besides the finding that the set of HR components in *P. patens* is different from that of other investigated organisms while displaying some interesting similarities to yeast, an additional interesting observation was made. For 33 out of the total of 120 recombination gene orthologs identified in *P. patens*, additional paralogs were found. Among those genes which were found in at least duplicate number are central components of homologous recombination like RAD54, RAD9, WRIP and PAXI1, in addition to a wide range of genes implied in a variety of other DNA repair processes. One well known source of such paralog genes are whole genome duplication events which are common in the evolutionary history of plants (Adams and Wendel 2005, Lee et al. 2013). Available evidence suggests that at least one such event has also occurred in *P. patens* (Rensing et al. 2007). While the majority of duplicated genes are generally lost after a duplication event (Blanc et al. 2003, Kellis et al. 2004), some are retained with altered function (Blanc and Wolfe 2004, Taylor and Raes 2004, Van de Peer et al. 2009). Interestingly, DNA repair genes are generally found to be among the most rarely retained gene duplications as has been determined in Arabidopsis (De Smet et al. 2013) as well as yeast (Scannell 2007). One possible explanation for the presence of a large number of duplicated DNA repair genes in *P. patens* could be that the duplication event may have occurred much more recently than that of Arabidopsis for example. However, there are studies claiming that the most recent whole genome duplication in Arabidopsis has occurred about 38 million years ago (Ermolaeva et al. 2003), thus being a more recent event compared to the 45 million years ago the *P.*

patens duplication has been dated (Rensing et al. 2007). This suggests that *P. patens* has a special predisposition towards retaining DNA repair genes. The important question in this regard surely is whether these DNA repair pathway gene duplicates in Physcomitrella have undergone functional diversification or if their functions are merely redundant. While not much related information is available, one of the key HR components, Rad51, of which there are 2 homologs available in *P. patens*, has been examined in this regard. To assess the contribution of each of the two homologs, single as well as a double knockout lines were generated and their sensitivity to genotoxic stress was tested. It was found that while each of the single mutants displayed a similar sensitivity as the wild type, the double mutant was markedly hypersensitive (Markmann-Mulisch et al. 2007). This implied that the two Rad51 homologs are in fact redundant in their DNA damage repair function.

Another possible reason for *P. patens* to retain additional copies of DNA repair genes might be related to its haploid nature. Having additional copies of key DNA repair factors would aid in preserving genome integrity should one copy suffer a loss of function mutation.

In summary, *P. patens* was found to show a number divergences in its in DNA repair and recombination gene complement. Whether the identified differences to Arabidopsis are indeed related to its much increased gene targeting efficiency remains to be seen. Nonetheless, this analysis provided a number of interesting candidate genes for functional analysis.

4.3 The ATM and ATR homologs of *P. patens*

Similar to their counterparts in other organisms *P. patens* ATM and ATR were found to be large proteins of 2897 and 2827 amino acid residues respectively. The *P. patens* orthologs also contain the conserved C-terminal domains, namely the FAT, PI3K and FAT-C domain. In addition, residues critical for function of the kinase domains, as determined by studies in mammals, are conserved as well. This was to be expected as these C-terminal domains are thought to be essential for the function of ATM as well as ATR (De Klein et al. 2000, Turenne et al. 2001, Madhusudan and Wilson III 2013). With respect to the generally much less conserved N-terminal region (Durocher and Jackson 2001, Seidel et al. 2008) the *P. patens* ATM homolog however displayed some interesting differences in comparison to the homologs of other organisms. According to domain predictions performed with the InterProScan software (Hunter et al. 2011) the very N-terminal sections of the human and yeast ATM homologues contain a TAN domain, the Arabidopsis homolog a PWWP domain and the *P. patens* homolog neither of the two. With regard to the TAN domain a study performed in yeast has shown it to be important for ATM's function in response to genotoxic stress (Seidel et al. 2008). While no study is available on the function of the PWWP domain, as it is found in the Arabidopsis ATM homolog, this domain is common in >60 eukaryotic proteins (Qiu et al. 2002). In studies

conducted in mammalian model systems it was found that the PWWP domain has a function in DNA-binding (Qiu et al. 2002, Ge et al. 2004, Lukasik et al. 2006) as well as protein binding, specifically histones (Wu et al. 2011). Proteins which carry this domain are generally found involved in DNA-repair, -transcription or -methylation (Qiu et al. 2002). The fact that the *P. patens* homolog of ATM displays neither of these two domains may therefore indicate an incomplete conservation of its function compared to its homologs. The second difference concerning the domain structure is that ATM in *P. patens* and also in yeast lack the Armadillo repeat domain which is present in the Arabidopsis and human homologs. The Armadillo type repeat forms a superhelical structure which is supposed to function in protein-protein interaction (Groves and Barford 1999). It is a rather common domain spread amongst a variety of proteins of plants and animals involved in signal transduction, cytoskeletal regulation, nuclear import, transcriptional regulation, and ubiquitination (Samuel et al. 2006). The lack of this domain in *P. patens* and yeast ATM may suggest an altered functional scope compared to the human and Arabidopsis homologs. Supporting this hypothesis is the fact that the yeast and *P. patens* ATM homologues are far less important for DNA DSB repair than their mammalian and Arabidopsis counterparts. This interesting difference in ATM function between these organisms will be discussed in detail in the following sections.

4.3.1 ATR is more important than ATM in response to DNA strand break induction in *P. patens*

Based on studies in yeast and mammals a key function of ATM and ATR is to maintain genomic integrity, especially through playing a role in repairing DNA DSBs (Craven et al. 2002, Cimprich and Cortez 2008, Shiotani and Zou 2009). They perform this function on multiple levels, sensing the DSB lesion, initiating cell cycle arrest and activating downstream effectors of the recombination based repair process (Durocher and Jackson 2001, Falck et al. 2005, Smith et al. 2010, Gobbini et al. 2013). To assess the importance of the *P. patens* homologues of ATM and ATR in DSB repair, the respective mutant lines were tested for sensitivity against different classes of genotoxic agents known to cause DNA DSBs.

One of the genotoxic agents used was the radiomimetic chemical bleomycin. Similar to ionizing radiation, bleomycin induces the formation of reactive oxygen species which then cut the DNA backbone (Pecinka and Liu 2014). The resulting breaks are two sided and may display single strand overhanging regions (Povirk et al. 1977, Povirk et al. 1989, Pastwa et al. 2001). While bleomycin is known to also generate DNA single strand breaks (Tounekti et al. 2001), these lesions do not require HR for repair (Caldecott 2003, Caldecott 2014) and are therefore unlikely to have affected the

evaluation of DSB repair in the ATM and ATR mutants.

It was found that in response to bleomycin treatment the *atr-1* single and *atmatr-1* double mutants were highly sensitive while *atm-1* was only marginally so. This implied that in *P. patens* ATR plays a much more important role in the repair of DSBs than ATM. This minor role of *P. patens* ATM in the repair of DNA DSB was unexpected for two reasons. First, in human cells as well as Arabidopsis, ATM and ATR mutants have both been found similarly sensitive to this type of genotoxic stress (Cliby et al. 1998, Wright et al. 1998, Culligan et al. 2006). Second, a large number of studies has been conducted on the mechanistic aspects of this repair process in human cells and according to the suggested model ATM and ATR function in sequence (Shiotani and Zou 2009). In short, it is claimed that in the initial stage following DNA breakage ATM and a number of other proteins localize to the break site. Subsequently the DNA ends at the break site are resected, yielding single stranded DNA overhangs. This structural change promotes ATM to be replaced by ATR at the break site, the latter initiating the next steps of the repair process. The fact that in *P. patens* ATM is much less important than ATR suggests that compared to its human and Arabidopsis counterparts its function is either reduced or redundant. It is possible that the role of ATM in this process, as observed in human cells and possibly Arabidopsis, is provided by another protein in *P. patens*, possibly even ATR. Interestingly the homologs of ATM and ATR in yeast, Tel1 and Mec1 respectively, are similar to their *P. patens* counterparts in that Mec1 plays a much more important role than Tel1 in response to this type of DNA DSB-inducing agent (Morrow et al. 1995). It is also worth mentioning that the reduced function of ATM/Tel1 in *P. patens* and yeast compared to the corresponding homologs in Arabidopsis and mammals coincides with the more similar N-terminal domain structure of ATM/Tel1, as discussed earlier.

To gain additional insight Mutant lines were also tested for their sensitivity to mitomycin c and methyl methanesulfonate (MMS). While these two agents also cause DNA DSBs they do so in a very different manner and the resulting DNA DSBs also differ in their structure from those generated by bleomycin or ionizing radiation. Initially the treatment with mitomycin C and MMS causes DNA interstrand crosslinks and DNA base methylations respectively (Beranek 1990, Norman et al. 1990, Warren and Hamilton 1996). If these lesions are not repaired prior to the onset of replication they may turn into DSBs if a replication fork collides with them and subsequently collapses (Bessho 2003, Niedernhofer et al. 2004, Groth et al. 2010, Nikolova et al. 2010). Owing to the fact that these DSBs originate from the collapse of a replication fork their structure is different in that they do not display two free DNA ends but just one(Helleday 2003, Shrivastav et al. 2007). Due to the lack of a second end it is also very unlikely that this type of DSB can simply be religated via NHEJ and instead is likely to rely solely on HR for repair.

In the experiments conducted as part of this thesis it was found that mitomycin C and MMS caused a hypersensitivity phenotype of similar magnitude in the *P. patens* ATR single and ATM/ATR double mutant line, but not in the ATM mutant. This implied that in *P. patens* ATR is required in the repair of DNA DSBs caused by replication blocking lesions while ATM is completely dispensable in this regard. While the important role of ATR in response to this type of DNA lesions is conserved in Arabidopsis, human cells and yeast (Morrow et al. 1995, Cliby et al. 1998, Wright et al. 1998, Garcia et al. 2003, Culligan et al. 2004) the relevance of ATM in this process differs considerably between these organisms. While in Arabidopsis *thaliana* the loss of ATM function does not lead to hypersensitivity to a crosslinking agent either, treatment with the methylating agent MMS did (Garcia et al. 2003). As both of these agents are similarly capable of causing replication specific DNA DSBs these are likely not the cause of the sensitivity to MMS in the Arabidopsis mutant. Instead the MMS specific sensitivity of the ATM mutant line can likely be explained by the recent finding that MMS is not only capable of inducing replication specific DSBs but also direct double ended DNA DSBs (Ma et al. 2011). Therefore it seems that, like in *P. patens*, ATM is dispensable in the response to replication blocking lesions in Arabidopsis. Concerning human ATM mutant cell lines derived from Ataxia telangiectasia patients there are conflicting reports. While some studies claim that ATM does not play any role in response to agents which cause replication blocking DNA lesions others suggest the opposite (McKinnon 1987, Hannan et al. 2002). One possible explanation for these conflicting results may be related to the diversity of ATM mutations in the cell lines used in these assays (Hannan et al. 2002). However in a more recent study conducted in human cells it has been shown that, in response to replication blocking lesions, ATM is phosphorylated at serine 1981 in an ATR dependent manner (Stiff et al. 2006). Since this modification is widely perceived as an indicator of ATM activation (Lee and Paull 2007, So et al. 2009) this study supports the notion that ATM is indeed involved in the DNA damage response to replication blocking lesions such as crosslinks and base methylations in human cells. In yeast loss of function of the ATM homolog Tel1 does not lead to increased sensitivity to replication blocking lesions (Morrow et al. 1995). However if *Tel1* is disruptedabled in a *Mec1* mutant strain the sensitivity of the latter to replication blocking lesions is increased further (Morrow et al. 1995). This implies that in yeast Tel1 does have a function in response to replication blocking lesions but is redundant to Mec1 in this regard.

In summary, the importance of ATR in response to replication blocking lesions seems to be conserved in *P. patens* and other major model organisms. While ATM appears to be generally less important in response to this type of lesion in all of the discussed organisms, *P. patens* is special in that its ATM homolog is apparently completely dispensable in this regard.

