Univerzita Karlova v Praze

Přírodovědecká fakulta

Mechanismy reparace DNA u mechu *Physcomitrella patens*

Mechanisms of DNA repair in moss *Physcomitrella patens*

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Disertační práce

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……

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OBSAH

Seznam zkratek

2-FA - 2 – fluoroadenin

6-4PP – 6‘-4‘ pyrimidin-pyromidon fotoprodukt

AMP – adenosin monofosfát

AP místo – místo DNA bez báze (apurinové/apyrimidinové místo)

APT – adenosin fosforibosyltransferáza

BER – excisní reparace bází (Base Excision Repair)

BLM - Bleomycin

CPD – pyrimidinový dimer vytvářející cyklobutanový kruh

DSB – dvouvláknový zlom DNA

HR – homologní rekombinace

IR – ionizující záření

NER – nukleotidová excizní reparace

NHEJ – nehomologní rekombinace, přímé spojování konců DSB

MMS – methyl methansulfonát

PCD – programovaná buněčná smrt (apoptóza)

PEG – polyetylenglykol

ROS – reaktivní kyslíkové radikály (Reactive Oxygen Species)

SSB – jednovláknový zlom DNA

SMC - proteinové komplexy udržující strukturu chromozómů (Structural Maintenance of Chromosome)

TLS – syntéza přes poškození (Translesion Synthesis)

UV – ultrafialové záření

wt - divoký typ (wild type)

Abstrakt

Genomy organismů jsou během životního cyklu vystaveny působení vnějších i vnitřních chemických, fyzikálních i biologických faktorů - genotoxinů. Genotoxiny způsobují změny jak struktury DNA tak jejích základních stavebních komponent – cukerných zbytků, fosfodiesterových vazeb i purinových a pyrimidinových bází. Vzhledem k rozmanitosti a četnosti možných poškození DNA si pro udržení stability genomu organismy v průběhu evoluce vyvinuly řadu reparačních mechanismů, které jsou často propojené s dalšími buněčnými dráhami, např. přestavbou - „remodelací“ chromatinu, replikací DNA, transkripcí, kontrolou buněčného cyklu či apoptózou - programovanou buněčnou smrtí (PCD).

Mechanismy reparace DNA jsou zatím nejlépe prostudovány u kvasinek a savčích buněk, u rostlin však stále zbývá řadu detailů a vztahů objasnit. I přes to, že základní mechanizmy reparačních drah jsou evolučně konzervovány, jsou mezi drahami živočišných a rostlinných buněk významné rozdíly.

Předkládaná disertační práce se zabývá a shrnuje výsledky zavedení rostlinného modelového organismu mechu *Physcomitrella patens* (*Physcomitrella*) a využití jeho unikátních vlastností jako je vysoká frekvence homologní rekombinace, haploidní vegetativní stav gametofytu a apikální růst filament protonemy při studiu reparace DNA. Studiem působení radiomimetika Bleomycinu indukujícího dvouvláknové zlomy DNA (DSB), alkylačního mutagenu methyl methansulfonátu (MMS) a UV záření je demonstrováno, že *Physcomitrella* je jedním z nejvýhodnějších modelových organismů.

Kombinovaným využitím studia reparace DNA a indukované mutageneze v kultuře dělících se buněk bylo ukázáno, že fenotyp citlivý k působení genotoxinů není přinejmenším u *Physcomitrelly* důsledkem neschopnosti eliminovat indukovaná poškození, ale naopak, rychlé a účinné reparace vedoucí k obnově struktury DNA, nicméně za cenu změny její sekvence v jejímž důsledku vznikají různé typy mutací. Pak zejména ty, které vznikají v životně důležitých genech, vedou k citlivému fenotypu. Zvláště dobře patrný je tento koncept u mutant *pprad50* a *ppmre11* komplexu MRN u kterých je mutací vyřazena dráha bezchybné homologní rekombinace (HR) a posílena k chybám náchylná dráha nehomologního spojování konců (NHEJ).

Abstract

Over the course of an organism's life, its genome is exposed to endogenous and exogenous chemical, physical and biological agents – genotoxins. These genotoxins alter DNA structure and also its basic structural components – sugar residues, phosphodiester bonds, and nitrogenous bases.

Organisms have therefore evolved a plethora of different strategies to both repair DNA lesions and maintain genomic stability. These DNA repair pathways are linked with several other cell pathways, including chromatin remodelling, DNA replication, transcription, cell cycle control, apoptosis – programmed cell death (PCD), thereby providing a coordinated cellular response to DNA damage.

Biochemical mechanisms of DNA repair are relatively well understood in yeast and mammals, however, far less so in plants. While these repair mechanisms are evolutionary conserved, significant differences still remain. Therefore, further investigation is required.

This thesis summarises the introduction of a novel plant model – the moss, *Physcomitrella patens* (*Physcomitrella*). As a haploid gametophyte with unique characteristics of high frequency of homologous recombination (HR), and apical growth of filaments, it is an ideal organism to study DNA repair in plants. Previous research on *Physcomitrella* regarding mechanisms of DNA lesion repair induced by radiomimetic Bleomycin, alkylating methyl methanesulfonate (MMS), and by UV irradiation has provided strong evidence of its capability to be one of the best plant models.

The combined DNA repair and induced mutagenesis study using a *Physcomitrella* culture of protonema dividing apical cells displays how the genotoxin-sensitive phenotype is not a consequence of a repair defect to eliminate induced damage. Rather this hypersensitivity is the result of rapid and effective DNA repair, thus allowing for the restoration of DNA structure at the cost of potential sequence changes prone to mutations. Mutations, particularly those occurring in essential genes, are then responsible for the sensitive phenotype.

This concept is well illustrated in the mutants, *pprad50* and *ppmre11,* of the MRN complex with an eliminated, error-free HR pathway and an enhanced, error-prone non-homologous end joining pathway (NHEJ)

Cíle práce

Studium reparace indukovaného poškození DNA u mechu *Physcomitrella patens*

- Vypracování účinné metody transformace mechu pro tvorbu transformant a cílených mutant.
- Studium indukce poškození a reparace DNA po působení mutageny s rozdílným mechanismem účinku u wt a reparačních mutant deficientních v reparaci DSB u *Physcomitrelly*.
- Studium indukované mutageneze a analýza mutací v genu pro adenin fosforibosyltransferázu (*PpAPT*) sloužícím jako pozitivní selekční marker mechu *Physcomitrella.*

1. Mechanismy reparace DNA

Poškození DNA představuje pro buňku vážný problém, který jí buď znemožňuje přímo vykonávat funkce spojené s její transkripcí, popř. translací nebo nepřímo vznikem mutací a tím možným ovlivnění funkce prakticky všech genů. Závažná poškození DNA vedou u savců, v případě saturace kapacity reparačních mechanismů, k apoptóze a eliminaci buněk s poškozenou DNA, popřípadě v důsledku fixace mutací vedoucích ke zvýšení proliferace, ke vzniku rakovinného bujení a s ním spojeným onemocněním.

Zlomy a další poškození DNA bránící replikaci a transkripci, mohou vést ke vzniku mutací a celkově ovlivňují buněčnou fyziologii. Vznik poškození DNA přitom není nijak výjimečnou událostí, podle Lindahla a Barnese (2000) dochází v jediné savčí buňce denně ke vzniku desítek tisíc defektů DNA.

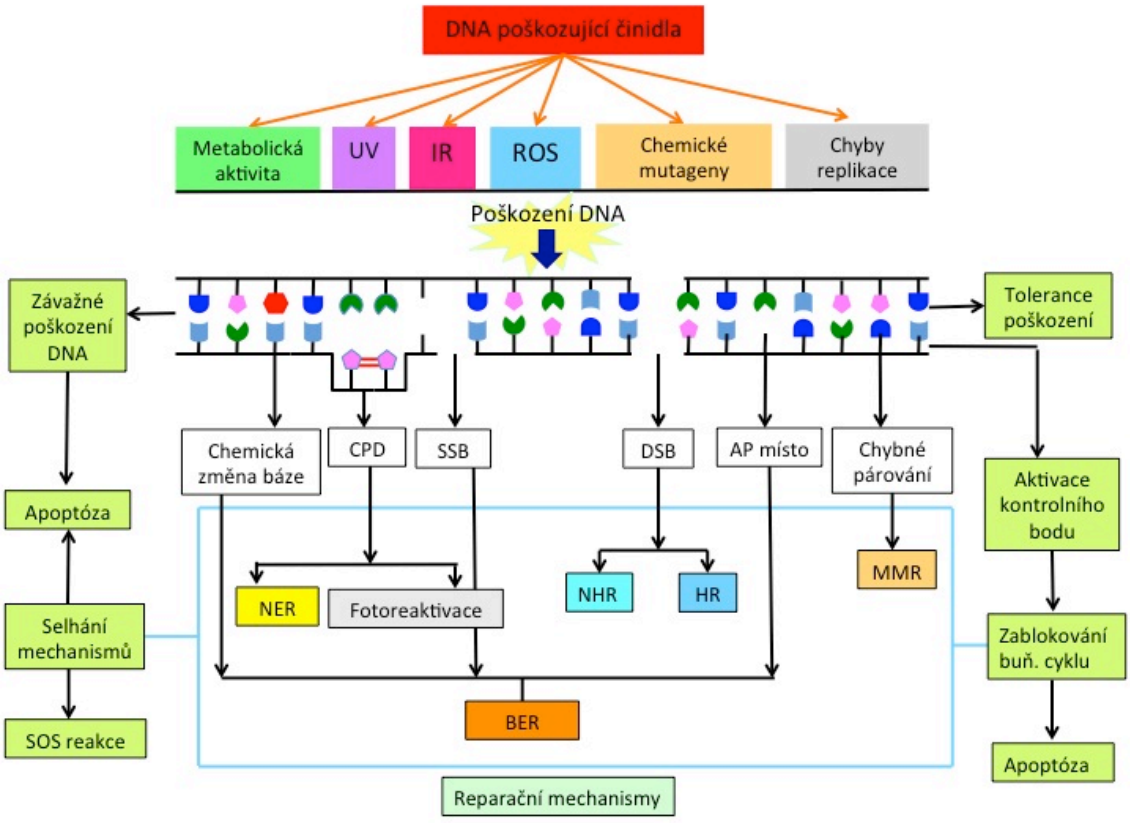
Jedním z hlavních zdrojů spontánního poškození DNA jsou reaktivní kyslíkové radikály (ROS), které vznikají během aerobního metabolismu, deaminace bází především ta, která vede k přeměně cytosinu na uracil, metylace a spontánní hydrolýza vedoucí ke vzniku abazických, tj. apurinních nebo apyrimidinových míst (Lindahl 1993).

Kromě toho může ke vzniku DNA poškození vést působení řady exogenních faktorů, jednak přirozeně přítomných v životním prostředí jako ionizační (IR) nebo UV záření, sucho, salinita, těžké kovy a dále řada chemických látek připravených lidmi a vyskytujících se jako průmyslové chemikálie, léčiva, odpady a znečištění životního prostředí jako např. MMS nebo benzo[a]pyren vznikající při spalování organických látek (např. tabákových listů) (Friedberg et al. 2006).

Široké spektrum možných poškození DNA vedlo k evoluci různých strategií, kterými se buňka může s poškozením vyrovnat (obr. 1). Tyto tzv. reparační dráhy zahrnují širokou škálu mechanismů od přímého zvratu poškození, např. štěpení UV fotodimérů fotolyázami, přes několik typů excisních reparací: nukleotidová excisní reparace, excisní reparace bází, oprava chybného párování bází po komplexní reparaci DSB buď HR nebo NHEJ mechanismem.

Kromě vlastní reparace existují mechanismy umožňující buňkám dočasnou toleranci poškození. Jeden z mechanismů tolerance spočívá ve schopnosti buňky replikovat DNA i přes poškozený templát, obsahující modifikované báze. Bez možnosti dočasné tolerance DNA poškození by musela buňka čelit riziku kolapsu

replikační vidličky vedoucího k translokacím, chromozomálním aberacím a případně buněčné smrti (Waters et al. 2009).



Obrázek 1: Poškození a reparace DNA. Podle typu poškození vznikajícím různě působícími genotoxiny jsou spouštěny rozdílné reparační mechanismy. S poškozením a reparací DNA jsou propojeny dráhy, které rozhodují o tom, zda a jak bude poškození reparováno. V případě tak závažného poškození genomu, že dojde k saturaci nebo selhání reparace dochází k aktivaci kontrolního bodu buněčného cyklu, která může vést k zablokování postupu buněčným cyklem, což poskytne buňce čas na opravu DNA nebo její zánik - PCD (upraveno podle Rastogi et al. 2010).

1.1 Přímá reverze poškození

Existuje několik mechanismů umožňujících opravovat určité typy poškození DNA přímo, bez nutnosti vyštěpovat a nahrazovat poškozenou část vlákna DNA.