Another interesting result of the sensitivity assays was that a *Rad51AB* double mutant tested parallelly with the ATR mutant was found less sensitive to the DNA DSB inducing agents mitomycin C and MMS than the ATR mutant. This was interesting as this type of DNA DSB requires HR for its repair and Rad51 is widely assumed to be a downstream component of ATR in the HR mediated pathway of DNA DSB repair (Sung 1994, Sigurdsson et al. 2001, Holthausen et al. 2010).While *P. patens* is special in that it contains two homologs of Rad51, Rad51A and Rad51B (Markmann-Mulisch et al. 2002), a previous study on their function has found that the *Rad51AB* double mutant is completely deficient in gene targeting (Schaefer et al. 2010). Since gene targeting is a genetic technique which relies on the cells homologous recombination machinery this finding suggested that HR is either heavily impaired or even completely dysfunctional in this mutant. It is therefore unlikely that the ATR mutants increased sensitivity to mitomycin C and MMS can be explained with the HR pathway being even more severely impaired in this line. Instead one should consider that this difference in sensitivity could be related to the initial lesions which cause this type of indirect DNA DSB. As previously discussed MMS and mitomycin C generate DNA crosslinks and base methylations as primary DNA lesions which subsequently may be transformed into DSBs during DNA replication. Thus one possible explanation for the increased sensitivity of the ATR mutant to these two agents could be that ATR plays a role in the repair of DNA crosslinks and base methylations. If that hypothesis were true, the loss of ATR function would mean that even more of the initially replication blocking lesions are transformed into potentially lethal DSBs, leading to an increased sensitivity to these genotoxic agents. ATR is generally not associated with the nucleotide excision repair (NER) or base excision repair (BER) pathways known to repair DNA crosslinks and methylations (Horton et al. 2000, Nilsen and Krokan 2001, Sarkar et al. 2006, Deans and West 2011). However, there are a number of studies which do support the notion that ATR may be involved in NER (Choi et al. 2007, Choi et al. 2009, Lindsey-Boltz et al. 2009, Shell et al. 2009, Lindsey-Boltz et al. 2014) as well as BER (Krokan and Bjørås 2013). In addition to a possible involvement of *P. patens* ATR in the NER and BER pathways, there are more indirect ways by which ATR could aid in preventing crosslinks or base adducts turning into potentially lethal DNA DSBs. As mentioned earlier, crosslinks and base adducts initially block replication fork progression and their subsequent collapse is what causes the DSB. A mechanism which may aid in preventing fork collapse is translesion synthesis. Translesion synthesis relies on a set of special polymerases which allow the replication machinery to bypass otherwise blocking lesions such as base adducts or crosslinks (Johnson et al. 2007, Kumari et al. 2008, Guo et al. 2009, Guainazzi et al. 2011). One important prerequisite for efficient translesion synthesis, as has been determined in yeast and E. coli, is a sufficient dNTP supply (Sabouri et al. 2008, Gon et al. 2011, Fuchs 2012). In yeast the ATR homologue Mec1 has been shown to promote dNTP production in response to DNA damage by phosphorylating and thereby deactivating SML1, a direct inhibitor of

Ribonucleotide reductase (Zhao et al. 2001, Zhao and Rothstein 2002, Chabes et al. 2003). Thus if *P. patens* ATR were likewise involved in dNTP regulation this could also explain the increased sensitivity of the *ATR* mutant compared to the *Rad51AB* mutant in response to replication blocking lesions such as crosslinks or base methylations.

These examples of ATR being involved in response to replication blocking lesions outside of HR mediated DSB repair in other organisms thus provide a number of possible explanations as to why the *P. patens* ATR mutant was found more susceptible to replication blocking lesions than the presumably HR defective *rad51AB* line. It also suggests that the broad spectrum of ATR function as observed in other organisms may be conserved in the *P. patens* homolog.

4.3.2 *P. patens* ATR is the major initiator of cell cycle arrest in response to DNA DSB induction

The previously discussed DNA damage sensitivities of ATM and ATR mutant lines provided a general overview of the importance of these two genes in the *P. patens* DNA damage response.

In another set of experiments *P. patens* ATM and ATR homologues were tested for one specific function in response to DNA damage, the arrest of the cell cycle (Vialard et al. 1998, Smith et al. 2010).

To test the importance of *P. patens* ATM and ATR in the activation of DNA damage checkpoints mutant plantlets were treated with the DSB inducing agent bleomycin. Subsequently any cells progressing through S-phase were marked by growing plantlets in presence of a base analog which was later labeled with an alexa488 fluorophore. In wild type plantlets treated with bleomycin hardly any alexa488 fluorescent nuclei, indicative of DNA replication, were observed. This suggested an overall tight cell cycle control in response to bleomycin induced DNA lesions. Compared to the wild type the *atm-1* mutant displayed a moderate but marked increase in fluorescent nuclei while *atr-1* and *atmatr-1* plantlets displayed ratios of fluorescent to nonfluorescent nuclei similar to mock treated samples. These results imply that ATR plays the critical role in facilitating cell cycle arrest in response to bleomycin induced DNA damage in *P. patens* whereas ATM has a minor function in this regard, which in addition is redundant to that of ATR. Having established the relative contributions to DNA damage induced cell cycle arrest of *P. patens* ATR and ATM another interesting aspect would be to discern which of the DNA damage responsive checkpoints may be under their control. From studies in mammals and yeast it has long been known that DNA damage checkpoints exist in all major cell cycle stages, G1, S and G2 (Elledge 1996, Longhese et al. 1998, Dasika et al. 1999). In regard to *P. patens* the experimental results obtained in this work suggest a critical role in the G1, S as well as G2 DNA damage checkpoints for ATR. This can be deduced from a number of observations. First, the fact that ATR mutants displayed the same ratio of fluorescent to nonfluorescent nuclei in

bleomycin and mock treated samples suggests that cell cycle progression was not slowed down in any way and thus all of the DNA damage checkpoints are likely to be dysfunctional. Second, the fact that fluorescence signals were always observed in pairs of neighbouring cells implies that cells did neither arrest in S- nor in the G2-phase of the cell cycle as otherwise isolated signals would have been observed. Third, a deficiency in the G1 DNA damage checkpoint can be deduced from the fact that the vast majority of *P. patens* cells is known to persist in the G2 stage of the cell cycle for most of the time (Schween et al. 2003). This means that to obtain asimilar ratio of fluorescent cells in treated and untreated samples, as was observed in the *ATR* mutants, the majority of cells is required to traverse the G1-phase.

At which stage of the cell cycle ATM may supplement the checkpoint function of ATR remains speculative. However, the fact that fluorescent signals in bleomycin treated ATM mutant plantlets appeared to be largely isolated suggested that the G2-phase checkpoint might be largely functional, leaving G1 and/or S-phase as a more likely target for ATM.

Further support for the G2 checkpoint being under ATR but not ATM control may be found in the result of the previously discussed DNA damage assay. There the *atm-1* mutant was shown to be only mildly hypersensitive to bleomycin while the *atr-1* and *atmatr-1* mutants displayed a much increased hypersensitivity. As mentioned earlier, arresting the cell cycle in response to DNA damage is an important aspect in the repair process (Abraham 2001). The G2 checkpoint is likely to be especially important in this regard as it provides the final barrier before entry of cells into mitosis. Its special significance lies in the fact that if cells complete mitosis in the presence of DNA DSBs the affected chromosomes will fail to segregate properly (van Gent et al. 2001, Kaye et al. 2004), likely leading to loss of genomic information and cell death. Therefore the fact *ATM* mutants were found less sensitive to DNA damaging agents than *ATR* mutants might be explained by ATM not being required for the critical S/G2-phase DNA damage checkpoint.

Interestingly such dominance of ATR in DNA damage checkpoint control that was found in *P. patens* has not been observed in other higher eukaryotes examined in this regard.

Human cells for example have been found to mainly rely on ATM in providing cell cycle arrest in the G1, S and G2 stages in response to a similar genotoxic agent (Painter and Young 1980, Kastan et al. 1992, Paules et al. 1995, Cliby et al. 1998). Human ATR meanwhile was found to only provide minor checkpoint function in this regard, likely restricted to the G2 phase (Cliby et al. 1998, Cortez et al. 2001). A similar dependence on ATM for effecting cell cycle arrest in response to directDNA DSBs has been observed in Arabidopsis. There it was found that ATM but not ATR is needed for a G2 arrest in response to treatment with the same class of genotoxic agents (Culligan et al. 2004, De Schutter et al. 2007). Interestingly, similarly to *P. patens*, the unicellular eukaryote yeast however appears to also

largely rely on its ATR homolog Mec1 and not its ATM homolog Tel1 to effect cell cycle arrest in response to this kind of DNA damaging treatment. It was found that upon treatment with phleomycin, a radiomimetic substance belonging to the bleomycin family, cell cycle arrest was dependent on Mec1 in G1, S and G2 phase while Tel1 was found to only contribute to S-phase arrest (Nakada et al. 2003).Thus the dominance of ATR over ATM in regard to DNA damage checkpoints in *P. patens* is another parallel to yeast, while human cells and Arabidopsis differ considerably in this regard.

4.3.3 *P. patens* ATM and ATR are not essential for HR

ATM and ATR are deemed to be key regulative components in the homologous recombination based repair of DNA DSBs (Shiotani and Zou 2009). It has however never been assayed in detail to what degree ATM and ATR are required for the homologous recombination process itself. *P. patens* offers a rare opportunity to directly assess the effect of ATM and ATR loss of function on homologous recombination. This is because *P. patens* is innately efficient in gene targeting, a method which relies on homologous recombination to modify a specific genomic locus. Since successful targeting events can be readily detected via PCR based analysis this approach should provide a direct measure of homologous recombination performance. While yeast may appear similarly suited for such an analysis, as it is also highly proficient in gene targeting, that model organism is hampered by the fact that loss of function mutants of the yeast ATR homolog Mec1 are not viable (Paciotti et al. 2001).

As it turned out *P. patens atm-1* and *atr-1* mutants did not display any decrease in gene targeting efficiency, however, in the double mutant *atmatr-1* successful targeting events were markedly decreased. This implied that fully functional ATM and ATR are no absolute requirement for homologous recombination, but either of the two is required to maintain it at peak efficiency. Two studies conducted in a vertebrate DT40 cell line which tested the effect of ATM loss of function obtained a similar result as gene targeting efficiencies were observed to be only slightly reduced (Takao et al. 1999, Wang et al. 2004). The findings in *P. patens* suggest that in this organism ATM and ATR possess redundant functions in facilitating homologous recombination. This conclusion raises a number of questions. First, considering that the current model of homologous recombination has ATM and ATR acting at different stages of the process, it is difficult to imagine how either of them could be sufficient to provide full gene targeting efficiency. However, one has to consider that the *P. patens* ATM and ATR mutant lines used in this study were generated by disrupting their respective C-terminal kinase domains. While the kinase domains are generally assumed to be vital for the function of ATM and ATR (Xu and Baltimore 1996, Cliby et al. 1998, De Klein et al. 2000, Park et al. 2001), the

remainder of the protein may still be produced and provide some degree of the original function. While the role of the N-terminal regions is hardly researched, available data suggest it to be important for protein-protein interaction (Chen et al. 2007, Takai et al. 2007, Cimprich and Cortez 2008, Seidel et al. 2008). Thus the kinase deficient ATM and ATR proteins of the respective mutants are likely to still be capable of direct interaction with other HR components. It has for example been shown that the loss of kinase function of the ATM homologue Tel1 in yeast did only slightly impair its function in telomere maintenance (Ma and Greider 2009). In addition to the phenomenon that kinase independent functions of ATM and ATR may provide basic HR activity in the single mutants, another possible explanation for either ATM or ATR sufficing for HR is a redundancy in phosphorylation substrates for ATM and ATR. In support of this is the fact that human ATM and ATR have been shown to share the same set of preferentially phosphorylated substrate motifs in in-vitro studies, Ser-Gln and Thr-Gln (Kim et al. 1999, O'Neill et al. 2000). In addition, there are a large number of presumed homologous recombination mediating proteins which have been verified to be phosphorylated by ATM as well as ATR. These include SMC1A (Yazdi et al. 2002, Wang and Qin 2003) BRCA1 (Gatei et al. 2000, Tibbetts et al. 2000) H2AX (Podhorecka et al. 2010) FancD2 (Ho et al. 2006)and WRN (Pichierri et al. 2003). Thus it could be that redundancy in phosphorylation substrates of ATM and ATR is what keeps homologous recombination functional in either of the single mutants.

4.3.4 ATM and ATR affect the balance between HR and NHEJ in *P. patens*

Another interesting result of the gene targeting experiment was that stable transformants, generated by random integration of the targeting construct, were increased significantly in the *atm-1* mutant. Since random integrations are mediated by the non homologous end joining pathway (NHEJ) (Roth and Wilson 1988) this would suggest that ATM loss of function promote this alternative recombination pathway. A similar effect has been found during a recent study of another homologous recombination gene mutant in *P. patens*. It was found that mutation of *Rad51*, a HR component situated downstream of ATM and ATR in the pathway, also led to an increase in random integrations (Schaefer et al. 2010). However, contrary to the *ATM* and *ATR* mutants, the *Rad51* mutant displayed a complete lack of targeted integrations, implying that HR was completely dysfunctional or at least highly impaired. Thus in the HR deficient *Rad51* mutant the increase in random integrations may simply be due to NHEJ being the only choice for recombination. In the *ATM* and *ATR* mutants however targeting events were not reduced, suggesting HR was still functional and thus the increase of random integrations was not merely the result of a switch from HR to NHEJ. It may instead suggest that especially ATM may have a direct function in repressing the NHEJ pathway. This is very interesting because in other systems, like Arabidopsis or mammalian cells, the technique

of gene targeting is innately inefficient because of the ratio of targeted to random integrations usually being in the range of 10^{-3} to 10^{-2} (Baker et al. 1988, Hanson and Sedivy 1995, Jasin 1996). The finding that the balance between NHEJ and HR can be hugely shifted by disabling key regulatory components of recombination in *P. patens* suggests that these key upstream regulatory elements like ATM and ATR could be manipulated to shift the balance from largely NHEJ based recombination to HR. So far people trying to improve the bias towards HR have usually tried to disable key downstream components of NHEJ such as Ku70 and Ku80. While this approach was successful in Fungi (Takahashi et al. 2006) and also in human somatic cells (Bertolini et al. 2009) it has not been successful in plants like Arabidopsis (Puchta and Fauser 2013). Thus it could be a worthwhile alternative to try and manipulate upstream regulatory components like ATM and ATR as a means of improving gene targeting in higher plants.

4.4 The transcriptional DNA damage response in *P. patens*

4.4.1 The transcriptional response to bleomycin involves a broad range of repair pathways

Besides arrest of cell cycle progression, the DNA damage response encompasses the induction of a large variety of DNA-repair proteins on a transcriptional and post-translational level (Elledge 1996, Huen and Chen 2007, Jackson and Bartek 2009). Past studies on the transcriptional response to DNA DSBs in yeast, mammalian cells or Arabidopsis have been conducted using microarray based approaches (Gasch et al. 2001, Heinloth et al. 2003, Watson et al. 2004, Elkon et al. 2005, Culligan et al. 2006). One of the limitations of microarrays is that low abundant transcripts or limited fold changes are difficult to detect/quantify (Wagner et al. 2003, Draghici et al. 2006). This is problematic since many components of the DNA damage response supposedly belong to this class of transcripts (Bachant and Elledge 1999, Watson et al. 2004). The sequencing based SuperSAGE technique provides a digital and more sensitive readout of transcriptional changes (Evans et al. 2003, Matsumura et al. 2008), which may aid in especially detecting low abundant transcripts (Gilardoni et al. 2010). Thus the latter technique was chosen to try and obtain an exhaustive list of DNA damage induced transcriptional changes among DNA repair genes in *P. patens*.

In response to bleomycin treatment close to 3000 genes were found to be transcriptionally regulated in the *P. patens* wild type. Checking the GO-term annotations of the induced/repressed genes it was obvious that the vast majority of these genes are not associated with a direct role in the DNA damage response. This however is a common phenomenon in this type of transcriptome studies and generally attributed to the pleiotropic effects of DNA damage (Gasch et al. 2001, Heinloth et al. 2003, Watson et al. 2004, Elkon et al. 2005, Culligan et al. 2006).

Focusing on genes with known functions in the DNA damage response, as per our previously discussed list of *P. patens* DNA repair/recombination genes, 119 were detected in the SuperSAGE transcriptome dataset. In response to treatment with the DSB inducing agent bleomycin 26 of these genes were found up- and 17 downregulated, more than 2 fold. Among the induced genes are prominent components of the homologous recombination pathway such as Rad51, Rad54, PaxI1 and WRIP1. Interestingly none of the three components of the MRN complex (MRE11, Rad50, NBS1) were found induced upon DNA damage. The MRN complex is supposed to function in the early stage of HR mediated DSB repair, recognizing the break site and subsequently recruiting ATM (Uziel et al. 2003, Lee and Paull 2005, Takeda et al. 2007). Also ATM and ATR themselves were not transcriptionally upregulated in response to genotoxic stress. This lack of upregulation in early HR components may have two reasons. First, the timepoint at which plant material was harvested for SuperSAGE analysis was 3 hours post genotoxic stress treatment. This might suggest that the initial transcriptional response may already be over at that point in time. However in a preliminary experiment (Reiss unpublished) it was found that even 1 hour post genotoxic treatment neither the MRN components nor ATM or ATR were induced. A likely explanation may be that early DSB responding/signaling components like MRN, ATM and ATR are constitutively expressed, independent of whether DSBs are present or not. Considering that DNA DSBs are a pervasive threat to the cells genome integrity the constant presence of key response elements may be required for rapid induction of repair once a lesion has occurred. The activity of such components might therefore rather be controlled on a post translational level. The human ATM homolog is a good example in this regard as it was found to persist in a dormant dimeric state until DNA damage induces post translational modifications transforming it into its active monomeric state (Lee and Paull 2005, Lavin and Kozlov 2007). In addition to the previously mentioned HR components, key components of NHEJ such as Ku70, Ku80 and XRCC4 were also found to be induced. Thus, even though a number of studies suggest that *P. patens* relies on mainly HR for DSB repair (Kamisugi et al. 2006, Markmann-Mulisch et al. 2007, Holá et al. 2013), the transcriptional changes in response to genotoxic stress suggest an important role for NHEJ also. One obvious explanation for this observed NHEJ response would be that it represents the DSB repair capacity of *P. patens* cells which are in the G1 phase of the cell cycle. Due to the haploid nature of *P. patens* HR based DSB repair would be impossible in G1 stage cells since no homologous chromosome is available to serve as a repair template. However, as was observed in our ploidy analysis of young protonema tissue, the amount of cells in the G1 stage is less than 5%, with the majority of cells being in G2 instead, a finding which is confirmed by a previous study (Schween et al. 2003). Considering that absolute transcript levels of key HR components like Rad51 were similarly abundant than important NHEJ factors such as Ku70 and Ku80 however makes a limitation of NHEJ to G1 cells seem unrealistic. Instead it appears far more likely that NHEJ does play an

important role in DSB repair in *P. patens* also.

Another interesting observation among the pool of DNA damage responsive genes was that one of the three SPO11 homologues, which were identified in the initial BLAST analysis, was induced upon genotoxic stress treatment. SPO11 is generally described as having a function in meiotic recombination only, inducing DSBs which are essential for the subsequent crossover events (Youds and Boulton 2011). In Arabidopsis for example none of the two available SPO11 homologues are induced upon DNA damage treatment (Culligan et al. 2006). Since the plant material used in the transcript analysis was in a developmentally premeiotic stage this finding suggested that one of the three available SPO11 homologues of *P. patens* may have acquired a function in somatic recombination.

Furthermore, it was found that also a number of genes from other DNA repair pathways, which are generally not thought to be involved in DSB repair, were induced. These were MLH3, a mismatch repair (MMR) component, as well as ERCC2 and ERCC6, two nucleotide excision repair (NER) factors. One possible explanation for the induction of these repair mechanisms may be related to the genotoxic agent bleomycin which was used to induce DNA DSBs in the samples. While bleomycin is generally used as a DNA DSB inducer (Chen et al. 2008), it is also thought to produce single strand breaks and abasic sites (Steighner and Povirk 1990). The previously mentioned Nucleotide excision repair factor ERCC6 for example is supposed to be involved in the repair of such abasic sites (Yu et al. 2003, Kim and Jinks-Robertson 2010). Furthermore it has been suggested that NER in general may play a role in the repair of abasic sites (Torres-Ramos et al. 2000). Therefore the upregulation of NER components may be related to the secondary DNA lesions produced by bleomycin. The involvement of the mismatch repair factor MLH3 in response to bleomycin mediated DNA lesions is more difficult to explain. Mismatch repair is a process which fixes mispaired DNA bases which naturally occur during DNA replication (Kolodner 1996), a type of lesion unlikely to be induced by bleomycin. However, it has been found that the expression level of MLH3 is increased in meiotic cells and the protein product itself was discovered to interact with components of meiotic recombination (Santucci-Darmanin et al. 2002). As even key components of the somatic HR based DSB repair like Rad51 (Ashley et al. 1995, Tarsounas et al. 1999) or ATM and ATR (Keegan et al. 1996, Barlow et al. 1998, Moens et al. 1999) are also involved in meiotic recombination, it appears possible that MLH3 may similarly not only have a function in meiosis but also in somatic recombination. In support of this, recent findings are suggesting that the various pathways for DNA repair are not as isolated as they were thought to be. It has been proposed for example that the NER and base excision repair (BER) pathways may be interconnected with DSB repair (Zhang et al. 2009). Thus the reason for a broad range of DNA repair pathways induced by the DSB inducing drug bleomycin might be due to the secondary lesions it causes as well as the proposed interconnectivity of the DNA damage

response pathways.

Comparing the transcriptional DNA damage response as found in our *P. patens* study with a similar approach performed by another group in Arabidopsis (Culligan et al. 2006) highlights an interesting phenomenon. While the central component of homologous recombination mediated DSB repair, Rad51, is found upregulated in the *P. patens* and Arabidopsis transcriptome datasets, core components of NHEJ mediated DSB repair, KU70, KU80 and XRCC4 were only found to be induced in *P. patens*. This lack of transcriptional induction of NHEJ components in Arabidopsis as compared to *P. patens* is highly unexpected. This is because Arabidopsis is presumed to rely on largely NHEJ to repair the type of DNA DSBs as were induced in the transcriptome assays (Tamura et al. 2002). This presumed preference of Arabidopsis for NHEJ is supported by the fact that Arabidopsis NHEJ mutants lacking functional Ku70 or Ku80 were found to be hypersensitive to this type of DSB inducing treatment while disruption of the HR component Rad51 did not lead to any increase in sensitivity (West et al. 2004, Bleuyard et al. 2005). One possible explanation for the lack of induction among key NHEJ components in the Arabidopsis microarray based approach could be the relative insensitivity of this technique. Indeed, transcription profiling of Ku70 and Ku80 via RT-PCR in a different study did show an at least threefold increase in expression after DNA DSB induction (Tamura et al. 2002). Regardless, the fact that the central HR component Rad51 was found induced almost 200 fold in the microarray based study, while NHEJ components did show no induction, highly suggests that activation of HR has precedent over NHEJ in Arabidopsis in response to random DSBs. Another possible explanation for this unexpected result may be that NHEJ components, due to their dominant role in DSB repair in Arabidopsis, are constitutively expressed. Evidence supporting this suggestion has been found in a number of studies based on RT-PCR based expression profiling. In untreated plants the NHEJ factors Ku70, KU80 and XRCC4 could be detected with this approach whereas the central HR component Rad51 could not, unless plantlets were subjected to genotoxic stress. (Tamura et al. 2002, Garcia et al. 2003, Wang et al. 2010).

In summary the transcriptional response of *P. patens* to treatment with the DNA DSB inducing agent bleomycin was highly diverse and not limited to DSB repair components. Furthermore, albeit *P. patens* is generally presumed to mainly utilize HR for the repair of DNA DSBs, the fact that key NHEJ components were also induced conflicts with this hypothesis.

4.4.2 ATM and ATR mutants are affected in their basal transcription profile as well as the induction /repression of genes in response to genotoxic stress

ATM and ATR are key regulators of the DNA damage response to DNA DSB inducing agents (Abraham 2001, Ciccia and Elledge 2010). In a human embryonic kidney cell line for example 700 proteins have been found phosphorylated at ATM and ATR consensus motifs in response to treatment with ionizing radiation (Matsuoka et al. 2007). Contrary to human cells the DNA damage response in plants is supposed to occur on a transcriptional level (Yoshiyama et al. 2014) and a study conducted in Arabidopsis has found that 163 genes were transcriptionally induced in response to ionizing radiation in an ATM dependent manner (Culligan et al. 2006).