Jedním z příkladů přímé reverze poškození je fotoreaktivace UV-dimerů. U rostlin je fotoreaktivace hlavní reparační drahou fotodimérů indukovaných UVC a UVB, kterou provádí fotolyázy specificky štěpící buď cyklobutanový kruh pyrimidinového dimeru (CPD) anebo 6‘-4‘ vazbu pyrimidin-pyromidonového

diméru (6-4PP) (Stapleton 1992). Po vazbě na dimér jejich aktivita závisí na přítomnosti a absorbci modrého světla (320-400nm), jehož energii využívají ke štěpení a monomerizaci dimerů (Carell et al. 2001).

Dalším mechanismem je dráha přímé reverze O^6–metylguaninu vznikajícího působením nitrosolátek zpět na guanin O^6–metylguanin DNA metyltransferázou, kerá se vyvinula u bakterií (Ada) a savců (O^6MeT) (Veleminsky et al. 1994).

1.2 Excisní reparace DNA

Všechny dráhy excisní reparace mají společný mechanismus zahrnující rozpoznání poškození, štěpení fosfodiesterové kostry v těsném, nebo vzdálenějším okolí poškození, odstranění úseku s poškozením a jeho nahrazení DNA syntézou *de novo* (reparační syntéza) a ligací nově syntetizovaného úseku se stávajícím (Ataian a Krebs 2006).

Excisní reparaci podle délky nahrazené části dělíme na tři typy: excisní reparaci bází (BER), nukleotidovou excisní reparace (NER) a reparaci chybného párování bází („miss-match“ repair).

Excisní reparace bází (BER)

Objemově malé adukty DNA, které jsou většinou směřovány do mělkého žlábku dvoušroubovice a nevedou ke změně struktury DNA, jsou odstraňovány excisní reparací bází (Lindahl a Barnes 2000). K poškození bází, které je rozpoznáváno BER, dochází zejména působením kyslíkových radikálů a alkylačních činidel, např. MMS.

BER je iniciována DNA glykosylázami, které rozpoznávají a odstraňují poškozenou bázi, čímž vzniká AP místo, které je následně štěpeno AP-endonukleázou. Vzniklý jednovláknový zlom (SSB) je dále opraven *de novo* buď syntézou pouze několika nukleotidů v místě zlomu, tzv. „short-patch“ repair nebo syntézou dlouhého úseku DNA tzv. „long-patch“ BER, kdy je nahrazeno 2-10 nukleotidů (Memisoglu a Samson 2000).

V savčích buňkách je závěrečná ligace prováděna v závislosti na buněčném cyklu buď ligázou 3 (LIG3) nebo ligázou 1 (LIG1) (Moser et al. 2007). U rostlin byly sice identifikovány tři geny pro DNA ligázy – *LIG1, LIG4* a *LIG6*, nicméně ekvivalent savčí LIG3 se u rostlin nevyskytuje (Bonatto et al. 2005). Waterworth et

al. (2009) ukázala, že u *Arabidopsis* je LIG3 při BER zastoupena LIG1. Holá et al. (2013) navíc zjistila, že u mechu *Physcomitrella* se na ligaci zlomů vzniklých během BER pravděpodobně podílí i LIG4. O specifitě LIG6 zatím nejsou údaje.

Nukleotidová excisní reparace (NER)

NER je univerzální a flexibilní reparační mechanismus, který dokáže odstranit široké spektrum strukturně odlišných poškození narušujících geometrii dvoušroubovice DNA a zabraňujících její funkci templátu, tj. blokujících replikaci a transkripci. Mezi taková poškození patří i fotodiméry CPD a 6-4PP. Pokud nejsou v buňce přítomny funkční fotolyázy, je NER hlavní reparační drahou odstraňující tato poškození.

Během opravy tímto mechanismem je vlákno s DNA lézí štěpeno po obou stranách poškození a 20-40 nt dlouhý oligonukleotid (2-4 závity dvoušroubovice) nesoucí poškození je z dvoušroubovice odstraněn. Vzniklá mezera je zaplněna reparační syntézou DNA a nově syntetizovaná DNA je připojena k původnímu vláknu ligací (Reardon a Sancar, 2005).

U člověka vedou defekty v genech účastnící se NER ke vzniku fotosenzitivních syndromů *Xeroderma pigmentosum* (XP) nebo Cockaynův syndrom (CS). Dosud bylo identifikováno osm geneticky komplementačních skupin pro XP (XPA-G, XPV) a dvě skupiny pro CS (CSA, CSB). Bioinformatická analýza NER proteinů u rostlin naznačuje, že se u nich nachází většina homologů savčích a kvasinkových proteinů této reparační dráhy (Schroeder 2011).

1.3 Oprava dvouvláknových zlomů

DSB vznikají přirozeně během fyziologických buněčných procesů jako je mitotická a meiotická rekombinace, V(D)J rekombinace, působením topoizomeráz nebo jako důsledek kolapsu replikačního komplexu. K endogenním činidlům indukujícím vznik DSB patří zejména IR a některé chemické látky, jako např. Bleomycin, které IR mimikují. DSB představují jedno z nejzávažnějších poškození DNA, protože neopravený zlom DNA může mít dalekosáhlé důsledky od ztráty genetické informace po přestavby chromozómů.

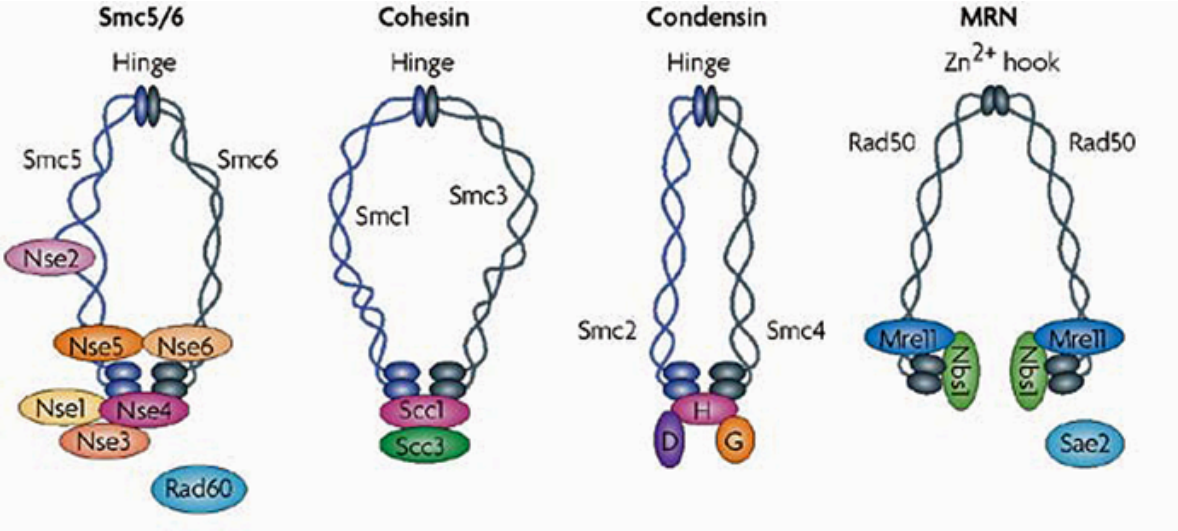
Proto se již záhy vyvinuly mechanismy sdílené všemi organismy, které dokáží dvouvláknové zlomy DNA rozpoznat, v závislosti na poškození regulovat buněčný cyklus, aktivovat opravu DNA, popřípadě iniciovat řízený rozpad buněk, apoptózu u rostlin popisovanou jako programovanou buněčnou smrt (PCD).

U eukaryot se vyvinuly dva principiálně odlišné mechanismy reparace DSB. HR, která k opravě využívá sekvence homologní alely, podle které se nahradí poškozený úsek DNA a dokáže tak kompenzovat případnou ztrátu genetické informace. Druhým typem opravy je přímé spojování konců DSB NHEJ, kdy není využívána žádná, nebo jen minimální homologie několika nukleotidů (mikrohomologie) v okolí zlomu.

SMC proteiny a MRN komplex a jejich význam v opravě DNA

Evolučně konzervovaná rodina *SMC* genů kóduje proteiny tří funkčních komplexů: kohesinu (SMC1/3) zajišťujícím kohezi sesterských chromatid, kondensinu (SMC2/4) účastnícím se kondenzace chromozómů během mitózy a komplexu SMC5/6, který má dosud ne zcela jasnou roli v rekombinaci a reparaci DNA (Harvey et al. 2002, Lehmann 2005).

Všechny SMCx/y komplexy jsou heterodiméry, ve kerých je každý SMC protein tvořen dlouhou aniparalelní superšroubovicí, tzv. „coiled-coil“ doménou, která má na jednom konci globulární ATPasovou doménu a na druhém tzv. „hinge“, oblast ohybu, která je důležitá jednak pro dimerizaci SMC proteinů a jednak pro vazbu komplexu k DNA. Ke globulárním ATPasovým doménám se připojují kleisiny, které heterodimér uzavírají a tím umožňují SMC komplexům vytvářet prstencové útvary kolem DNA (Hirano 2006) (obr. 2).

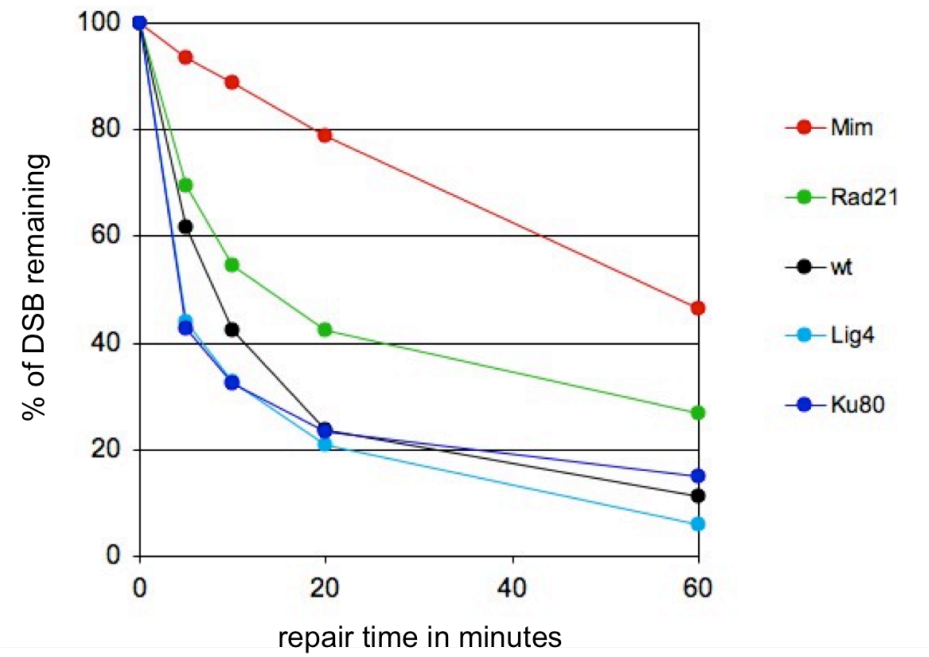


Obrázek 2: Struktura SMC a MRN komplexu (Murray a Carr, 2008).

O SMC5/6 komplexu je známo, že u kvasinek stabilizuje replikační vidličku, a je důležitý pro reparaci DSB homologní rekombinací (Murray & Carr 2008). Předpokládá se, že SMC1/3 a SMC5/6 komplexy váží a stabilizují poškozené molekuly DNA v kontaktu s odpovídajícími sesterskými chromatidami, a tím umožňují a podporují homologní rekombinaci mezi nimi (Lehmann 2005).

U *Arabidopsis* se SMC5/6 komplex podílí na rychlé opravě DSB mechanismem nezávislým na klíčových proteinech NHEJ dráhy, heterodimeru KU70/80 a LIG4. Oproti tomu mutant *smc6b* (*atmim*, Mengiste et al. 1999; *atsmc6b,* Watanabe et al. 2009) vykazuje na rozdíl od *atlig4* a *atku80* výrazný defekt opravy DSB, přičemž především zcela postrádá pro rostliny typickou rychlou 1. fázi reparace DSB (obr. 3).

Částečná porucha reparace DSB se také projevuje u *atrad21.1* (Kozak et al. 2009). RAD21/SCC1 patří do rodiny kleisinů, které jsou podjednotkou komplexu kohesinu a interagují s SMC1/3 proteiny. Mutant v genu *RAD21* kvasinek je vysoce citlivý vůči činidlům poškozujícím DNA (Birkenbihl a Subramani, 1992). U *Arabidopsis* se RAD21 vyskytuje ve třech alelách, které vzájemně kooperují v průběhu reparace DSB (da Costa-Nunes et al. 2014).