Evaluating the effect of ATM and ATR loss of function on the transcriptional DNA damage response of *P. patens*, two main effects were observed. First, in the *atr-1* single and even more so the *atmatr-1* double mutant a number of DNA repair genes showed an increased level of basal expression. This suggested that parts of the DNA damage response are constitutively induced in these mutant lines. A possible explanation for this phenomenon may be related to the hypersensitivity phenotype observed in especially the ATR mutant lines in response to various genotoxic agents as discussed in a previous chapter. It was suggested that this hypersensitivity may have been caused by impaired repair of the induced DNA lesions. Now one has to consider that a wide array of DNA lesions including DSBs, single strand breaks, abasic sites or base adducts also occur as a natural byproduct of cellular metabolism (Helbock et al. 1998, Okano et al. 2003, Vilenchik and Knudson 2003, Swenberg et al. 2010). If repair of these lesions would be impaired, as suggested for the ATR mutant lines, they would persist for an extended time. This in turn would imply that the time they serve as an elicitor of the transcriptional DNA damage response is also increased, resulting in the observed basal increase of transcripts involved in the DNA damage repair.

The second effect observed in mutant lines was that induction of a number of DNA repair genes was reduced or even absent. This effect was again least prominent in the *atm-1* mutant while the *atr-1* and *atmtr*-1 mutant lines were markedly more affected. This implies that in *P. patens* ATR but not ATM plays the major role in the transcriptional DNA damage response to bleomycin induced lesions. It should also be noted that not only were *ATM* and *ATR* mutants defective for the transcriptional induction of key HR components like Rad9 or Rad51 but also central NHEJ components such as Ku70 and XRCC4. This finding implies that the key HR components ATM and ATR also play an important role in the transcriptional regulation of the NHEJ pathway for DSB repair in *P. patens*. The fact that even in the *atmatr-1* double mutant a number of DNA repair genes were still induced upon genotoxic stress treatment or displayed an increased basal level of expression is also interesting to note. It suggests that there is another regulatory component capable of inducing parts

of the DNA damage response besides ATM or ATR.

Comparing the importance of ATM and ATR in inducing the transcriptional DNA damage response between *P. patens* and Arabidopsis, there is a marked difference. While in *P. patens* ATR and to a lesser degree ATM both are required to ensure proper transcriptional induction of the DNA damage response, Arabidopsis appears to solely rely on ATM (Culligan et al. 2006). It is interesting to note that in yeast the ATR homolog Mec1 was also found to play an important role in the DNA damage response to a similar DNA double strand break inducing agent (Gasch et al. 2001). This highlights another parallel in the function of ATM and ATR homologs between yeast and *P. patens*.

4.5 *P. patens ATM* and especially *ATR* mutants are affected in vegetative and reproductive growth

4.5.1 *ATM* and *ATR* mutants show disturbed growth pattern during the juvenile stage

Observing the juvenile developmental stage in the *P. patens* mutant lines, *atm-1* and *atr-1* single mutants were found to display a marked increase in caulonemal growth. As caulonema in the aforementioned mutants did not displayany increased growth speed this phenomenon suggested that mutation of either *ATM* or *ATR* promoted differentiation of cells into caulonema. In humans or mice ATM and ATR have until now not been accredited with any direct role in the control of cellular differentiation. Therefore, the previously described phenomenon is likely an indirect effect of ATM and ATR loss of function. In this regard it is important to note that the abundance of caulonema in wild type plantlets is easily affected by a change in individual growth parameters. For example, omitting ammonium tartrate, a nitrogen compound, from the media has been found to increase caulonemal growth in experiments presented in this work as well as studies conducted by another lab (Schween et al. 2003). Nitrogen is the most important inorganic nutrient in plants, being a major component of proteins, nucleic acids, cofactors and secondary metabolites (Scheible et al. 2004). As would be expected from such a widely required component, lack of nitrogen inhibits plant growth (Taiz and Zeiger 2002). In maize it was specifically shown that low nitrogen availability promotes root growth (Mi et al. 2008), presumably to improve substrate coverage and thus nitrogen access. While *P. patens* does not possess real roots, its caulonema type filaments, are thought to provide root functionality (Reski 1998, Schaefer and Zrÿd 2001, Cove 2005). Thus it seems reasonable to assume that increased caulonemal growth in the absence of ammonium tartrate in *P. patens* wild type is a response to nutrient stress. The latter is supported by the fact that wild type plantlets were found to indeed grow markedly slower in the absence of ammonium tartrate (this work, data not shown). While *P. patens* ATM and ATR are very unlikely to be involved in nutrient uptake, their functionality in the DNA damage response is presumably also of considerable importance for cellular homeostasis.

This is because DNA damage is a normal byproduct of cellular metabolism (Helbock et al. 1998, Okano et al. 2003, Vilenchik and Knudson 2003, Swenberg et al. 2010) and it is very likely that impaired repair will disturb cellular processes on a large scale which may be perceived as stress. Such a scenario is supported by the results of our proteome as well as transcriptome analysis of mutant lines. In the former it was found that a few proteins associated with primary metabolic pathways such as the RuBisco subunit or activase as well as proteins implied in plant stress response were differentially expressed in the mutant lines. The SuperSAGE based transcriptome analysis of mutant lines meanwhile identified changes in basal expression levels of thousands of genes, their individual annotation suggesting a wide variety of cellular processes being affected. Thus it appears quite likely that loss of ATM and ATR function may be perceived as cellular stress, possibly invoking the caulonemal growth response.

This of course leaves the question as to why there was no marked increase in caulonemal growth in the *atmatr-1* double mutant. Considering the loss of either ATM or ATR function on its own was found sufficient to induce caulonemal growth, the simultaneous loss of both was expected to at least provide a similar if not stronger effect. This unexpected phenomenon can very likely be explained by a negative growth phenotype of *ATR* mutants which will be discussed in the following chapter.

4.5.2 Loss of ATR and much more so ATR and ATM function simultaneously impairs proliferation of fast growing cells and complex structures

Besides the increase of caulonemal growth observed in the single mutant lines there were also two distinctly negative growth phenotypes. It was found that if plantlets were grown in conditions which restricted filamentous growth to largely fast growing caulonema type filaments, *atr-1* and much more prominently *atmatr-1* displayed markedly impaired growth. As there was no distinct increase in dead or shortened cells in the filaments this phenomenon was likely related to a reduced rate of cellular proliferation. Looking back at the previously discussed lack of increased caulonemal growth in the *atmatr-1* double mutant the severe impairment of caulonema proliferation itself is likely the reason. Thus while loss of ATM and ATR function simultaneously is still likely to induce caulonemal growth, as it did in the single mutants, the effect is masked by the markedly impaired proliferation of this filament type in the double mutant.

A second growth defect was observed during the adult stage of *P. patens* gametophytic growth. The hallmark of this growth stage is the switch from exclusively 2-dimensional filamentous growth of caulonema and chloronema to the formation of 3-dimensional leafy structures, the gametophores. In *atr-1* and even more so *atmatr-1* plantlets these gametophores were found to be malformed and generally impaired in growth.The first interesting thing to emphasize is that while *atm-1* unlike *atr-1* single mutants did not produce any of the previously mentioned growth impairments, the latter were

noticeably more severe in the *atmatr-1* double mutant. This suggested two things. First, ATM and ATR likely provide a similar function in *P. patens* in relation to growth. Second, the function of ATM appears to be largely redundant to ATR in this regard. This leaves the question how loss of ATR and even more so ATR and ATM simultaneously could affect growth in *P. patens*. The main function ATM and ATR are generally associated with is their role in the DNA damage response (Shiotani and Zou 2009, Shiloh and Ziv 2013), a role which according to experiments performed as part of this thesis is conserved in the *P. patens* ATM and ATR homologs. Considering DNA damage is a natural byproduct of the cellular metabolism (Helbock et al. 1998, Okano et al. 2003, Vilenchik and Knudson 2003, Swenberg et al. 2010) it is likely that *P. patens ATM* and *ATR* mutants will at the very least be less efficient in dealing with this endogenous genotoxic threat. As a result it is likely that cellular homeostasis will be disturbed. That cellular homeostasis is indeed greatly affected in the mutant lines is, as previously mentioned, confirmed by especially our transcriptome analysis. Befitting the severity of growth phenotypes mutant lines displayed deregulation of thousands of transcripts increasing in number from *atm-1* to *atr-1* and *atmatr-1*.

The observation of ATR being more important than ATM in the DNA damage response of *P. patens*, together with the fact that the growth deficiencies were only observed in the *atr-1* single and *atmatr-1* double mutant further support the hypothesis that growth defects are the result of deficiencies in the DNA damage response. This leaves the question why especially caulonema filaments and gametophores, but not chloronema filaments, would be markedly affected by an impaired DNA damage response. To answer this question one has to consider the differences between chloronema on the one hand and caulonema and gametophores on the other. One central difference between caulonema and chloronema is their rate of proliferation. While caulonema divide every 6-12 hours chloronema divide only every 24 hours (Cove and Knight 1993, Jang and Dolan 2011). While no dedicated study has been done on the proliferation time of gametophore cells, a study on gametopore development contains data suggesting cell cycle times are equally short than those of caulonema (Harrison et al. 2009). On top of that gametophores differ from protonema in that their growth pattern is far more complex. While protonemal growth is defined by individual tip growing filaments, gametophores are three-dimensional structure whose growth and shape relies on its individual cells growing in a concerted and interdependent manner. Taking this into account it seems reasonable to assume that any deficiencies in the DNA damage response and the interconnected disturbance of cellular homeostasis may affect especially these fast cycling and complex growing cells. Interestingly this phenomenon of fast proliferating cells being especially affected upon loss of ATR function is also observed in mammals. Cre-recombinase mediated deletion of *ATR* in adult mice for example has been found to especially affect tissues which are known for high

proliferation rates such as the bone marrow and the intestinal walls, whereas organs known for slow proliferation like the brain do show less severe cellular degeneration (Ruzankina et al. 2007).
There are a number of additional arguments which support the claim that an impaired DNA damage response affects especially caulonema but not chloronema. First, when observing growth of wild type plantlets on media supplemented with various genotoxic agents they did not produce any visible caulonema but only chloronema. Second, flow cytometric analysis of *P. patens* has indicated that choronema cells are predominantly at the G2 stage whereas caulonema are at G1 (Hohe et al. 2002, Schween et al. 2003). This implies that caulonema have a shortened G2 stage as compared to chloronema. In this regard it is important to consider that *P. patens* is haploid and HR based repair of DNA damage, the central responsibility of ATM and ATR in the DNA damage response, can only occur in the G2 phase. It is therefore reasonable to assume that caulonema, with their comparably short G2 stage, may suffer more severely from loss of ATM and ATR function than chloronema.
In summary, *P. patens* ATR and to a lesser extent ATM are required for proper somatic growth and development, a phenomenon which is likely related to their functionality in the DNA damage response.

Comparing the importance of ATM and ATR for vegetative growth between *P. patens* and other organisms reveals a number of differences. Contrary to *P. patens,* Arabidopsis displays no vegetative growth phenotypes at all, in either *ATM* or *ATR* single or even double mutant plants. In mice targeted knockout of *ATM* produces a number of disease symptoms such as neurodegeneration, immune deficiency and growth retardation (Barlow et al. 1996, Elson et al. 1996), similar symptoms as are observed in the human disease associated with mutation of the ATM gene, Ataxia telangiectasia (Chun and Gatti 2004, McKinnon 2004). ATR meanwhile appears to be essential in mammals as targeted mutation of *ATR* in human cell lines is lethal and generation of *ATR* knockout mice was unsuccessful due to early embryonic lethality (Brown and Baltimore 2000, Cortez et al. 2001).
These discrepancies in the requirement of ATM and especially ATR for vegetative growth beckon the question as to why that may be. In principle there are two possible explanations for this phenomenon. Either the function of ATM and ATR differ in between organisms or the differences between organisms themselves may affect the impact of ATM and ATR loss on vegetative growth. The fact that loss of ATR functionality can lead to loss of viability in mammals while affecting *P. patens* only slightly and Arabidopsis not at all is particularly surprising. This is because the role of ATR in the DNA damage response, which has been proposed as a critical functionality for viability of human and mouse cells (Cliby et al. 1998, Wright et al. 1998, De Klein et al. 2000), appears to be principally conserved in all the aforementioned organisms. The latter is deduced from the observation that ATR mutant lines of all these organisms have been found hypersensitive to similar

types of genotoxic stress such as DNA crosslinking or alkylating agents (Cliby et al. 1998, Wright et al. 1998, Culligan et al. 2004).

However, more recent studies in mammalian model organisms have shown that ATR also exerts important functionality outside of the DNA damage response. For example, ATR has been implied in the regulation of origin firing and stabilization of replication forks during S-phase (Friedel et al. 2009, Ammazzalorso et al. 2010) and it has been suggested that this functionality of ATR might also be essential for survival of proliferating cells (Cimprich and Cortez 2008). In yeast where loss of function of the ATR homolog Mec1 is also lethal it was found that Mec1 is required for upregulating dNTP production during S-phase (Zhao et al. 2001, Chabes et al. 2003). This suggests that there are functionalities of ATR outside of the DNA damage response which could be essential for survival of proliferating cells. If these functions were indeed essential and would not be performed by the *P. patens* and Arabidopsis ATR homologs it may explain the comparably minor effects on vegetative growth observed upon their loss of function. At this point in time however it is impossible to make any such claims as studies on the functionality of ATM and ATR in *P. patens* and Arabidopsis are still at an early stage and these aspects have not yet been examined.

The second possible explanation for the lack of a lethal phenotype in *P. patens* and Arabidopsis ATR mutants is related to a fundamental difference between plants and animals. That is while DNA damage sustained by individual plant cells may only be detrimental to the affected cell and the immediate surrounding tissue, a similar damage to mammalian cells may lead to the death of the entire organism through cancer. Cancer is a disease which is triggered by the mutation of genes involved in the regulation of cellular growth and differentiation (Loft and Poulsen 1996, Vogelstein and Kinzler 2004). This results in the uncontrolled proliferation of cells which may impair the functionality of for example vital organs, eventually causing death. As DNA damage is one of the elicitors of cancer animal cells have developed an apoptotic response to persistent DNA lesions (Orren et al. 1997, Roos and Kaina 2006), thus removing potential cancer cells early. While tumorous growths also occurs in plants there they are far less detrimental as cells are confined by the rigid cell wall which prevents the spreading of tumorous cells to other parts of the organism (Doonan and Sablowski 2010). It has furthermore been shown that increased proliferation rates of cells do not necessarily lead to tumour formation in plants, cells instead being properly incorporated into the growing tissue (Doonan 1996). Thus even if individual plant cells start uncontrolled proliferation it is unlikely to produce a major problem and preemptive cell death of DNA damaged cells may therefore be uneconomic. This might be an especially important trait due to the sessile nature of plants which exposes them much more to abiotic stresses which either directly or indirectly may induce DNA damage (Tuteja et al. 2009). In conclusion, the severe phenotype of ATR loss in mammals as opposed to plants may be related to a stricter apoptotic response to persistent DNA damage, as can be

expected from the loss of function of a key DNA damage repair factor. This hypothesis is further supported by the observation that loss of function of a number of other DNA repair factors such as Rad51, NBS1, BRCA1, BRCA2, XRCC4 and LigaseIV are also embryonally lethal in mice (Lim and Hasty 1996, Ludwig et al. 1997, Barnes et al. 1998, Gao et al. 2000, Zhu et al. 2001), but produce no or hardly any effect on vegetative growth in Arabidopsis (Riha et al. 2002, van Attikum et al. 2003, Li et al. 2004, Reidt et al. 2006, Abe et al. 2009).

4.5.3 Loss of ATR function induces sterility

During extended culture of *P. patens ATM* and *ATR* mutant lines it was found that *atr-1* and *atmatr-1* are practically sterile. This implies that ATR but not ATM is required for reproduction in *P.patens*. This requirement for ATR for sexual reproduction while ATM is dispensable is not observed in other organisms. While in humans and mice the functional evaluation of ATR in reproduction is difficult, due to its loss of function being lethal (De Klein et al. 2000), ATM loss of function has been found to induce sterility in mice as well as humans (Barlow et al. 1996, Xu et al. 1996, Lavin 2008). In Arabidopsis, where *ATM* and *ATR* loss of function mutants are viable, *ATR* mutants were observed to be fertile, *ATM* mutants partially sterile and a double mutant was found to be completely sterile (Garcia et al. 2003, Culligan et al. 2004). While similar to humans and mice complete loss of ATR function is lethal in yeast, a second mutation in the Sml1 gene suppresses this lethality phenotype (Zhao et al. 1998). Using this approach it could be assessed that in yeast ATR but not ATM is required for sexual reproduction (Greenwell et al. 1995, Grushcow et al. 1999, Carballo et al. 2008). These findings once again mark an interesting similarity between *P. patens* and yeast regarding their ATM homologs, both being dispensable for reproduction as opposed to their mammalian and Arabidopsis counterparts. This lack of requirement for the ATM homolog in sexual propagation of *P. patens* and yeast could be directly related to the minor role ATM was found to play in response to DNA DSBs in these organisms (Morrow et al. 1995). This hypothesis is based on the fact that the exchange of genetic information via recombination between homologous chromosomes during meiosis requires the formation of DNA DSBs (MacQueen and Hochwagen 2011). While ATM and ATR are generally known for their function in the repair of somatic DNA DSBs (Culligan et al. 2006) there is evidence that they are also involved at meiotic DNA DSBs. For example, both proteins have been detected at meiotic chromosomes in mouse gametocytes (Keegan et al. 1996, Moens et al. 1999, Perera et al. 2004). In addition to that the sterility phenotype of Arabidopsis and mice *ATM* mutants has been accredited to chromosomal fragmentation as it was observed in meiotic cells (Xu et al. 1996, Barlow et al. 1997, Garcia et al. 2003, Culligan and Britt 2008), a phenomenon indicative of failed DSB repair. The hypothesis that the DNA DSB repair capacity of ATM and ATR homologs may indeed be coupled

to their importance for sexual reproduction is also supported by the situation as it is observed in Arabidopsis and mammals. In these ATM is of similar importance to ATR in response to DNA DSB induction (Cliby et al. 1998, Wright et al. 1998, Culligan et al. 2006) and as mentioned previously, loss of ATM function is also associated with a sterility phenotype.

However there is one aspect of the *P. patens* sterility phenotype which suggests that in this organism sterility is not a result of failed DNA DSB repair during meiosis. This is because the *atr-1* and *atmatr-1* mutants sterility phenotype manifested before onset of meiosis. This is evident from the fact that the structure in which meiosis takes place in *P. patens*, the sporophyte, was practically not formed at all. In *P. patens* the Sporophyte is the only diploid structure in the lifecycle and it is formed after fusion of the male and female gametes. As visually intact gametangia were observed in *atr-1* and *atmatr-1* the sterility of these lines may either be derived from a deficiency of the gametes themselves, the process of fertilization, or the development of the diploid sporophyte. At present it is difficult to estimate which of these aspects may be affected in the ATR mutants and thus produce the sterility phenotype. The only experimental data related to this comes from the crossing of ATM and ATR single mutants. There it was found that both of the double mutant lines were generated from fertilization of ATM egg cells by ATR spermatocytes. This may suggest that the male gametes are intact and the sterility is due to a defect of the female gametes. However this would have to be verified in a quantitative manner in an additional set of experiments.

5 Appendix

5.1 cDNA sequences of *P. patens ATM* and *ATR*

ATM cDNA (green and red highlights indicate start and stop codons respectively):

CAGTTAGATATGTACTTTTGATTAACAATGTCGTTGTTGCTATCGTGCCACCGATTCTCCAATTTTTGAATTCCATTCACTAC
GTAGCAGGAAGAATGAGACGACTTTGTGTAGCCGGGCTCTGATTCTGGATAGGATGTGGACCTCAGGGGTCAAGGTGAA
TTATGGTCTGCATGCGATGGTCATGAAGGTTCATAAATCATTGGGCAGAATTGGGGAGCTGAACCATGGTTGTTCTGGTTG
AGGATGTTCGGGATTATTTGAGAGAACTTTTTATTGCCAGCTCCAAGATTCAGCGCTGAAGCTAAGGTGAATCGGAACGT
GGGTCCAGTACATCTTTTTTGGGAATGTGAGATAGGAAAATTGGTTTGATGGAGATGGTAAGCGTGGAGGATGTGGAGG
ATCTCAACATCAAGCTTGTTTCGGATCGCGCTAAAACTCGGGAGGAAGGCGTGCGAGCTTTGGCAAGCTACTTGGAGAGC
AATGCTGGGTTGAGTATTTGCCCTTTGTTGGATCAGCAGACTGTGCTCTTGCGTCCCAACGACCGCATTCCCTCTGCAACAT
GGCCAGGGGTTTTACACGCACTGTGCGATTGCATACTAATGGACGTTCTGGCCAGCAAAAAGCGGGGTCCAAAACCTATC
CTGGCCAAGACTCTAAGGAATTTTATCCACAAAGCGGAAGACAAAAGTCGTTCAGGGAAAAGCCACTTTCTCTTACGGAA
AATCAAAAGATTGTTTCAGCATATACTGGATATGCTTCAAGAGGTGCCGGCTTTTTCGTCCGACTATAGCCACATTCCGCAT
GAGCTACTTCCTGATGTTGAATATCGCATGCGTATGGGCAAGAAGATCTACAACGATCTCGTGATCCTTTATTTAACGAAG
GCAAAGGAGATTATACACACCCGCTCGTTTGAGGCCCCTTTAGCTAAAGAAGAAGCTTTTAGAAATACTTTAACACTACTTA
TTCTGTTGAAAAATCCACCCGGGGACTTAGCAGCGCATGTCAAGGAGGATGTCGTCGATGGCTTTTGTGACATCTTTGCAT
TCAACAGAGATGAAGACCGGATAACTAAGAAGCTTGTCTCATCATTAAATGCTTTTTTGTTAATAGACGGATTGAATTTGG
GAGATAAAGTAGCAAAGCTTCATTGTAGTTTTCGACCATTTATGGTGCGAACATGGTTAACTACACGAGATCGAGACTTGA
AGGATGAACTTGTGTTATATGCTCGAATTCAGTTGAAGCTTCAATACATTTTAACATTAGATGAAGGAATCGTGGTGGAAG
AGCTCCTCGGGCTAGTTGAGAAGGAGCTTGATCAGATTGGAATTGTTCTTACTGCATACAATAGATACGATATCATGCGTG
AGGACAAAGTTGGTTCTGTCAGCCGTAGTGAACGAGGCTTTCTTGAATTTGCTGCGGCGGTTCTTTTTGAGGTTGGCAAGA
GAGATGAAATTGGGAGGTTGTATTGGAGTGCCAGTAAGCGCAAGAAAAGGGATCCTGTTTACACTCTTGTGGGAGAGAA
GATAATTGCTGCCAAGACCTTGTGGGATGGAGCTTTCTGCATTTTGATTCGTAATTTCGGGCCTCAATTACCCCCTAAAAGT
CTTGAAGCCTGGCTTGATGGTTTATCTGGGAATTTAGAACGAGTGATGAGTGAAGGTGTGAACACCCGAAGCTTTGGAAC
TCCTGTTTGGACTCTCCGGTGCCTCCAAGAGTTGAGCAGTCTTTGGTGTGATGCGGTCAATTCTTCAGCTGACACGTTGAG
CAGGAAATCAAGCGTATGGCAATCCATTTGGGAATCCGTGCTGCATTGGCTACCTTTGTTCATAAACGTTTCTATCATGGTG
GATGAGGCATTTCGTCTACTTGGACTTATAGCTACCATGAGATTGGTGCCAGTGTACAACGTTTCTCCAGATTTTTGGGAGC
TTCAAGTATTTTCAGAGGTGCCTTCTGTAAATATACTCTTTTTCACTGCAGCGTTCTTTACCAGTGCCGGAAGCCAGGTCGT
AACAAAAGGTGACCTTGACCTTCGCAGCAAGCTTCTGAAATGGTCATTGACATATCTGAATCCCCAGGAGACTGAGAAAG
CAAAAGTAGCAGCTTGGAATGAAAAGTCGTTGATTCTTCTCAGCGGATCAGTATTAGCGCTTAGTGTGGGCTTTGTTCCAC
TGCGTCCTAACGACAAGTTATCACTTCAAAACTCAACTTGCGTGTCACTGGATGTAACAAAAGAATTGTCTCACTGGGCAG
AGTGCAAGGAACCAGAGGTGGAGCTTGATTCTATGGACCGCTCTTCATACACATCCTCCAGCATGGGGGTTATTATCACTC
ACACGGATGCATCCGATACTCACACACGGAGCAACCCTTTGCCACGGTCAAGCAGTGACGTTCTTCTGCAGCGCCTATCTG
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ATTGCTTGCTAACTGCATCTATGGAGTTGAGTATTTAATGCAAAGAAGGGGTCAACGACTTCCAGATTGGTGTCCTAATGG
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GATTCATCCAGAAGTTCTTTGTCTGCTTCTTTGAATACATTTTTTCATGAGTTTACACCAGCAGCACTATCAGCCTTTTCATGT
TTTTTATCATCACCGGGGCGGGCGACCTTGGTATGCAGTGGAGGGCTCGCTTATGAACTGGACACATCAGTTGCTGTTCTT
CCGATACTGACCAGGTTTTTGGAATCGATTTCTAGTGCGCTGACCTTCACTGCAGCTGTGAAATCTTCAGCTCAAGAAGAG
ATTCCAGGCAGTCCTGTAACATTGACTGAGGAGCGTGAGGGGAAAGTCAAGTTGATGGATGTTGATTTAGATGAGTTCTC
AGAGATGGGTCCCCAGGGTTCCCAAAAGCTTGAAGGGTTACGCTCGAAAATGAGAGGTGACAACTGTCTGTACGATCAAT
GGAAGGAGCTTTGTTTGAAAATTGTTGCTGACATTGGGCGTATTTTGCCAGAGACAACGTACAATGTTCTTTTTAAACTACT
AGACAATGAGGAAGATATCAAGGTTCGGTGGAAAATTTTGCTTATTTTATGCGACCTTACGAAGGAGAAAACTATTGAAC
ATCTACCTGAATTGGTGCGCTGCATGGAGAGTTTTCGCGAAGTCTGCCTGCATAGTGAGTCTGGGCGATTTAATATTTTGT
CAGGCATCGATGCTATACTGGTTAACTTACTGATGCAAGATCAGGGTGCTGCCAAAGTTGGTTCCTTCAAAGATATGCAGC
TAGTTACACAAGAGGCTTTGCCTCAGTTGGCTTCTTTCATTTCAGATTTGGAGAATATTAATCTGCTTTGGCCTACTCGAATT
AAGTTTGTTAACACGGTCTTTAACTATATTCTGGTCTCTCCAAGTACAGCTCAGGTGTTAACCGAGAAACTGCTGAGTTTTA