Obrázek 3: Kinetika opravy DSB po působení 50 µg/ml bleomycinu u *Arabidopsis*. Maximální poškození je normalizováno jako 100% v čase t = 0 pro všechny mutanty. Divoký typ a *atku80* a *atlig4* (dokonce rychleji) opravují většinu indukovaných DSB již během prvních 10 minut. Oproti tomu *atrad21.1* a *atmim* vykazují zřetelně pomalejší reparaci DSB s výrazným defektem u *atmim*, který zcela postrádá pro rostliny typickou rychlou 1. fázi reparace DSB. (Kozák et al. 2009)

Strukturně podobný SMC komplexům je komplex MRN (obr. 2), který vystupuje ve většině, pokud ne ve všech, procesech spojených s konci dvouvláknové DNA, včetně signalizace poškození, homologní i nehomologní rekombinace, udržování telomer a meiotické rekombinace (Lamarche et al. 2010).

Skládá se ze tří proteinů z nichž dva – MRE11 a RAD50 – lze nalézt u všech fylogenetických domén (Lammens et al. 2011). Třetím, v evoluci méně konzervovaným proteinem komplexu je NBS1 (Nijmegen Breakage Syndrome). Celý komplex je heterohexamer, ve kterém je každý z proteinů zastoupen vždy dvakrát ($M_2R_2N_2$) (Williams et al. 2010).

MRE11 nese na svém N-konci Mn^{2+}/Mg^{2+} dependentní fosfodiesterázovou doménu a na svém C-konci dvě DNA vazebné domény. *In vitro* tvoří stabilní dimery, které mají schopnost vázat se ke koncům dvouvláknové DNA a vykazují

endo- i exonukleázovou aktivitu u jednovláknové i dvouvláknové DNA, postrádají však 5‘-3’ exonukleázovou aktivitu nezbytnou pro tvorbu dlouhých 3’ jednovláknových konců vyžadovaných pro homologní rekombinaci (Lamarche et al. 2010). Nicméně pro vznik dlouhých jednovláknových 3’-konců je MRN komplex esenciální jako řídící faktor, který *in vivo* zajišťuje jejich vznik v součinnosti s dalšími 3‘-5’ exonukleázami (Mimitou a Symington 2008).

Protein RAD50 zastává v MRN komplexu podobnou strukturní roli jako SMC proteiny tím, že vytváří cirkulární jádro MRN komplexu podobné SMC komplexům (obr. 2). Walker A motiv na N‘-konci a Walker B motiv na C‘-konci spolu interagují a vytváří ATPasovou doménu s afinitou k dvouvláknovým DNA koncům (Hopfner et al. 2001). Oblast mezi těmito motivy vytváří antiparalelní superšroubovici, která je zakončená zinkovým háčkem (Zn-hook) (Hopfner et al. 2002). K MRE11 se váže RAD50 ATPasovou doménou a společně vytvářejí globulární „hlavu“, která asociuje s konci dvouvláknové DNA (Hopfner et al. 2001). Zinkovými háčky se mohou MR komplexy propojovat a díky tomu udržují konce DNA ve vzájemné juxtapozici a nedovolí jim, aby se od sebe vzdálily.

Třetím proteinem v MRN komplexu je NBS1. Prostřednictvím flexibilního řetězce se připojuje k MRE11 a tím stimuluje vazbu k DNA, nukleázovou aktivitu MR komplexu a je také zodpovědný za translokaci MRN do jádra. V centrální oblasti nese několik SQ motivů, které jsou fosforylovány ATM kinázou. Na C‘-konci nese doménu, která interaguje s ATM kinázou a přitahuje ji k DSB (Williams et al. 2009). NBS1 je také důležitý pro indukci apoptózy u savců a PCD u rostlin jako odpovědi na masivní poškození DNA.

U savců vede nulová mutace některé z komponent MRN komplexu k letalitě (Lamarche et al. 2010), zatímco u rostlin tomu tak není. Např. u *Arabidopsis* jsou mutanty *atrad50* a *atmre11* životaschopné, i když vykazují růstové poruchy, částečnou sterilitu, defekty v opravě DSB, meiose a udržování telomer (Gallego et al. 2001; Puizina et al. 2004). NBS1 deficientní rostliny nevykazují růstové poruchy ani poruchy reprodukce (Najdekrová a Široký, 2012).

Podobně jako u *Arabidopsis* jsou i u *Physcomitrelly* mutanty *ppmre11*, *pprad50* a *ppnbs1* životaschopné. Nicméně *ppnbs1* oproti *ppmre11* a *pprad50*, které jsou extrémně citlivé k poškození DNA IR a radiomimetiky indukujícími DSB a je u nich výrazně snížena schopnost cílené integrace transgenní DNA, vykazuje z tohoto hlediska téměř divoký fenotyp.

Překvapivě u všech mutant *MRN* komplexu probíhá reparace DSB stejně rychle a efektivně jako u divokého typu, ale zřejmě mechanismem nezávislým na MRN komplexu, během něhož dochází k chybám reparace na úrovni sekvence DNA (error-prone) a tím vzniku a akumulaci mutací (Kamisugi et al. 2012).

Homologní rekombinace (HR)

Jednou z cest opravy dvouvláknových zlomů je HR. Jde o velmi přesný mechanismus reparace, při němž je při odstraňování DSB jako templát využita nepoškozená sesterská chromatida nebo alela na homologním chromozomu. Proto je tento způsob reparace omezen pouze na dobu, kdy je homologní DNA v buňce přítomná, což u somatických buněk haploidních organismů znamená pozdní S a G_2 fázi buněčného cyklu. U diploidních organismů i přesto, že je homologní chromozom přítomen stále, je HR během G_1 fáze výrazně redukována, zřejmě jako prevence ztráty heterozygosity (Mao et al. 2008; Rothkamm et al. 2003).

Ostatní mechanismy opravy DSB mohou s homologní rekombinací spolupracovat, nebo s ní o opravdu DSB soutěžit (Jasin a Rothstein 2013). O mechanismu opravy DSB tak rozhoduje řada faktorů. Jedním z nich je způsob úpravy konců zlomu tak, aby byly kompatibilní pro následnou ligaci. Pro iniciaci HR je potřeba generovat 3‘ jednovláknové konce DNA resekcí 5‘ konců (Symington a Gautier 2011). Počátku úpravy DNA konců se účastní mimo jiné i MRN komplex, který interaguje s KU proteiny, které naopak konce DNA před resekcí chrání a jsou jedněmi z klíčových proteinů NHEJ dráhy. Tím se může MRN komplex podílet na výběru způsobu opravy DSB (Mimitou a Symington 2009; Garcia et al. 2011).

Dalším klíčovým krokem pro opravu DSB mechanismem HR je navázání DNA – dependentní APTázy RAD51. RAD51 se váže k jednovláknovému konci DNA, kde za podpory BRCA2 a paralogů RAD51, nahrazuje replikační protein A (RPA). Zprostředkovává vstup/invazi jednovláknové DNA do templátové, tedy nepoškozené, dvoušroubovice a vyhledání homologní sekvence (Baumann a West 1998; Effrossyni Boutou 2011; McIlwraith et al. 2000).

Zřejmě proto, že se jedná o jeden z nejdůležitějších proteinů v mechanismu HR, je mezi eukaryoty velmi dobře konzervován. Ortology RAD51 byly identifikovány i u několika rostlin, včetně *Arabidopsis* a *Physcomitrelly*. Zatímco u *Arabidopsis* byl nalezen jeden paralog RAD51 (Doutriaux et al. 1998), *Physcomitrella* kóduje dva proteiny RAD51 – PpRAD51.1 a PpRAD51.2, které jsou

velmi podobné a v průběhu HR zřejmě spolupracují (Ayora et al. 2002). Na rozdíl od savčích mutant jsou mutanty *atrad51* i *pprad51* životaschopné (Li et al. 2004; Schaefer et al. 2010).

Po nalezení homologie prodlouží DNA polymerázy 3'-konec invazního vlákna syntézou nové DNA, čímž dochází k tvorbě Hollidayova kříže (Szostak et al. 1983; Sung a Klein 2006). Po reparační syntéze DNA pokračuje HR připojením druhého 5'-konce zlomu k templátovému vláknu. Toto připojení zajišťuje protein RAD52, který je schopný k templátové dvoušroubovici připojit DNA vlákno i s navázaným RPA proteinem (Sugiyama et al. 1998; Sugiyama et al. 2006). Výsledný Hollidayův kříž je pak substrátem pro komplex BLM helikázy a topoizomerázy IIIα (Bizard a Hickson, 2014), nebo pro specifické endonukleázy, u člověka je to resolváza A a komplex Mus81-EmeI (Boddy et al. 2001; Constantinou et al. 2002).

Homologní rekombinace je někdy chápána v užším slova smyslu jako klasická oprava dvouvláknových zlomů. Oprava DSB založená na vyhledávání homologie však zahrnuje několik podobných drah, které jsou až do kroku reparační syntézy DNA shodné (Paques a Haber 1999).

Specifickým způsobem opravy DSB homologní rekombinací lišícím se od předchozích tím, že k vyhledání homologie nevyužívá ani vlákno sesterské chromatidy ani homologního chromozomu je model SSA (z angl. Single-Strand Annealing). Vyhledávání homologie probíhá v rámci vlákna, na dvoušroubovici u které došlo ke vzniku zlomu. Tento mechanismus je sice efektivní, ale vysoce mutagenní, protože při něm dochází ke ztrátě DNA, která leží mezi homologními úseky (Helleday et al. 2007).

SSA společnš s dalším modelem opravy - SDSA (z angl. Synthesis-Dependent Strand Annealing) jsou považovány za mechanismy opravy DSB v somatických rostlinných buňkách. Podle SDSA modelu je po reparační syntéze DNA invazní vlákno uvolněno z D-smyčky a spojeno s druhým koncem dvouvláknového zlomu, takže z principu nemůže dojít ke crossing-overu (McMahill et al. 2007).

BIR (z angl. Break-Induced Replication) přispívá k opravě DSB v případě, že v genomu může homologii nalézt pouze jeden konec zlomu. Může hrát důležitou roli při kolapsu replikační vidličky a je nezbytná pro udržování konců chromozómů (Malkova a Ira 2013).

Kromě opravy DSB je homologní rekombinace jedním z mechanismů, kterým může být do genomu buňky začleňována transgenní DNA. U vyšších

eukaryot, jak rostlin tak obratlovců, se vkládaná DNA integruje do genomu prostřednictvím NHEJ dráhy, což vede k inzerci DNA do náhodných míst. Nicméně integrace homologní rekombinací u savců i rostlin také probíhá (Siebert a Puchta 2002; Liang et al. 1998), byť s mnohem nižší frekvencí – u *Arabidopsis* je to asi jedna integrace homologní rekombinací na 3000 integrací NHEJ dráhou (Kempin et al. 1997).

Oproti tomu u *Physcomitrelly* je většina transgenní DNA inkorporována do genomu homologní rekombinací (Kamisugi et al. 2006). Jakým způsobem bude transgenní DNA integrována závisí pravděpodobně na expresi proteinů, které se účastní opravy DSB. U *Physcomitrelly* vede například ztráta funkce MRE11 a RAD50 k silnému potlačení cílené integrace DNA, zatímco nehomologní integrace zůstává prakticky nezměněna (Kamisugi et al. 2012). Podobný vliv má ztráta funkce obou proteinů RAD51 (Schaefer et al. 2010). MSH2 – centrální protein opravy chybného párování bazí – působí naopak proti rekombinaci a jeho vyřazením frekvence HR stoupá (Trouiller et al. 2006).

Nehomologní rekombinace (NHEJ)

Dalším způsobem reparace DSB je tzv. nehomologní rekombinace, která spočívá v přímém, nehomologním spojování volných konců DNA – NHEJ (Non-Homologous End Joining). Na rozdíl od HR je účinnější, rychlejší, ale z principu vede ke vzniku mutací (Mao et al. 2008). Bylo popsáno několik drah nehomologní rekombinace: klasicky míněné přímé nehomologní spojování konců (C-NHEJ), mikrohomologií zprostředkované spojování konců DNA (MMEJ) a tzv. alternativní spojování konců DNA (Alt-NHEJ). Tyto dráhy jsou charakterizovány především jejich závislostí respektive nezávislostí na KU komplexu (Mladenov a Iliakis, 2011).

KU nezávislá je Alt-NHEJ a MMEJ dráha. MMEJ podobně jako homologní SSA, vyhledává homologie na protilehlém vlákně DNA, na rozdíl od SSA ale využívá k opravě výrazně kratší homologie, u *Arabidopsis* 1–16 nukleotidů (Gorbunova a Levy 1997; Windels et al. 2003). Studium na kvasinkách ukázalo, že touto dráhou jsou přednostně opravovány zlomy DNA s nekompatibilními konci. Jde o reparační dráhu často vedoucí k rozsáhlým delecím a tím letálním mutacím (Frit et al. 2014; Ma, Kim et al. 2003).