TGCACGATGTAGAGTACAGAGTTCGACGTTTAATGTGTATGCGAACCAACGTCTTCTTCCAGACCTGGGATGGTCATCATG
GACTATTCCGTGATGTGTGCGCTAATTTTGGAGTCAAGATGGTGATGGTGACAAAAAGCAAGGTTGTCCGGGCATCAGAA
GTTGAATTGGCTGGTTCACAAGATACTCCCTTTACAGAAACTGCCTTGTTAACATTAGCAGAGATTGCTGCGTGTAGTGAC
AAAGTGGAAGGCGAGGCATTGTTTATGCTATGTGCGCATGCTGCCTTGAATCCATCACAAAGGTCTCTTGTCAGAAATGTC
CTTGACAGAGTAGCAAGTCAACTACAATATCCACATCGCTGGACGTACTTCGAGTACGTGATGGGAGCGCTACTGAGCAG
ATGGGTCATAGCACGTCTGTCAATACCGTCACTTGTTGAGATCAAGGACTTACTCAGTGAAAGATCAGCCGTAGCGATGTT
CCTCGAGCAATGCTGTCCTTGGCTGCTATCATCTCTTTTTCTTCATGATGACAAGGATGAACTGCAATGGATTGCCAAGAGC
ATGTCTCTTTCGCTCTCATCATTGGTAAGGCAAAATTTTGCTTCCATCTTCGCAGGCCTTTTGCCTATACATTCCCATGGAAC
GGAGGATGAACAGGATAAGGCTGGAGCTGTTTTACAATTGCACATGCTGACAGCAGCTGAGTTAACAGAGGATGAGCGT
GATACTCTCATTCGGAAGCACATGATTTCTATTGTGAGCTTTCTTTTCAGACTATGTGGTGCCGCGGAGACACCAGAGCTTC
CATACTTCTCAAAAGCTGCCATTTCGTCAGCTGTACGAACTGTTGTGGATGGATTTTTGGATACAGAACAAGTCTCGGAAG
AGGGGGGAGTTGTTGACAAGATGCAGGTTTTCCGACCCGATCGTGTATTCATGCTTCTGCTCCACCTTCACTACGAGATCG
ACTCGGCATACCACCCACGGCATCGACGCCATTTGTTGGCTAGCTTGATGGCTATAATGACTGTAATAGATAAACGTGTGA
TCGTCTCTAGCACTTTTCGTTACATCATACATATCATCTTGCAGTCTATAAATGTCAATGAACTTCAAGATCAATGTTGCGGC
CTCTTAGGATACCTCCTAGACCAAGTGGAATCTTCGCCATTAAGCGCAAATACCCTTGATGACCAGCTTCAGTCAATCGTCT
CGAAGCTAGTGTCTTGCATCACAGCATATGACCAAGTTTCTCTTGATGTTGATGGGCTTCAATCGAATCCTTCACATCAACT
GCACATACTGGTAGGTTTGCTGGAGCGGTTAACAGTTAAAGCCAGTCGCTCTCTACATGATTCAATAAAAGAATTGAACCC
TTTTCCGAAGCTGCCAATTTTTGAGCCCATGCGAGCCTTGCATAACTCCTTGTGTAGTGGAAGAAGTCTTGCAGATGAATTT
GTCCAGTTTGTCAAAAGAGCGCCGAGCCTTCCACCTCAACTTCAACTTATCAGCTTACAAAGTCTTGCAAATGAGCTCAAAC
ATCGAAAGTGCGAGTTGTACGCGCATCCATTTATTAGTAAAGACGGGACCAGGTGGCGATGTAGTTCTAAAGTTGTGTCA
GCGACCTGGAGATTAGTGCAACTATGTGACGAACAAAACTTTACTGACATGCGTGATCTTGCGGGCATGTTCTTGGCTGCT
GTTGGAATGGGTAACCCTCACGCTGTTGTGTTTCATTTGCCTGAAGAGCCTGAAGAGGATCTTTCTCGGTCATCTGCAATA
ACAGATGTTACGATGAACACAGAGTTGGGTCCATCAGACATCTTTTTGAAGAAGATCATGATACAAATGCGAGGTTACCTC
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TGAACACATCAAAGCAACAGTCAGCAAGTGTAGCAACCCCAGTTGAGGAGCCACAACTCTGGAGAACAACTGGCAAAGC
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***ATR* cDNA (green and red highlights indicate start and stop codons respectively):**

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5.2 Targeting plasmids for ATM (pKOATM) and ATR (pKOATR) disruption

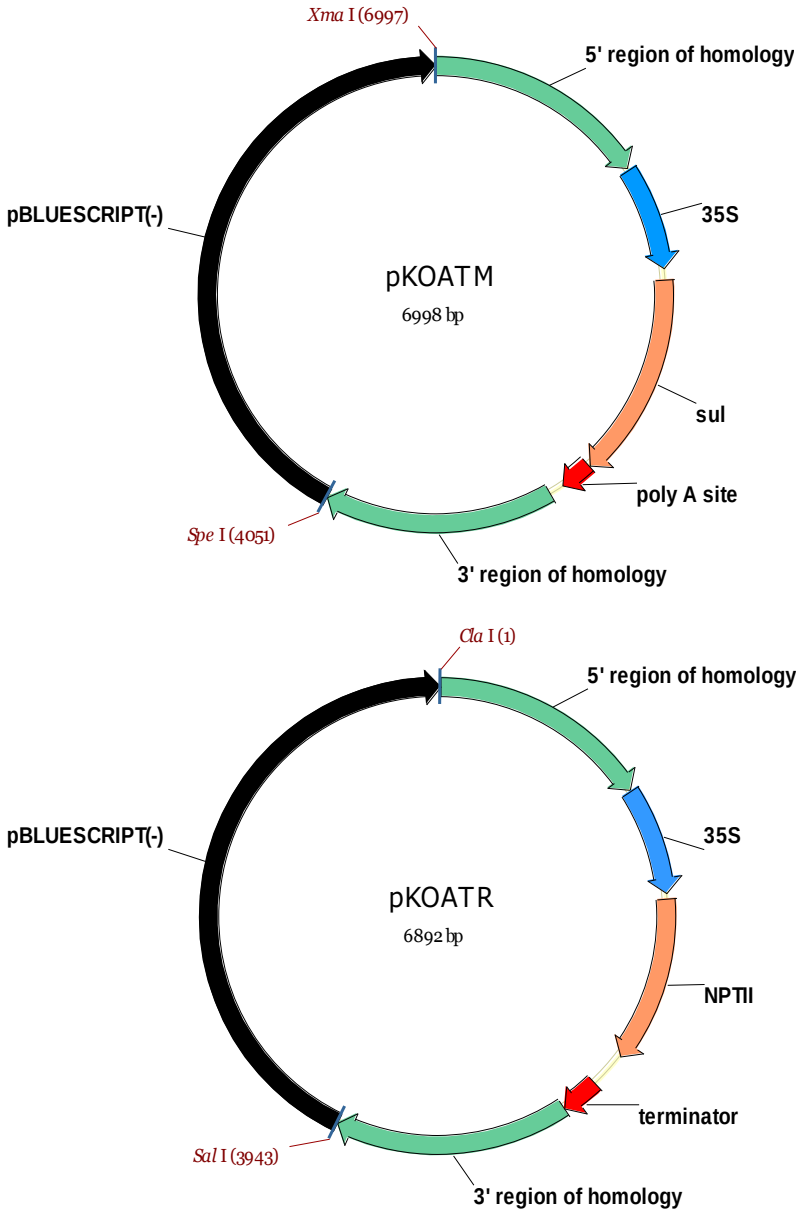

Figure 5.2-1: Maps of pKOATM and pKOATR gene targeting vectors.
a) 5' region of homology, 1087 bp sequence cloned from the *P. patens ATM* genomic sequence located upstream of the targeted kinase domain. **35S**, cauliflower mosaic virus promoter; **NPTII**, Neomycin phosphotransferase; **terminator**, terminator sequence of the octopine synthase gene from the agrobacterium tumefaciens ti-plasmid; **3'region of homology**, 1138 bp sequence cloned from the *P. patens ATM* genomic sequence located downstream of the targeted kinase domain. **pBLUESCRIPT(-)**, vector backbone; ***Spe*I/*Xma*I**, restriction sites used to release gene targeting construct from vector backbone.
b) 5' region of homology, 1074 bp sequence cloned from the *P. patens ATR* genomic sequence located upstream of the targeted kinase domain. **35S**, cauliflower mosaic virus promoter; **NPTII**, Neomycin phosphotransferase; **terminator**, terminator sequence of the octopine synthase gene from the agrobacterium tumefaciens ti-plasmid; **3'region of homology**, 1140 bp sequence cloned from the *P. patens ATR* genomic sequence located downstream of the targeted kinase domain. **pBLUESCRIPT(-)**, vector backbone; ***Cla*I/*Sal*I**, restriction sites used to release gene targeting construct from vector backbone.

5.3 Gene targeting in ATM and ATR mutant lines

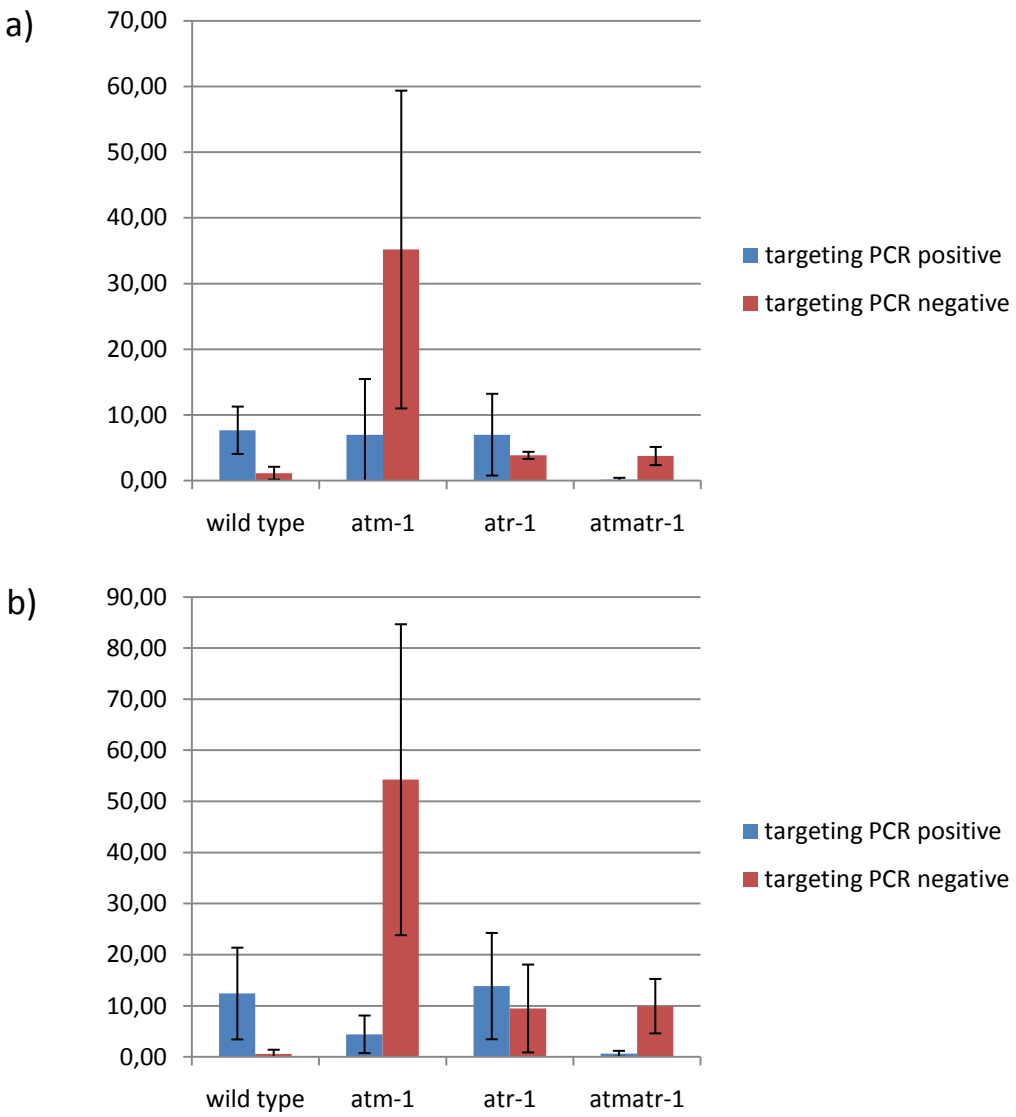

Figure 5.3-1: Percentage of targeting PCR positive and negative transformants is similar in fully (a) and partially PCR analyzed Transformation experiments.

5.4 Top regulated genes in response to bleomycin treatment

Table 5.4-1: Top 50 bleomycin-induced genes in *P. patens* wild type according to fold change.

Cosmoss gene id	Fc	BLAST inferred homolog including description from swissprot database
Pp1s10_51V6.2	484.92	GATLA_ARATH Probable galacturonosyltransferase-like 10
Pp1s34_238V6.1	216.54	Y5828_ARATH Putative B3 domain-containing protein At5g58280
Pp1s316_9V6.1	186.29	DNJ15_ARATH Chaperone protein dnaJ 15
Pp1s183_29V6.1	65.44	CKX11_ORYSJ Cytokinin dehydrogenase 11
Pp1s421_1V6.1	39.47	RTNLE_ARATH Reticulon-like protein B5
Pp1s477_11V6.1	35.28	AMP1A_ARATH Methionine aminopeptidase 1A
Pp1s41_228V6.1	33.28	-
Pp1s34_308V6.1	32.94	DCE_SOLLC Glutamate decarboxylase
Pp1s129_85V6.1	28.72	HSP11_PEA 18.1 kDa class I heat shock protein
Pp1s2_413V6.1	27.57	FAO1_ARATH Long-chain-alcohol oxidase FAO1
Pp1s93_114V6.1	26.61	BH054_ARATH Transcription factor bHLH54
Pp1s106_185V6.1	24.78	RPIA_ARATH Probable ribose-5-phosphate isomerase
Pp1s314_65V6.1	22.38	-
Pp1s94_114V6.2	21.78	-
Pp1s130_202V6.1	20.98	ATL52_ARATH RING-H2 finger protein ATL52
Pp1s162_128V6.1	19.25	-
Pp1s202_5V6.1	19.02	SNC1_ARATH Protein SUPPRESSOR OF npr1-1, CONSTITUTIVE 1
Pp1s137_199V6.1	19.02	MYBF_ARATH Putative Myb family transcription factor At1g14600
Pp1s89_161V6.1	18.38	ALF_MAIZE Fructose-bisphosphate aldolase, cytoplasmic isozyme
Pp1s9_16V6.1	18.03	-
Pp1s162_133V6.1	17.93	FH3_ORYSJ Formin-like protein 3
Pp1s41_12V6.1	15.97	-
Pp1s12_307V6.1	15.97	KPYC_SOLTU Pyruvate kinase, cytosolic isozyme
Pp1s126_37V6.1	15.40	UBC23_ARATH Probable ubiquitin-conjugating enzyme E2 23
Pp1s192_22V6.1	15.39	KEA2_ARATH K(+) efflux antiporter 2, chloroplastic
Pp1s311_41V6.1	14.83	CUL3A_ARATH Cullin-3A
Pp1s11_99V6.1	11.41	-
Pp1s47_69V6.1	11.41	QORL_ARATH Quinone oxidoreductase-like protein At1g23740, chloroplastic
Pp1s412_30V6.1	11.41	-
Pp1s298_31V6.1	11.09	-
Pp1s79_51V6.1	10.85	-
Pp1s1_97V6.1	10.70	Y2144_ARATH Putative leucine-rich repeat receptor-like ser/thr-protein kinase
Pp1s54_54V6.1	10.61	-
Pp1s43_101V6.1	10.37	TRXH_BRARA Thioredoxin H-type
Pp1s35_401V6.1	10.27	-
Pp1s252_5V6.1	9.92	AGP19_ARATH Lysine-rich arabinogalactan protein 19
Pp1s56_52V6.1	9.90	RS27A_LUPAL Ubiquitin-40S ribosomal protein S27a
Pp1s166_109V6.1	9.51	-
Pp1s79_129V6.1	9.40	-
Pp1s43_67V6.1	9.40	PALY_PINTA Phenylalanine ammonia-lyase OS
Pp1s317_10V6.1	9.36	-
Pp1s21_400V6.1	8.87	-
Pp1s73_118V6.1	8.82	MYBF_ARATH Putative Myb family transcription factor At1g14600
Pp1s23_51V6.1	8.57	PEXLP_TOBAC Pistil-specific extensin-like protein OS
Pp1s79_92V6.2	8.30	-
Pp1s40_23V6.1	8.27	YCF2_OENGL Protein ycf2 OS
Pp1s5_77V6.1	8.24	CMT2_ARATH DNA (cytosine-5)-methyltransferase CMT2
Pp1s19_24V6.1	8.19	TPC1_ARATH Two pore calcium channel protein 1
Pp1s20_346V6.1	7.99	-
Pp1s299_4V6.1	7.87	KU70_ARATH ATP-dependent DNA helicase 2 subunit KU70

Table 5.4-2: Top 50 bleomycin-repressed genes in *P. patens* wild type according to fold change.

Cosmoss gene id	Fc	BLAST inferred homolog including description from swissprot database
Pp1s93_123V6.1	356.07	-
Pp1s218_93V6.1	-92.79	RPIA_ARATH Probable ribose-5-phosphate isomerase
Pp1s216_49V6.1	-77.67	AMP1A_ARATH Methionine aminopeptidase 1A
Pp1s104_88V6.1	-49.55	ALF1_PEA Fructose-bisphosphate aldolase, cytoplasmic isozyme 1
Pp1s359_39V6.1	-47.16	Y5977_ARATH Probable leucine-rich repeat receptor-like protein kinase
Pp1s44_257V6.1	-23.05	GATLA_ARATH Probable galacturonosyltransferase-like 10
Pp1s545_8V6.1	-14.55	-
Pp1s253_16V6.1	-13.81	ACO2M_ARATH Aconitate hydratase 2, mitochondrial
Pp1s22_76V6.1	-12.44	-
Pp1s5_324V6.1	-12.01	-
Pp1s234_16V6.2	-11.95	-
Pp1s298_59V6.2	-10.34	XT1_ARATH Xyloglucan 6-xylosyltransferase
Pp1s96_61V6.1	-10.08	-
Pp1s223_97V6.1	-9.82	-
Pp1s88_195V6.1	-9.82	-
Pp1s336_28V6.2	-9.25	NNJA4_ORYSJ Ninja-family protein Os05g0558800
Pp1s49_214V6.1	-9.12	-
Pp1s41_281V6.1	-9.06	PSBQ2_ARATH Oxygen-evolving enhancer protein 3-2, chloroplastic
Pp1s106_27V6.1	-8.85	-
Pp1s236_54V6.1	-8.74	HSDD2_ARATH 3beta-hydroxysteroid-dehydrogenase/decarboxylase isoform 2
Pp1s165_119V6.1	-8.59	CSK2A_MAIZE Casein kinase II subunit alpha
Pp1s225_61V6.1	-8.52	RS16_GOSHI 40S ribosomal protein S16
Pp1s6_191V6.1	-8.24	-
Pp1s141_116V6.1	-8.22	TPS9_ARATH Probable alpha,alpha-trehalose-phosphate synthase 9
Pp1s480_12V6.1	-7.80	TINY_ARATH Ethylene-responsive transcription factor TINY
Pp1s59_195V6.1	-7.80	-
Pp1s69_123V6.1	-7.71	-
Pp1s59_319V6.1	-7.68	-
Pp1s32_328V6.1	-7.67	AGP9_ARATH Classical arabinogalactan protein 9
Pp1s74_224V6.1	-7.63	NEED_PINRA Floricaula/leafy-like protein FL1
Pp1s152_167V6.1	-7.54	-
Pp1s33_139V6.1	-7.45	-
Pp1s91_140V6.1	-7.34	NUD20_ARATH Nudix hydrolase 20, chloroplastic
Pp1s111_153V6.1	-7.19	-
Pp1s62_221V6.1	-7.19	-
Pp1s61_296V6.1	-7.10	4CLL3_ARATH 4-coumarate--CoA ligase-like 3
Pp1s98_136V6.1	-7.07	-
Pp1s27_149V6.1	-6.92	-
Pp1s211_143V6.1	-6.92	MYB86_ARATH Transcription factor MYB86
Pp1s96_101V6.1	-6.92	-
Pp1s49_102V6.1	-6.88	AGP9_ARATH Classical arabinogalactan protein 9
Pp1s51_136V6.1	-6.79	-
Pp1s106_65V6.1	-6.75	-
Pp1s56_236V6.1	-6.75	SYNC1_ARATH Asparaginyl-tRNA synthetase, cytoplasmic 1
Pp1s6_306V6.1	-6.70	-
Pp1s113_81V6.1	-6.66	BT1_MAIZE Protein brittle-1, chloroplastic/amyloplastic
Pp1s22_94V6.1 or	-6.57	-
Pp1s67_234V6.1	-6.49	-
Pp1s130_197V6.1	-6.40	-
Pp1s259_41V6.1	-6.40	-