O opravě Alt-NHEJ mechanismem je toho zatím známo velmi málo. Zdá se, že tato dráha nevyužívá ke spojování konců DNA žádné homologie a že se na tomto

způsobu opravy podílí DNA-ligáza 1 (LIG1). Alt-NHEJ i MMEJ jsou v přítomnosti funkčního KU komplexu potlačeny a slouží zřejmě jako „záložní“ opravné dráhy (Decottignies 2013). Pokud se ukáže, že v této reparační dráze jsou volné konce DSB stabilizovány místo KU SMC komplexy, lze předpokládat, že dříve popsaná KU/LIG4 nezávislá dráha rychlé reparace DSB u *Arabidopsis* (Kozák et al. 2009) patří do této skupiny.

Hlavním mechanismem opravy DSB nehomologní rekombinací u většiny eukaryotních organismů je NHEJ. Klíčovými proteiny této dráhy jsou: heterodimer KU70/80, katalytická podjednotka DNA dependentní proteinkinázy (DNA-PKcs), endonukleáza Artemis, protein XRCC4, DNA ligáza 4 (LIG4) a protein XLF. Homology těchto proteinů se nacházejí u savců (Williamset al. 2014) a s výjimkou DNA-PKcs i u rostlin (Friesner a Britt, 2003; Tamura et al. 2002; West et al. 2000).

Klíčovým proteinem NHEJ dráhy je heterodimer KU70/80, který rozpoznává a váže konce dvouvláknové DNA (Tamura et al. 2002). V savčích buňkách se ke KU komplexu na DSB váže DNA-PKcs a tento DNA-PK komplex chrání konce DNA před nukleázami a fosforyluje celou řadu dalších proteinů. Zároveň k DSB přitahuje endonukleázu Artemis se kterou DNA-PKcs vytváří komplex schopný štěpit 5’ a 3’ přesahující konce DNA a DNA vlásenky (Ma et al. 2002) a komplex XRCC4-LIG4, který dokončuje reparaci DSB spojením konců DNA (Hsu et al. 2002).

LIG4 u *Physcomitrelly* je jak se zdá nejen proteinem dráhy C-NHEJ, ale je významná i pro reparaci objemově malých poškození mechanismem BER. Kinetika reparace jednovláknových zlomů a poškození DNA indukovaných ROS u *pplig4* mutanta ukazuje, že odstraňování tohoto typu poškození u něj probíhá pomaleji než u wt (Holá et al. 2013).

1.4 Tolerance poškození DNA

Mechanismy tolerance poškození DNA se od reparačních mechanismů liší v principu tím, že jejich cílem není obnovení původní struktury DNA, ale překonání dočasně přetrvávajícího poškození DNA ve formě neinstrukčního templátu (AP-místo) a různých typů aduktů. Mechanismy tolerance poškození DNA jsou optimalizovány pro podporu přežití buňky umožněním dokončení replikace DNA, nikoliv pro zachování přesné genetické informace. Jeden z hlavních mechanismů

tolerance poškození DNA spočívá ve schopnosti replikovat DNA i přes stávající poškození, jde o tzv. syntézu přes poškození (translesion synthesis, TLS).

Během syntézy přes poškození je DNA léze překonána inkorporací nukleotidu proti poškození. V tomuto kroku vystupují specifické polymerázy, tzv. TLS polymerázy, protože replikační DNA polymerázy, optimalizované pro přesnou replikaci DNA, nejsou obvykle schopné poškození překonat (Waters et al. 2009).

TLS polymerázy postrádají korekční 3‘-5’exonukleázovou aktivitu a oproti replikačním polymerázám jsou mnohem méně procesivní a jejich aktivní centrum je větší a více otevřené, aby mohly obsáhnout objemná poškození DNA (Prakash et al. 2005; Curtis a Hays 2007). V důsledku toho, že TLS polymerázy přednostně v místě neinstrukčního nebo poškozeného templátu inkorporují adenin je TLS silně mutagenní dráha (Rabkin et al. 1983). To je zejména dobře patrné v případě UV mutageneze (Holá et al. 2015).

2. Mutageneze – modelové genotoxiny

Aby bylo možné studovat určitou dráhu reparace je potřeba indukovat vznik poškození, které daná dráha reparuje. K tomuto účelu jsou využívány v laboratorních podmínkách genotoxiny se známým mechanismem účinku.

V disertační práci bylo použito radiomimetikum Bleomycin působící v místě účinku mnohačetná poškození DNA včetně DSB prostřednictvím generovných ROS, alkylační činidlo methyl methansulfonát (MMS) k indukci objemově malých alkylačních poškození bází odstraňovaných BER mechanismem a UV záření pro indukci objemných DNA lézí odstraňovaných NER.

2.1 Bleomycin (BLM)

Bleomycin je glykopeptid hojně využívaný jako protinádorové léčivo. Je produkován bakterií *Streptomyces verticillus.* V laboratorních podmínkách simuluje Bleomycin efekt působení ionizačního záření, protože se dokáže vázat k DNA a za přítomnosti redukčních iontů (Fe^{2+}, Cu^{2+}) a kyslíku indukovat štěpení DNA (Stubbe a Kozarich 1987).

Fe^{2+}-Bleomycin komplex interkaluje DNA a poté reaguje s O_2 (Buettner a Moseley 1993). Vzniká tzv. aktivovaný Bleomycin, který generuje ROS a štěpí vlákno DNA. Pokud je komplex reaktivován redukcí oxidovaného iontu, může indukovat další štěpení a způsobit tak vznik DSB (Hecht 1986; Steighner a Povirk 1990). Poměr jednovláknových a dvouvláknových zlomů indukovaných Bleomycinem je přibližně 10:1(Povirk et al. 1977).

Aktivovaná forma Bleomycinu je vysoce oxidační činidlo, které může z DNA odčerpávat vodíkové radikály (H•) a generovat hydroxylové radikály (HO•), díky čemuž způsobuje oxidativní poškození DNA (Oberley & Buettner 1979).

2.2 UV záření

Mutagenem zevního prostředí, se kterým přicházejí do kontaktu všechny živé organismy možná nejčastěji je UV záření, které je přirozenou součástí slunečního světla.

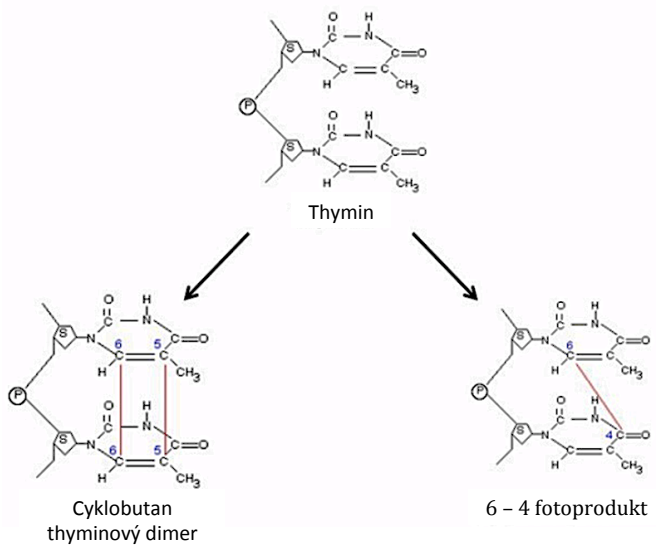
Podle vlnové délky rozlišujeme tři druhy UV. UVC má nejkratší vlnovou délku (< 280 nm). Na biotickou sféru Země nemá vliv, protože jeho složka je ve

slunečním záření kompletně pohlcována zemskou atmosférou obdobně jako složka UVB (280-315 nm), která je také z velké části pohlcena, nicméně část jí proniká. Největší dávku UV záření, které dopadá na zemský povrch, tvoří UVA o vlnové délce 315-400 nm.

Nežádoucí účinky slunečního záření jsou připisovány hlavně UVB, které je absorbováno buněčnou DNA. UVA nativní DNA absorbováno není a proto je při přímé indukci DNA poškození méně účinné než UVC a UVB. Může však být příčinou sekundárních fotoreakcí již existujících DNA fotoproduktů nebo poškozovat DNA nepřímo generováním ROS (Sinha a Häder 2002).

UVC a UVB záření indukuje převážně vznik fotodimérů CPD a 6-4PP (obr. 4). CPD tvoří kolem 75% a 6-4PP asi 25% všech poškození DNA indukovaných UV zářením. Oba dva typy poškození způsobují distorzi dvoušroubovice DNA a blokují transkripci i replikaci DNA (Sinha a Häder 2002).

Nepřímé působení UV, především UVA způsobuje i další typy poškození DNA, jako je vznik dvouvláknových zlomů při kolapsu replikační vidličky zablokované neopravenými fotoprodukty nebo oxidativní poškození DNA způsobené ROS, vznikajícími převážně působením UVA záření a vedoucí jak k SSB a DSB, tak ke vzniku křížových vazeb DNA-protein (Heck et al. 2003; Rastogi et al. 2010; Rapp a Greulich 2004).



Obrázek 4: Cyklobutan pyrimidinové dimery a 6‘-4‘ fotoprodukty vznikají mezi sousedními pyrimidiny působením UVB záření. Způsobují distorzi dvoušroubovice DNA a jsou rozpoznávány jako objemné léze DNA (bulky lesions) mechanismem NER (Horrell 2015).

2.3 Methyl methansulfonát (MMS)

MMS je elektrofilní alkylační činidlo, které způsobuje modifikaci DNA přidáváním metylační skupiny mechanismem SN2 na řadu nukleofilních míst v DNA. Přednostně jsou metylovány pozice N^7 a N^3 v adeninu, protože se jedná o místa s nejvyšším negativním elektrostatickým potenciálem v DNA (Wyatt a Pittman 2006). Jedná se převážně o objemově malá poškození bází směrovaná do mělkého žlábku dvoušroubovice DNA, která nevedou k výrazné změně struktury a proto je tento typ poškození odstraňován mechanismem BER (Kondo et al. 2010).

3. Metody detekce poškození a studium reparace DNA

Existuje řada metod, kterými lze studovat mechanismus reparace poškození DNA na různých úrovních od kvantitativního měření opravy jednotlivých lézí DNA po zjišťování změn na molekulární úrovni DNA (Kumari et al. 2008). Řada metod je založena na sledování projevů reakce na poškození, např. na remodelaci chromatinu (Price a D'Andrea 2013), fosforylaci H2AX (Redon et al. 2009), translokaci a modifikaci reparačních proteinů (Polo a Jackson 2011), změny v replikaci, transkripci či translaci atd.

V současné době oblíbená a přednostně využívaná metoda detekce a studia kinetiky reparace DSB převážně v savčích buňkách, je založena na kvantifikaci γH2AX ohnisek (foci), vznikajících fosforylací, která je důsledkem rychlé buněčné reakce na indukci DSB (Mah et al. 2010). γH2AX vznikají fosforylací C'-koncového serinu histonu H2AX a to až do vzdálenosti 2Mb od poškození DNA (Rogakou et al. 1999). Jedná se o nepřímou metodu detekce poškození založenou na sledování kinetiky fosforylace histonů γH2AX (Chowdhury et al. 2005).

Mezi metody monitorující přímé fyzické poškození DNA patří gelová elektroforéza jednotlivých buněk nebo jader, tzv. kometový test (comet assay). Jde o citlivou a rychlou metodu detekce SSB a DSB genomové i organelové DNA (Ostling a Johanson 1984; Koppen a Verschaeve 1996; Angelis et al. 1999).

Elektroforéza jednotlivých jader ukotvených v agarózovém gelu na mikroskopickém sklíčku vede k pohybu fragmentované DNA a rozvolněných nukleoidů v elektrickém poli z kruhových jader a vzniku objektů ne nepodobných

kometám. Poškození DNA je stanoveno jako poměr signálu hlavy a ohonu komety, pozorovaný fluorescenční mikroskopií (Collins 2004). Inkubace jader před elektroforézou s endonukleázami rozpoznávajícími specifická poškození umožňuje detekovat i léze DNA, které primárně nevedou ke vzniku zlomu DNA, např. oxidační poškození bází (Collins et al. 1996).

Samotný kometový test indikuje přítomnost zlomů DNA a nevypovídá nic o průběhu a důsledcích reparace. Ke zjištění vlivu reparace na sekvenci DNA je nutné použít jiný přístup, např. analýzu indukce mutací založenou na měření frekvence vzniku mutant, popřípadě kombinovanou se zjištěním vzniku konkrétního typu mutace sekvenováním. U *Physcomitrelly* je možné sledovat frekvenci vzniku 2-fluoroadenin (2-FA) rezistentních mutant, nesoucích mutaci v genu pro adenin fosforibosyltransferázu (APT), umožňujícím pozitivní selekci a, následnou sekvenací *APT* lokusu, určení typu mutace.

Kometový test společně s mutační analýzou je kombinací kvalitativního a kvantitativního pohledu na opravu DNA (Kamisugi et al. 2012; Holá et al. 2013; Holá et al. 2015).

4. Modelový organismus *Physcomitrella patens*

Physcomitrella patří mezi mechy (*Bryophyta*), které společně s lišejníky (*Marchantiophyta*) a játrovkami (*Anthocerotophyta)* představují jednu z nejstarších rostlinných skupin (Kenrick a Crane 1997). Na rozdíl od pozdějších cévnatých rostlin mají mechy poměrně jednoduchou morfologii, vytvářejí jen několik druhů pletiv s omezeným počtem buněčných typů.

U *Physcomitrelly* stejně jako u ostatních mechů dochází během životního cyklu ke střídání generací (obr. 5). Minoritní sporofyt vznikající po oplození vaječné buňky pohyblivým spermatozoidem představuje diploidní stádium vývoje. Sporofyt produkuje haploidní spóry, ze kterých vyrůstá gametofyt, který, na rozdíl od semenných rostlin kde je tvořen jen několika buňkami, u mechu převládá (Cove et al. 1997).

Gametofyt je haploidní a dimorfní – zpočátku roste ve formě filament, která vytvářejí dvoudimenzionální síť nazývanou protonema. Vlákna protonemy se prodlužují díky dělícím se apikálním buňkám na obou koncích každého vlákna. Později dochází k větvení filament a diferenciaci protonemy a vzniká charakteristická struktura – gametofóra, na které se posléze vytvářejí pohlavní orgány: samčí antheridia produkující pohyblivé spermatozoidy a samičí archegonia, ve kterých se tvoří vaječné buňky (Cove 2005).

V laboratorních podmínkách se využívá téměř výlučně gametofyt ve stádiu protonemy, který má řadu vlastností, jenž z něj dělají velmi výhodný rostlinný model. Mezi výhody patří nenáročná kultivace na jednoduchém anorganickém médiu (Cove 1992), kdy *Physcomitrella* může být pěstována jak na médiu zpevněném agarem, tak v kapalných kulturách, které jsou vhodné i pro velkoobjemové bioreaktory (Hohe a Reski 2005). Tato možnost je zajímavá pro biotechnologické využití mechu (Šmídková et. al 2012)

Kromě spor lze dlouhodobě uchovávat také gametofyt *Physcomitrelly*. Při snížené teplotě a za omezeného osvětlení může kultura mechu přežívat ve sterilním prostředí na dostatečné vrstvě média řadu měsíců. V případě většího počtu mutant nebo pro dlouhodobější skladování je možné mech uchovávat pomocí kryokonzervace (Schulte a Reski 2004).

Physcomitrellu lze jednoduše vegetativně propagovat. U jakéhokoli pletiva – gametofor nebo protonemat, lze mechanickým rozrušením indukovat v narušených oblastech přeměnu buněk v chloronemální apikální buňky, které dále

rostou ve formě filament (Prigge a Bezanilla 2010). Ranné stádium protonemy ve fázi růstu filament je pro experimentální práci nejvhodnější a proto se také nejčastěji využívá.

Působením enzymy degradujícími buněčnou stěnu protonemat lze izolovat protoplasty, které v osmoticky vhodném médiu buněčnou stěnu opět regenerují. Na rozdíl od cévnatých rostlin nevyžadují pro regeneraci fytohormony, ani nevytvářejí kalus, ale po vytvoření buněčné stěny začínají růst jako filamenty protonemy (Schween et al. 2003).

Protoplasty *Physcomitrelly* se rutinně využívají pro transformaci pomocí polyethylenglykolu (PEG) (Hohe et al. 2004). Alternativou protoplastů jsou krátké fragmenty jednodenních protonemat. Ty jsou připravována důkladnou homogenizací mladého (většinou týdenního) pletiva. Jednodenní protonemata jsou složena z fragmentů filament obsahujících až 50% dělících se apikálních buněk (Holá et al. 2013; Holá et al. 2015). Taková protonemata lze využít k řadě operací podobně jako protoplasty. Jedním z využití je transformace mechu biolistickou metodou, při které je transgenní DNA vnášena do buňky na kovových mikročásticích (Šmídková et al. 2010).

Integrace transgenní DNA do genomu rostlin se uskutečňuje rekombinací prostřednictvím mechanismů reparace dvouvláknových zlomů DNA. U kvetoucích rostlin se transformující DNA zavedená do buňky začleňuje do genomu převážně náhodně a cílená integrace DNA mechanismem homologní rekombinace (HR) probíhá s velmi nízkou frekvencí (10^{-4}–10^{-5}) (Britt a May 2003). Náhodná integrace vyžaduje enzymy nehomologní rekombinační dráhy NHEJ, během které jsou konce DNA spojovány přímo, popřípadě bez vyhledávání rozsáhlých homologních úseků.

Unikátní vlastností *Physcomitrelly* je vzhledem k vysoké účinnosti HR, že vnášená DNA, pokud nese úseky shodné s genomovou DNA, se přednostně integruje v místě homologie. Podobný jev je známý u kvasinky *Saccharomyces cerevisiae* a naznačuje, že HR je při opravě dvouvláknových zlomů DNA upřednostňována i přesto, že u *Physcomitrelly* je přítomna také reparační dráha NHEJ (Kamisugi et al. 2005; Kamisugi et al. 2006).

Další unikátní vlastností *Physcomitrelly* je haploidní genom gametofytu, který umožňuje snadnou identifikaci transformant, protože ztráta funkce nemůže

být kompenzována funkční alelou homologního chromozomu a proto je vyřazení či modifikace genu haploidní *Physcomitrelly* ihned manifestována změnou fenotypu.

Pro identifikaci a selekci transformant jsou často s transformující DNA vnášeny také selekční markery rezistence k antibiotikům (kanamycin, hygromycin), popřípadě reportérové geny jako β-glucuronidáza (GUS), nebo různé formy fluoreskujících proteinů (GFP a jeho deriváty, RFP atd.).

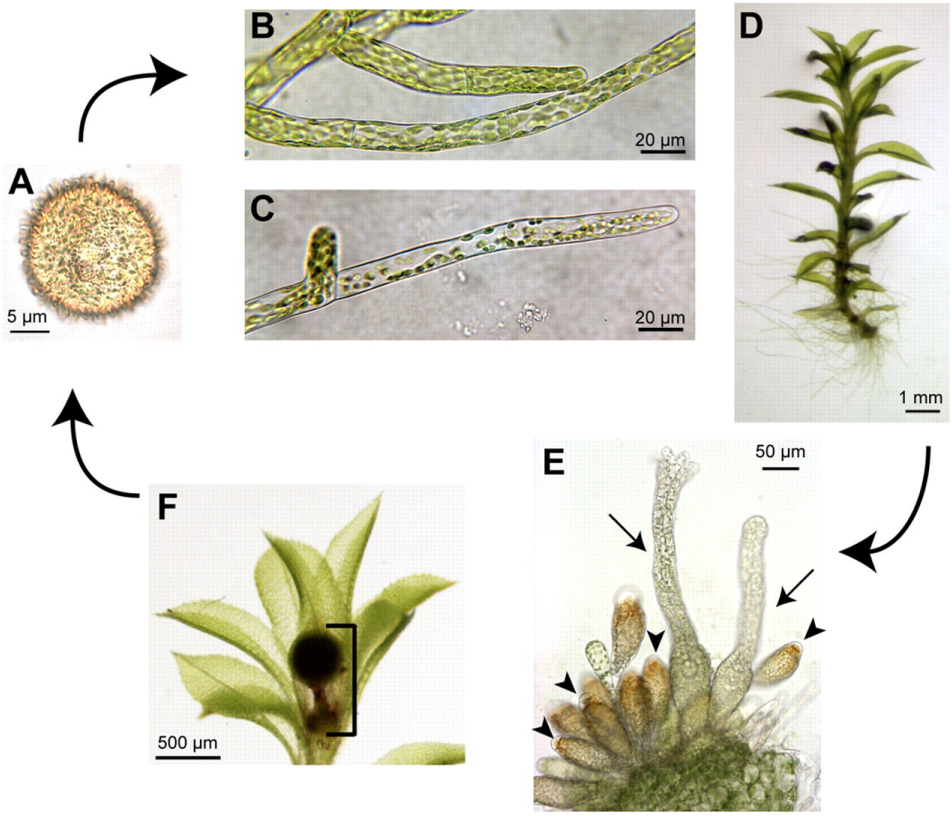
Jako pozitivní selekční marker, kromě vnesení např. externího genu pro bakteriální cytosin deaminázu (*codA*), lze využít i přirozeně se vyskytující geny metabolismu nukleových kyselin, po jejichž inaktivaci přestává organismus vytvářet toxické metabolity a získává tak rezistenci vůči selekčnímu činidlu. Jako příklad může sloužit u *Physcomitrelly* gen pro adenin fosforibosyltransferázu (*APT*), která se účastní syntézy adenosin monofosfátu (AMP) z adeninu a fosforibosyl pyrofosfátu. Ztráta funkce APT dovoluje rostlině přežívat na médium obsahujícím halogenované analogy adeninu např. 2-FA, které jsou pro rostliny s funkční APT toxické (Gaillard et al. 1998; Trouiller et al. 2007). Proto lze gen *APT* využít pro vyhledávání a identifikaci mutací vznikajících po působení genotoxinů a není potřeba sekvenovat celý genom (Holá et al. 2013; Holá et al. 2015).

Physcomitrella představuje modelový organismus vhodný jak pro „přímou" tak reverzní genetiku, kdy lze díky vysoké frekvenci HR a haploidnímu stádiu snadno připravovat a izolovat životaschopné mutanty v požadovaných genech. Cílenou mutagenezí lze také připravit mutanty s upravenou posttranslační modifikací glykosylací tak, aby odpovídala modifikaci savčí či lidské. Pro biotechnologické využití lze z těchto mutant připravit transformací expresní kazetou produkční linie, které budou v kapalné kultuře v bioreaktoru produkovat „humanizované" formy bioaktivních proteinů (Šmídková 2012; Reski et al. 2015).

Dále je *Physcomitrella* také vhodným modelem pro studium buněčných procesů jako např. reparace DNA. Zejména ve formě kultury krátkých fragmentů filament protonemy, která dobře aproximuje kulturu dělících se savčích nebo kvasničných buněk.

Technicky jde o jednodenní kulturu po pasáži do ukončení jednoho buněčného cyklu (20-24 hodin). Kultura, podle stupně fragmentace filament obsahuje až 50% dělících se apikálních buněk, a představuje tak dělící se pletivo, které se u vyšších rostlin nachází pouze ve velmi omezeném množství ve

vrcholových, listových nebo kořenových meristémech (Holá et al. 2013; Holá et al. 2015).



Obrázek 5: Životní cyklus *Physcomitrelly*. A) haploidní spóra vyklíčí v B) protonemu tvořenou buňkami chloronemy, které pokračují v růstu a mění se v C) buňky kaulonemy. Z protonemy vyrůstají D) „lístky“ gametofor, na kterých se vytvářejí E) samičí archegonia (šipky) a samčí antheridia (hroty). Pohyblivá spermie oplodní vajíčko a vznikne F) diploidní sporofyt. (Prigge a Bezanilla, 2010).

5. Materiál a metody

V průběhu disertační práce byla pro studium reparace a indukované mutageneze výhradně používána jednodenní kultura protonemy *Physcomitrelly*, pěstovaná *in-vitro*, připravovaná důkladnou homogenizací sedmidenního pletiva. Pro izolace DNA byla používána sedmidenní kultura protonemy pěstovaná na agarovém médiu.

Seznam metod

Izolace DNA

Kometový test

Klonování DNA – příprava transformačních vektorů

Kultivace *Physcomitrelly*

Mutageneze jednodenní kultury mechu působením genotoxinů

PCR

Sekvenování

Transformace biolistickou metodou

Zpracování sekvenačních dat, vytváření kontigů a porovnávání sekvencí pomocí programu MacVector, Inc.

6. Prezentované publikace

Publikace 1

Genotoxin induced mutagenesis in the model plant *Physcomitrella patens*

HOLÁ, M., J. KOZÁK, R. VÁGNEROVÁ a K. J. ANGELIS

BioMed Research International. 2013, **2013**: 1-7

IF_{2013}: 2,706

Příspěvek autora: mutageneze, sekvencování APT genu u 2-FA rezistentních klonů jednotlivých linií mechu, analýza mutací

Publikace 2

Mutagenesis during plant responses to UVB irradiation

HOLÁ, M., R. VÁGNEROVÁ a K.J. ANGELIS

Plant Physiology and Biochemistry. 2015, (93): 29-33

IF_{2014}: 2,352

Příspěvek autora: sekvencování APT genu u 2-FA rezistentních klonů jednotlivých linií mechu a analýza mutací

Publikace 3

Efficient biolistic transformation of the moss *Physcomitrella patens*

ŠMÍDKOVÁ, M., **M. HOLÁ** a K. J. ANGELIS

Biol Plantarum. 2010, **54**(4): 777-780

IF_{2010}: 1,582

Příspěvek autora: příprava a údržba mechu, adaptace biolistické metody, transformace biolistickou metodou

Publikace 4

MRE11 and RAD50, but not NBS1, are essential for gene targeting in the moss *Physcomitrella patens*

KAMISUGI, Y., D. G. SCHAEFER, J. KOZAK, F. CHARLOT, N. VRIELYNCK, **M. HOLA**, K. J. ANGELIS, A. C. CUMING a F. NOGUE

Nucleic Acids Research. 2012, **40**(8): 3496-3510

IF_{2012}: 8,278

Příspěvek autora: izolace *apt* mutant po působení bleomycinu, identifikace mutací v sekvenci lokusu genu *APT* u wt a *pprad50* 2-FA rezistentních mutant.