5.5 Verification of key phenotypes with auxiliary *ATM* and *ATR* mutant lines

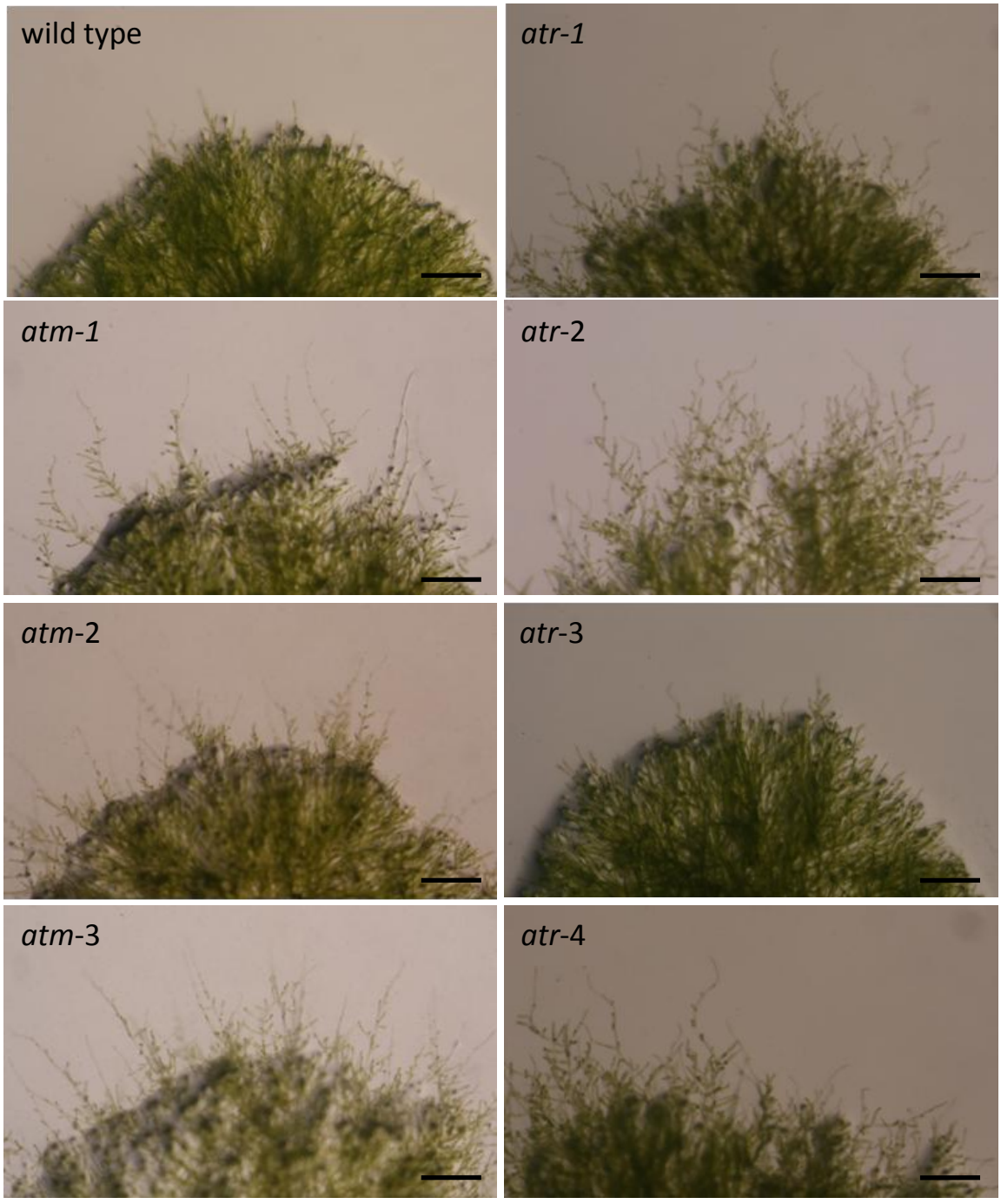

Figure 5.5-1: Caulonemal growth is increased in *ATM* and *ATR* mutant lines. Starting from pregrown 1 week old plantlets, similar sized ones were selected from wild type and independent *ATM* as well as *ATR* mutant lines. These were transferred onto fresh plates with PPNH4 standard medium and grown for two more weeks under permanent light before pictures were taken. Pictures showcase actively growing fringe regions of plantlets, protruding filaments resembling caulonema. Scale bar = 1 mm

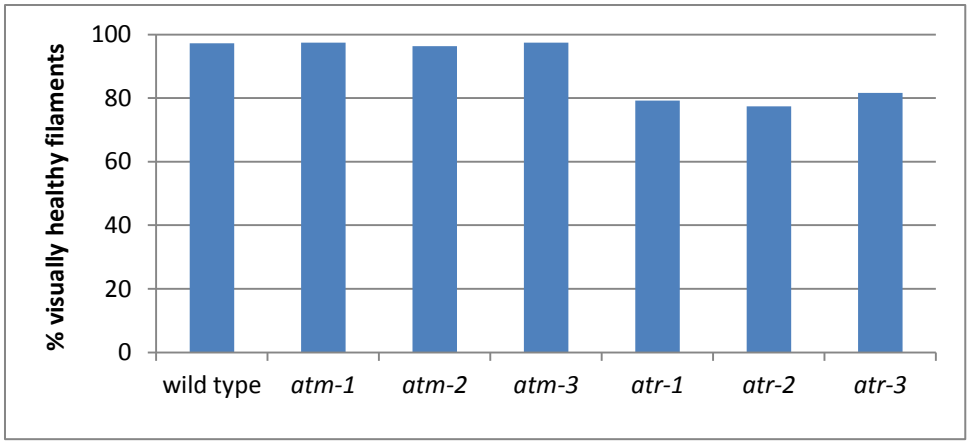

Figure 5.5-2: Viability of early regenerating filaments is reduced in ATR mutant lines. Freshly homogenized protonema fragments of wild type and mutant lines were plated on standard PPNH4 medium and the number of visually healthy filaments was assessed. After six days of growth visually healthy filaments were counted once more and the percentage of filaments surviving until this point was calculated.

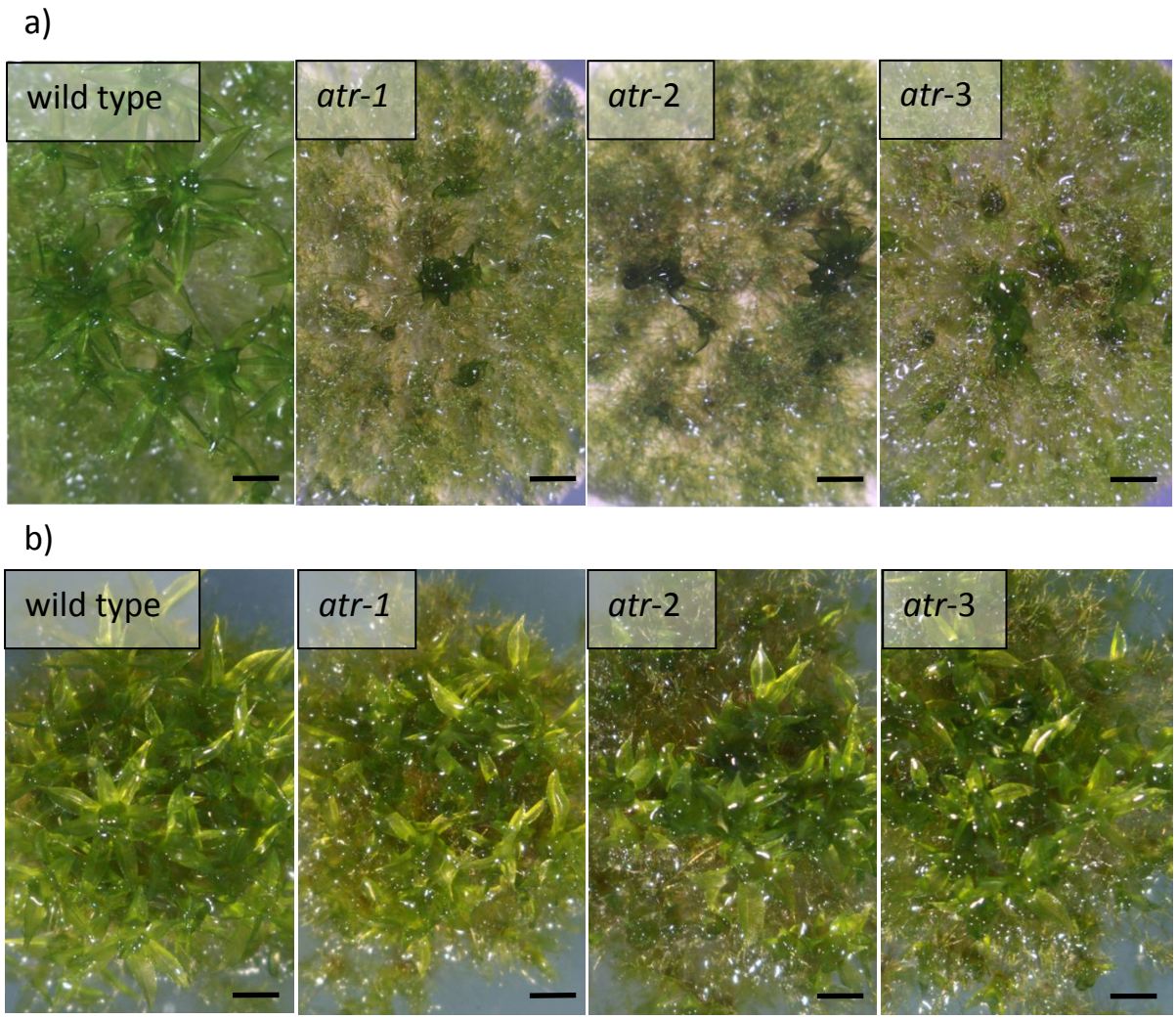

Figure 5.5-3: *ATR* mutant lines display impaired gametophore growth which can be alleviated however by limiting nitrogen availability in the growth media. Wild type and *ATR* mutant lines were grown for 5 weeks on standard PPNH4 medium (**a**) or on PP- medium (**b**), the only difference between the two being the absence of ammonium tartrate as an additional source of nitrogen in the latter. Scale bar = 1 mm.

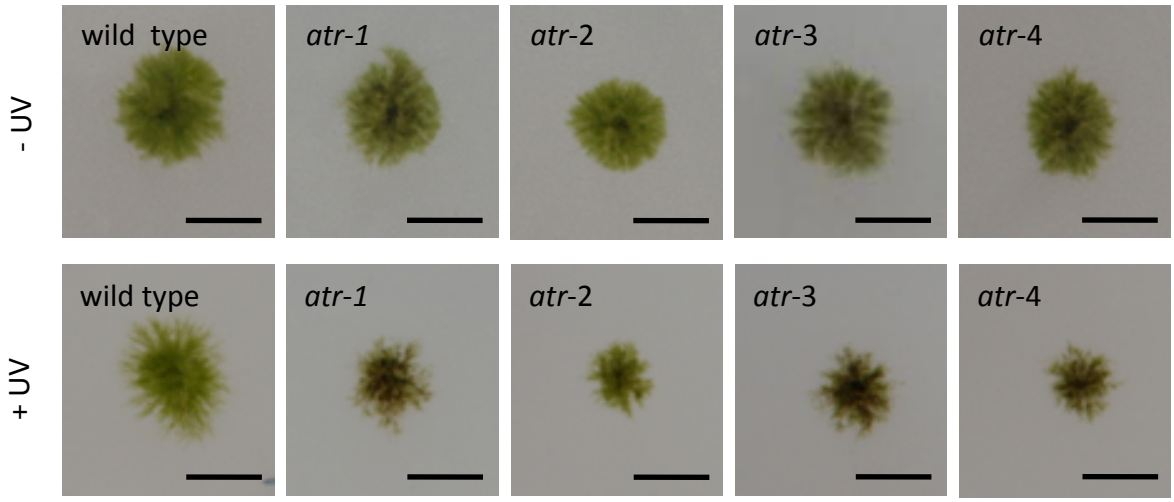

Figure 5.5-4: ATR mutant lines display reduced growth if exposed to UV light. Small 7 day old plantlets of similar size of wild type, *atr-1*, *atr-2* and *atr-3* were transferred onto fresh plates containing standard PPNH4 media and grown for two more weeks while being exposed to UV (0.05-0.06 mW/cm^2). Scale bar = 0.5 mm

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