Publikace 5

DNA repair in plants studied by comet assay

ANGELIS KJ, KOZÁK J, VÁGNEROVÁ R AND **HOLÁ M**

Front. Genet 2015, Conference Abstract: ICAW 2015 - 11th International Comet Assay Workshop.

doi: 10.3389/conf.fgene.2015.01.00067

Příspěvek autora: příspěvek k tvorbě manuskriptu

Publikované práce nesouvisející s tématem disertační práce:

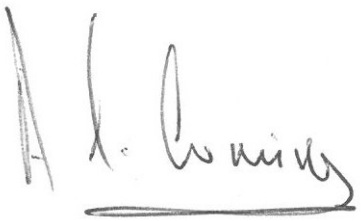
Plant production of vaccine against HPV: A new perspectives

M ŠMÍDKOVÁ, **M HOLÁ**, J BROUZDOVÁ AND K J. ANGELIS

Human Papillomavirus and Related Diseases - From Bench to Bedside

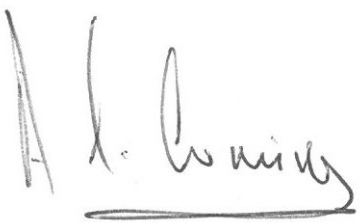
– A Clinical Perspective, 2012, D. Vanden Broeck, ed., InTech.
ISBN 978-953-307-860-1

I declare that the contribution of Marcela Holá to the presented results in Kamisugi et al. *Nucleic Acids Research*, 2012, as stated in "Presented publications" chapter, is true.



Leeds, 23.6.2015 Andrew C Cuming

Potvrzuji, že příspěvek Marcely Holé k prezentovaným výsledkům v Kamisugi et al. *Nucleic Acids Research*, 2012, tak jak je uvedeno v kapitole "Prezentované publikace", je pravda.



Leeds, 23.6.2015 Andrew C Cuming

Potvrzuji, že příspěvek Marcely Holé k prezentovaným výsledkům ve Šmídková et al., Biol Plantarum, 2010, tak jak je uvedeno v kapitole "Prezentované publikace", je pravda.

Praha 16.7.15

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Karel J. Angelis

Potvrzuji, že příspěvek Marcely Holé k prezentovaným výsledkům v Angelis et al., Front. Genet. 2015, tak jak je uvedeno v kapitole "Prezentované publikace", je pravda.

Praha 16.7.15

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Karel J. Angelis

Genotoxin induced mutagenesis in the model plant *Physcomitrella patens*

HOLÁ, M., J. KOZÁK, R. VÁGNEROVÁ a K. J. ANGELIS

BioMed Research International. 2013, **2013**: 1-7
IF_{2013}: 2,706

Příspěvek autora: mutageneze, sekvenování APT genu u 2-FA rezistentních klonů jednotlivých linií mechu, analýza mutací

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Research Article

Genotoxin Induced Mutagenesis in the Model Plant *Physcomitrella patens*

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The moss *Physcomitrella patens* is unique for the high frequency of homologous recombination, haploid state, and filamentous growth during early stages of the vegetative growth, which makes it an excellent model plant to study DNA damage responses. We used single cell gel electrophoresis (comet) assay to determine kinetics of response to Bleomycin induced DNA oxidative damage and single and double strand breaks in wild type and mutant *lig4 Physcomitrella* lines. Moreover, *APT* gene when inactivated by induced mutations was used as selectable marker to ascertain mutational background at nucleotide level by sequencing of the *APT* locus. We show that extensive repair of DSBs occurs also in the absence of the functional LIG4, whereas repair of SSBs is seriously compromised. From analysis of induced mutations we conclude that their accumulation rather than remaining lesions in DNA and blocking progression through cell cycle is incompatible with normal plant growth and development and leads to sensitive phenotype.

1. Introduction

Plants developed several strategies to protect integrity of their genome against various environmental stresses. Common denominator of most of them is oxidative stress mediated by reactive oxygen species (ROS). The origin of ROS within the cell could be a consequence of physical or chemical genotoxic treatment, as well as byproduct of internal oxygen metabolism often triggered by external stimuli as drought and salinity. To be able to cope with oxidative stress we have to assess all faces of this challenge for plants; in particular, how it affects genetic material of the cells and how eventual changes are temporarily or permanently expressed in plant phenotype. This is why we need a flexible and robust model system, which experimentally enables the use of reverse genetics for genotoxic and biochemical studies. In this paper we describe a novel system to be considered for genotoxicity testing in plants.

The moss *Physcomitrella patens* is an emerging model plant [1] with the following differences/advantages as compared to other plant test systems: efficient homologous recombination (enabling reverse genetics of virtually any gene), dominant haploid phase (enabling assessment of mutation phenotype), small size plantlets colonies with a quick and during early vegetative stage also filamentous growth, easy cultivation in inorganic media and several options of long term storage. Here we describe and validate a system of small protonemata fragments with high fraction of apical cells primarily developed for the purpose of genotoxicity testing. However, these one-day-old protonemata could be used as a substitute of protoplasts for other purposes, for example, for moss transformation [2].

APT (adenine phosphoribosyltransferase) is an enzyme of the purine salvage pathway that converts adenine into AMP and its loss of function generates plants resistant to adenine analogues, for example, 2-FA (2-Fluoroadenine) [3]. Mutational inactivation can be used as selectable marker for mutator genotyping as well as analysis of mutations in *APT* locus on nucleotide level [4–6].

This paper is an extension of our previous study of *Physcomitrella* knockout mutants of a key MRN (MRE11, RAD50, and NBS1) complex [6] with a pleiotropic effect on DSB repair in whole. We explore and validate the above outlined moss model system for genotoxicity testing in

plants. We describe a parallel use of SCGE (single cell gel electrophoresis, comet) assay for detection of DNA damage and its repair and of APT assay with sequencing analysis of mutants. On example of *lig4*, mutated in a key component of nonhomologous DSB-end joining pathway (C-NHEJ), we show consequences of mis repair. For strengthening the model concept we also present preliminary results of *pprad51AB* sensitivity to genotoxin treatment and on *ppku70* mutation rate.

2. Material and Methods

2.1. Plant Material. Physcomitrella patens (Hedw.) B.S.G. "Gransden 2004" wild type and *pplig4* were vegetatively propagated as previously described [7]. The *lig4* and *ku70* mutants of C-NHEJ were generated by D. G. Schaefer, Neuchatel University, Switzerland, and F. Nogue, INRA, Paris, France, and kindly provided by F. Nogue. Detailed characteristic of this mutant will be published elsewhere. Mutant in both alleles of *Physcomitrella RAD51* gene (*pprad51AB*, clone 721) is described elsewhere [8] and was kindly provided by B. Reiss, MPIZ, Cologne, Germany.

Individual plants were cultured as "spot inocula" on BCD agar medium supplemented with 1 mM $CaCl_2$ and 5 mM ammonium tartrate (BCDAT medium) or as lawns of protonemal filaments by subculture of homogenized tissue on BCDAT agar medium overlaid with cellophane in growth chambers with 18/6 hours day/night cycle at 22/18°C.

One-day-old protonemal tissue for repair and mutation experiments were prepared from one-week-old tissue scraped from plates, suspended in 8 mL of BCD medium, and sheared by a T25 homogenizer (IKA, Germany) at 10 000 rpm for two 1-minute cycles and let 24 hours to recover in cultivation chamber with gentle shaking at 100 rpm. This treatment yielded suspension of 3–5 cell protonemata filaments, which readily settle for recovery. Settled protonemata could be handled without excessive losses by tweezers on glass Petri plates.

2.2. Bleomycin Treatment and Sensitivity Assay. For treatments was used Bleomycin sulphate supplied as Bleomedac inj. (Medac, Hamburg, Germany). All solutions were prepared fresh prior treatment from weighted substance in BCDAT medium.

Protonemal growth was tested by inoculating explants of wild type and 5 mutant lines onto 6 × 4 multiwell plates organized to allow in line comparison of the effect of increasing Bleomycin concentrations. The wells were filled with 2 mL of standard growth BCDAT agar medium without or with 0.01, 0.1, and 1 $\mu g\,mL^{-1}$ Bleomycin. The experiment was carried in 3 independent replicas and monitored up to 3 weeks for growth of "spot inocula."

Treatment of one-day-old protonemata was performed on glass 5 cm Petri plates with the aid of bent tweezers to handle tissue and pipettes to remove excess liquids. Opening of yellow tips is generally small enough to avoid suction of moss filaments when drawing majority of liquid from tissue prior blotting of collected tissue on filter paper to remove the rest.

In dose-response and repair kinetic experiments, one-day-old protonemata were after the Bleomycin treatment thoroughly rinsed in water, blotted on filter paper, and either flash-frozen in liquid N_2 (dose response and repair $t = 0$) or left to recover on plates in liquid BCDAT medium for the indicated repair times, before being frozen in liquid N_2.

For induction and regeneration of *apt* mutants one-day-old protonemata were after Bleomycin treatment thoroughly rinsed with H_2O, suspended in 2 mL of BCDAT medium, and spread on cellophane overlaid BCDAT agar plates, which were for selection supplemented with 2-FA (2-Fluoroadenine, Sigma-Aldrich, cat. Nr. 535087) and further incubated in growth chamber.

2.3. Detection of DNA Lesions. DNA single and double strand breaks were detected by a SCGE assay using either alkaline unwinding step A/N [9, 10] or fully neutral N/N protocol [11, 12] as previously described. In brief, approximately 100 mg of frozen tissue was cut with a razor blade in 300 μL PBS + 10 mM EDTA on ice and released nuclei transferred into Eppendorf tubes on ice. 70 μL of nuclear suspension was dispersed in 280 μL of melted 0.7% LMT agarose (GibcoBRL, cat. Nr. 15510-027) at 40°C and four 80 μL aliquots were immediately pipetted onto each of two coated microscope slides (in duplicate per slide), covered with a 22 × 22 mm cover slip and then chilled on ice for 1 min to solidify the agarose. After removal of cover slips, slides were immersed in lysing solution (2.5 M NaCl, 10 mM Tris-HCl, 0.1 M EDTA, and 1% N-lauroyl sarcosinate, pH 7.6) on ice for at least 1 hour to dissolve cellular membranes and remove attached proteins. The whole procedure from chopping tissue to placement into lysing solution takes approximately 3 minutes. After lysis, slides were either first incubated 10 minutes in 0.3 M NaOH, 5 mM EDTA, pH 13.5 solution to allow partially unwind DNA double helix to reveal SSBs (A/N protocol) or without unwinding step (N/N protocol) directly equilibrated twice for 5 minutes in TBE electrophoresis buffer to remove salts and detergents. Comet slides were then subjected to electrophoresis at 1 V cm^{-1} (app. 12 mA) for 3 minutes. After electrophoresis, slides were placed for 5 min in 70% EtOH, 5 min in 96% EtOH, and air-dried.

Comets were viewed in epifluorescence with a Nikon Eclipse 800 microscope stained with SYBR Gold (Molecular Probes/Invitrogen, cat. Nr. S11494) according to manufacture recommendation and evaluated by the LUCIA Comet cytogenetic software (LIM Inc., Czech Republic).

2.4. SCGE Assay Data Analysis. The fraction of DNA in comet tails (% tail-DNA, % T DNA) was used as a measure of DNA damage. Data for the wild type and the mutant *pplig4* line analysed in this study were obtained in at least three independent experiments. In each experiment, the % T DNA was measured at seven time points: 0, 5, 10, 20, 60, 180, and 360 min after the treatment and in control tissue without treatment. Measurements included four independent gel replicas of 25 evaluated comets totalled in at least 300

comets analysed per experimental point. The percentage of damage remaining as plotted on Figure 2(b) after given repair time (t_x) is defined as

$$\%\ \text{damage remaining}\ (t_x) = \frac{\text{mean \% tail-DNA}\ (t_x) - \text{mean \% tail-DNA (control)}}{\text{mean \% tail-DNA}\ (t_0) - \text{mean \% tail-DNA (control)}} * 100. \quad (1)$$

Time-course repair data were analysed for one- or two-phase decay kinetic by Prism v.5 program (GrafPad Software Inc., USA).

2.5. Isolation and Analysis of apt Mutants after Bleomycin Treatment. Mutation rates were measured as the number of *apt* mutants that appeared as green foci of regenerating clones resistant to 2-FA (Figure 3). Treated protonemata were first incubated approximately 3 weeks on plates with 2 or 3 mM 2-FA until first green foci start to emerge. Then whole cellophane overlay was transferred to a new plate with 8 mM 2-FA and emerging clones were allowed to form colonies. Stable clones were then counted.

Some clones were further propagated on plates with 8 mM 2-FA and their *APT* locus was subsequently PCR amplified and sequenced to identify the mutation(s) responsible for the resistance. Approximately 100 mg of tissue was used to isolate genomic DNA with DNeasy Plant Mini Kit (Qiagen, cat. Nr. 69104) using "ball" mill Retsch MM301 to homogenize the tissue in 2 mL round bottom Eppendorf tubes. *APT* locus was amplified from isolated genomic DNA with KOD Hot Start DNA Polymerase (Millipore/Novagen, cat. Nr. 71086), purified with the QIAquick PCR Purification Kit (Qiagen, cat. Nr. 28104) and used as a template for sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, cat. Nr. 4337455). Locations of PCR primers used for *APT* amplification and sequencing are depicted on Supplementary Figure 2 and their sequences are listed in Supplementary Table 1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/535049). To keep sequencing cost down only half volume of the BigDye Ready Reaction Mix was used in a standard sequencing reaction and combined with the same volume of Half-Term-Dye-Termination mixture (Sigma-Aldrich, cat. Nr. H1282).

2.6. Analysis of Sequencing Data. Sequences of each clone obtained on genetic analyser Prism 3130x1 (Applied Biosystems, USA) were stiched together with MacVector program Assembler 12.7.5 (MacVector, USA) into contigs and aligned to the latest annotated *APT* sequence Pp1s114_124V6.1 in the COSMOSS—the *Physcomitrella patens* resource database (https://www.cosmoss.org/).

3. Results and Discussion

In all experiments a model *Physcomitrella patens* has been used as one day recovered fragments of 3–5 cell size derived from the lawn of growing protonema filaments by extensive shearing. Such one-day-old protonemata represent a unique system among plants to study plant tissue with up to 50% of apical dividing cells. Convenient mechanical handling enables quick processing of tissue after the treatment to address short repair times and with fine tip tweezers also for uniform spotting to test sensitivity. In this respect one-day-old protonemata are preferred system to so far widely used protoplasts, which could be collected only by centrifugation. Another reason for using protonemata is possibility their mechanical disintegration by razor blade chopping for rapid release of nuclei for SCGE assay. In this way direct use of protoplasts for comet assay is obstructed by nearly instant regeneration of the cell wall within 4 hours after the release from cellulase treatment (unpublished observation), because cell wall prevents DNA movement from nuclei during electrophoresis.

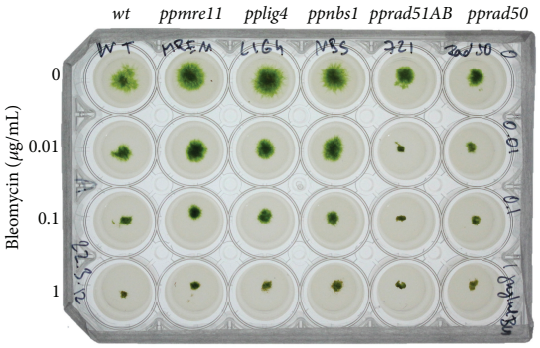

Figure 1: Sensitivity of the *Physcomitrella patens* repair mutants *mre11*, *lig4*, *nbs1*, *rad51AB*, and *rad50* to chronic exposure of Bleomycin. *Physcomitrella* explants were inoculated as "spot inocula" onto BCDAT-agar plates supplemented with 0, 0.01, 0.1, and 1 μg mL^{-1} Bleomycin and photographed 10 days after inoculation.

3.1. Sensitivity to Bleomycin Treatment. Moss wild type and *pplig4*, *mre11*, *nbs1*, *rad51AB*, and *rad50* [6, 8] mutant lines were analysed for their sensitivity to radiomimetic Bleomycin in chronic "survival" assay when test plates with various concentrations of Bleomycin were inoculated with equal tissue "spots" of one-day-old protonemata and incubated up to 3 weeks (Figure 1). Only *rad51AB* and *rad50* strains displayed one order of a magnitude higher sensitivity in comparison to other tested lines. The survival growth of *ppmre11* is somehow in contradiction with pervious results of Kamisugi et al. [6], but one has to realize different assay conditions, for example, acute versus chronic exposure and protoplast cells versus protonemata. In protonema tissue under permanent genotoxic stress *mre11* express phenotype similar to wild type, *nbs1*, and also *lig4*. One can speculate that 3′ to 5′ exonuclease and endonuclease activity associated with MRE11 is dispensable for tissue survival, but proteins RAD50 and RAD51 supporting DNA structure are not. Kozak et al. [12] previously showed crucial role of structural maintenance

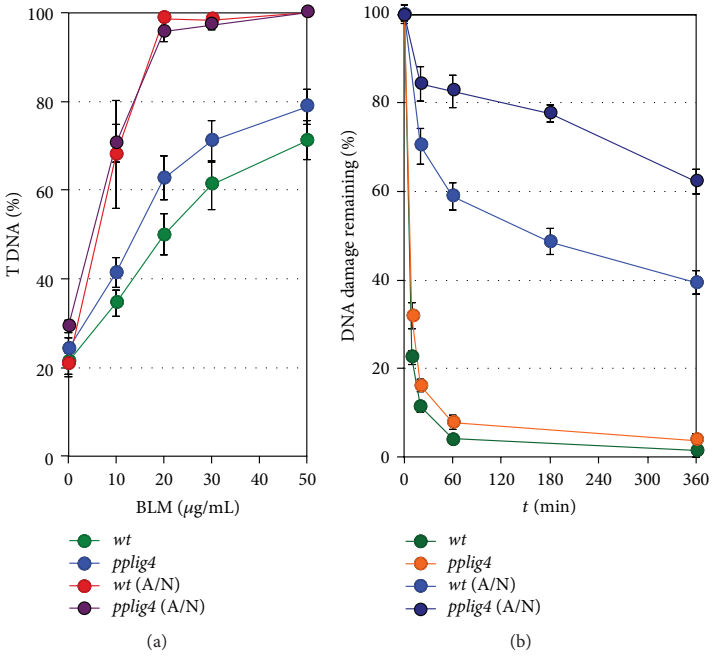

Figure 2: SSB and DSB repair kinetics determined by SCGE. One-day regenerated protonemal tissue from wild type and *pplig4* lines was treated with Bleomycin for 1 h prior to nuclear extraction and the analysis. (a) Dose-response as the percentage of the free DNA moved by electrophoresis into comet tail (% T DNA) at the indicated Bleomycin concentrations. DSBs were determined by N/N protocol: green: wild type, blue: *pplig4*, whereas SSBs were determined by A/N protocol: red: wild type, dark purple: *pplig4*. (b) Repair kinetics is plotted as % of DSBs remaining over the 0, 5, 10, 20, 60, 180, and 360 min period of repair recovery. Maximum damage is normalised as 100% at $t = 0$ for all lines. SSBs were induced by 1-hour treatment with 2 μg mL^{-1} Bleomycin; bright blue: wild type, dark blue: *pplig4*, and determined by A/N protocol. DSBs were induced by 1-hour treatment with 30 μg mL^{-1} Bleomycin, green: wild type, orange: *pplig4*, and determined by N/N protocol. (Error bars-standard error).

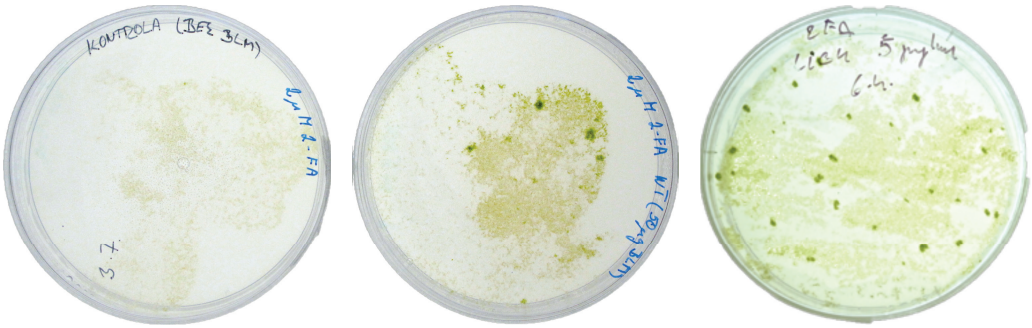

Figure 3: Plates with 2-FA resistant foci of wild type *Physcomitrella* (a and b) and *pplig4* (c) after 3 weeks of selection. (a) Untreated *Physcomitrella* wild type, (b) 50 μg mL^{-1} Bleomycin treated wild type protonemata for 2 hours, and (c) 5 μg mL^{-1} Bleomycin treated *pplig4* protonemata for 1 hour prior being spread on plates with BCDAT medium supplemented with 2 μM 2-FA and cultivated for 3 weeks.

of chromosome complex SMC5/6 in the repair of Bleomycin induced DSBs. In this context RAD50s have similar structural role in MRN complex as SMCs in the structure of the SMC5/6 complex [13]. Both these complexes can function in tethering of broken ends in close proximity.

3.2. Induction of DNA Lesions and Their Repair. Bleomycin, an ionizing radiation mimicking agent, functions as a catalyst activated by interaction with DNA and attachment of Fe^{2+} to produce oxygen radicals leading to lesions as SSBs, DSBs, AP-sites, and damaged bases [14, 15], which all could be

readily detected by SCGE [16]. DNA breaks and other lesions converted to breaks lead to DNA fragmentation and nucleoid unwinding allowing relaxed DNA to move in electric field from nuclei out to form a "comet" like object in which increased quantity of fragmented DNA in comet tail (% T DNA) is proportional to breakage. DSBs are detected by an N/N assay when pH of lysing and electrophoretic solutions is kept under pH 10, whilst for the detection of SSBs DNA after the lysis is allowed to unwind DNA double-helix in alkali [17] to separate individual strands and expose their fragmentation (A/N protocol [9]).

Bleomycin fragmentation of genomic DNA by induction of SSBs and DSBs is documented on Figure 2(a). Ten times higher efficiency to induce SSBs than DSBs is in agreement with generally accepted ratio of 1 : 10, DSBs versus SSBs, induced by ionizing radiation. Evidently this also applies for Bleomycin treatment of *Physcomitrella*. The background level of genomic DNA damage in wild type and *pplig4* is similar, between 20 and 25% T DNA, indicating that the repair defect has no significant effect on natural levels of genomic DNA fragmentation. Nevertheless, in comparison with wild type, *pplig4* is vulnerable to Bleomycin induction of DSBs and SSBs.

In both wild type and *pplig4* lines, the Bleomycin induced DSBs are repaired with a rapid, biphasic kinetics (Figure 2(b)). Half-lives of DSB survival $\tau_{1/2}$ 1.5 min for wild type and 2.5 min for *pplig4* are similar to $\tau_{1/2}$ 2.9 min for *pprad50*, $\tau_{1/2}$ 4.1 min for *ppmre11*, and $\tau_{1/2}$ 1.9 min for *ppnbs1* previously reported in [6].

Contrary to DSBs, SSBs are repaired far less efficiently. Slow SSB repair might be common feature of plants since Donà et al. [18] recently observed similar phenomenon in *Medicago truncata* cell culture irradiated with different doses of γ-ray. The SSB repair kinetic in wild type *Physcomitrella* is clearly biphasic and in this respect parallels repair of MMS induced SSBs in *Arabidopsis* [19]. In comparison to DSBs, substantially smaller fraction of SSBs is repaired with fast kinetics; the defect even more manifested in *pplig4*. It suggests an important role for LIG4 in the repair of DNA lesions like modified basis, AP sites that are usually detected as SSBs and are repaired via BER (base excision repair). It is noteworthy that LIG3, the ligase finishing BER pathway, is not represented in plants. We showed earlier that principal substitute for LIG3 in *Arabidopsis* is LIG1 [19]. The repair kinetic of MMS induced SSBs in *atlig1* posed an exceptional route. After the treatment the number of breaks continues to increase during the first hour of repair and after 3 hours returns to the level at the end of treatment. Then repair continues similarly as in the wild type (see Figure 4 in [19]). Because *atlig1* is an *RNAi* line with only 40% of remaining LIG1 activity, such repair course is a consequence of unbalanced BER due to attenuated ligation step. Evidently the knockout mutation in *pplig4* does not have such severe effect on repair of SSBs; nevertheless, the defect clearly shows that LIG4 is also involved in the repair of SSBs in plants.

3.3. Induction and Analysis of apt Mutants. The mutator phenotype was assessed as the loss of function of the *APT* gene [4] due to presence or error prone repair of endogenous DNA damage in the wild-type moss and *lig4*, *mre11*, and *rad50* repair mutant lines.

We found dramatic, over two orders of magnitude, variation of mutator phenotype in response to mutagenic treatment. While wild type *Physcomitrella* with low mutator phenotype needed 2 hours and 50 μg mL^{-1} Bleomycin treatment to induce any *apt* clone, in *pprad50* with high mutator phenotype 1 hour treatment with only 0.1 μg mL^{-1} Bleomycin was enough for massive induction of *apt* clones. Other lines, *pplig4* and *ppmre11*, assumed as having "moderate" mutator phenotype, were mutagenized either with 5 or, respectively, 1 μg mL^{-1} Bleomycin for 1 hour. Mutagenesis and clone selection in *Physcomitrella* wild type and *pplig4* is depicted on Figure 3. For comparison we normalised the yield of 2-FA resistant clones to 1 μg mL^{-1} Bleomycin treatment per 1 g dry tissue weight in each line as "relative number of *ppapts*." The values of these normalised yields range from 9 in wild type to 875 for *pprad50* (see Supplementary Figure 1 where are plotted summarized results of Bleomycin mutagenesis in *Physcomitrella* wild type and *lig4, ku70, rad50, mre11*, and *nbs1* mutants).

Randomly picked *apt* clones from selection plates were further propagated on 2-FA media to provide enough material for isolation of genomic DNA and sequencing analysis of *APT* locus. Results of sequencing analysis are pictured in Figure 4 and detailed annotations of identified mutations are summarized in Supplementary Table 2. In total were analysed 5 clones of *Physcomitrella* wild type, 4 clones of *pplig4*, 3 clones of *ppmre11*, and 6 clones of *pprad50* and identified 48 mutations. Mutations were according to assumed mechanism of formation classified as reversions, single base insertion or deletion, and insertions or deletions larger than 2 bases either in coding (exons) or noncoding regions of *APT* locus.

Most of the identified mutations are as expected localised within CDS, in particular within exon 4 that is annotated as coding for adenine salvage activity (see Figure 4). Nevertheless, in *wt:1*, *lig4:1*, *lig4:2*, and *mre11:6 apt* clones, mutations were identified only in the noncoding region and their contribution to mutated *APT* phenotype has to be established. Majority of mutations in CDS are point mutations (base substitution, single base insertions, and deletions) and it is difficult to dissect the route of their formation. Some of single base deletions could come from classical or altered NHEJ repair of DSBs [20], but more likely they represent along with other point mutations outcome of processing base oxidative damage. Interesting point is that only single base insertions were identified in *APT* CDS of *pplig4*. Insertion of extra base might imply defect in BER repair of oxidative damage in the absence of LIG4 and could be associated with defective repair of SSBs in *pplig4*.

Long deletions are clearly associated with NHEJ repair of DSBs, because, besides one rather short (8 bp) deletion in *wt:3* clone, all appear in clones derived from *mre11* and *rad50* background. This supports our working hypothesis that MRN-unsupervised repair generates more severe forms of genomic damage [6].

Only one 4 base insertion was identified in noncoding region of *wt:2*.

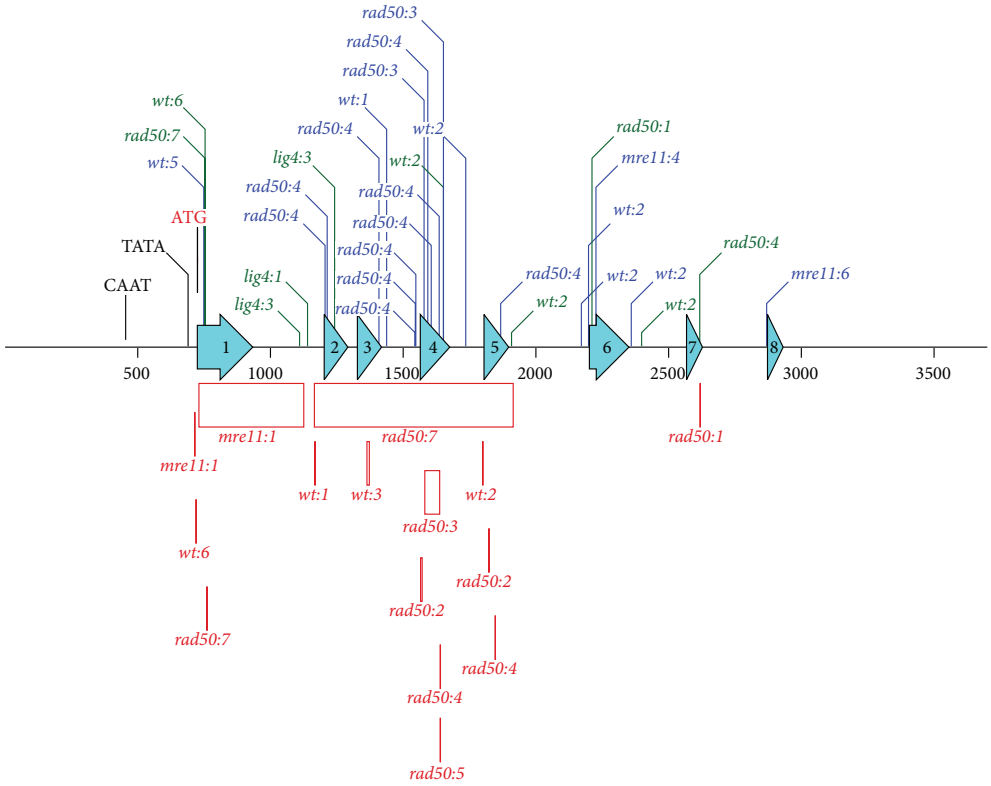

Figure 4: Map of identified mutations within *APT* locus. Bleomycin induced mutations are identified by color (blue: substitutions, green: insertions, and red: deletions) and tagged according to background as *wt*, *lig4*, *mre11*, and *rad50* and the number of line in which mutation was detected. Deletions are shown as boxes of size proportional to their length. On the locus map are depicted 500 nucleotide size markers and eight turquoise hollow arrows representing exons of *APT* CDS. Detailed description of each mutation is provided in Supplementary Table 2.

4. Conclusions

We validated the use of regenerating one-day-old protonemal tissue of *Physcomitrella patens* for complex analysis of genotoxic stress by parallel study of DNA damage, its repair, and mutagenic consequences in wild type and *lig4* mutant plants. From experimental point of view we developed a novel model system where 3–5 cell protonemata filaments with up to 50% of apical cells can substitute and surplus protoplasts use. Bleomycin was used to model DNA oxidative genotoxic stress with all its consequences as SSBs and DSBs, which can be followed by SCGE. We confirmed in *Physcomitrella* as previously in *Arabidopsis* rapid DSB repair even in the absence of LIG4, the key ligase of major DSB repair pathway by NHEJ mechanism [12]. Moreover, we showed crucial role of LIG4 in the repair of SSBs by BER mechanism, where it can substitute along with LIG1 [19] in plants missing LIG3. We selected and analysed by sequencing 2-FA resistant clones with Bleomycin mutated *APT* locus and found out that mutation spectra of *lig4* mutant reflects rather the defect of SSB than DSB repair. Nevertheless, as previously described [6], we interpret that mutations due to the error prone repair in *pplig4* rather than unrepaired lesions within DNA and interfering with progression through the cell cycle are responsible for *pplig4* sensitive phenotype.

Abbreviations

A/N: Comet assay with alkaline unwinding step
AP: Apurinic/apyrimidinic (site)
APT: Adenine phosphoribosyltransferase
BER: Base excision repair
CDS: Coding DNA sequence
DSB(s): DNA double-strand break(s)
2-FA: 2-Fluoroadenine
HR: Homologous recombination
MMS: Methyl methanesulfonate
NHEJ: Nonhomologous end joining
N/N: Neutral comet assay
ROS: Reactive oxygen species
SCGE: Comet assay
SSB(s): DNA single-strand break(s)
$\tau_{1/2}$: Half-life.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Mutagenesis during plant responses to UVB irradiation

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Mutagenesis during plant responses to UVB radiation

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ABSTRACT

We tested an idea that induced mutagenesis due to unrepaired DNA lesions, here the UV photoproducts, underlies the impact of UVB irradiation on plant phenotype. For this purpose we used protonemal culture of the moss *Physcomitrella patens* with 50% of apical cells, which mimics actively growing tissue, the most vulnerable stage for the induction of mutations. We measured the UVB mutation rate of various moss lines with defects in DNA repair (*pplig4*, *ppku70*, *pprad50*, *ppmre11*), and in selected clones resistant to 2-Fluoroadenine, which were mutated in the adenosine phosphotrasferase gene (*APT*), we analysed induced mutations by sequencing. In parallel we followed DNA break repair and removal of cyclobutane pyrimidine dimers with a half-life $\tau = 4$ h 14 min determined by comet assay combined with UV dimer specific T4 endonuclease V. We show that UVB induces massive, sequence specific, error-prone bypass repair that is responsible for a high mutation rate owing to relatively slow, though error-free, removal of photoproducts by nucleotide excision repair (NER).

1. Introduction

The majority of UVB photoproducts, pyrimidine dimers (CPD) and pyrimidine(6–4)pyrimidinone dimers (6–4 PP), are removed in plants by blue light (320–450 nm) induced direct dimer reversal with photolyases specific for CPDs as well as 6–4 PP (Britt, 1995). Light repair is efficient and error free, nevertheless half-life of CPDs removal is about 1 h (Pang and Hays, 1991) and for complete elimination of 6-4PPs are needed at least 2 h (Waterworth et al., 2002; Chen et al., 1994), during which other mechanisms can take place. In contrast to photoreactivation, dark repair pathways do not directly reverse DNA damage, but instead replace the damaged DNA with new, undamaged nucleotides. There are recognized to be two possible mechanisms relying either on excision of dimers or on their tolerance by trans-lesion synthesis, of which replicative polymerases are also capable (Rabkin et al., 1983). CPDs and 6–4 PP are recognized and removed due to their "bulky" distortion of the DNA double helix by nucleotide excision repair (NER), a repair mechanism able to cope with a broad spectrum of DNA lesions that disturb the conformation of DNA, by error-free replacement of the DNA strand containing the lesion in a range of 2–4 helical turns (20–40 nucleotides) by a newly synthesized patch. Both photoreactivation and NER are error free, and so we asked which mechanism underlay the generally observed high mutagenic as well as severe carcinogenic risk caused by UV irradiation. The most relevant form of UV for the induction of biological effects is UVB, since UVC hardly penetrates the Earth's atmosphere. In the present research we used a recently-described approach employing regenerating one-day-old protonemal tissue of *Physcomitrella patens* (Hola et al., 2013) for complex analysis of UVB genotoxic stress in laboratory conditions by parallel study of DNA damage, its repair and its mutagenic consequences in wild type and *pplig4*, *pprad50*, *ppmre11*, *ppku70* mutants, to ascertain the nature of observed high rates of UV mutagenesis.

2. Materials and methods

Detailed description of Materials and Methods is in Appendix A.

2.1. Plant material

P. patens (Hedw.) B.S.G. "Gransden 2004"wild type and *pplig4*, *pprad50*, *ppmre11* were described previously (Hola et al., 2013; Kamisugi et al., 2012) along with cultivation and treatment conditions. The *ppku70* mutant in the canonical non-homologous DSB repair pathway (C-NHEJ) was generated and kindly provided by D.

Abbreviations: A/N, comet assay protocol with alkaline unwinding step; *APT*, adenine phosphotrasnferase; BER, base excision repair; CPD, cyclobutyl pyrimidine dimer; 2FA, 2-Fluoroadenin; NER, nucleotide excision repair; N/N, neutral comet assay; 6–4 PP, pyrimidine(6–4)pyrimidinone dimer; SSB, DNA single strand break; τ, half-life; T4EndoV, T4 Endonuclease V.

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0981-9428/

G. Schaefer, Neuchatel University, Switzerland.

2.2. UV and Bleomycin treatment

Laboratory broadband UVB irradiation was carried in a Hoefer UV crosslinker with the unwanted UVC fraction filtered out by a cellulose acetate sheet (Britt et al., 1993) overlaying samples and a crosslinker UV gauge (Figure A.2). Insertion of the sheet increased by about 20% the time needed for the crosslinker to achieve the set irradiation dose; e.g. from 40 to 50 s to deliver dose 3 kJ m^{-2}. To block light repair, irradiation and recovery cultivation were performed in dark and collection and freezing of samples under red light in a darkroom. In mutation experiments, samples were kept in the dark for 24 h after irradiation to allow induction of mutations.

Bleomedac inj. (Medac, Hamburg, Germany) was used for Bleomycin treatment as previously described (Hola et al., 2013).

All studies were performed with protonemata 1 day following homogenisation, having approximately 50% of actively dividing cells (Fig. A.1).

2.3. Detection of DNA lesions

DNA single strand breaks (SSBs) were detected by an A/N comet assay using neutral protocol with an alkaline unwinding step (Angelis et al., 1999; Menke et al., 2001; Olive and Banath, 2006). For specific detection of CPDs, T4 endonuclease V (T4EndoV) digestion step was included in the protocol after cell lysis. T4EndoV enzyme was prepared as a crude lysate from overexpressing bacteria (Collins, 2011; Valerie et al., 1985). Thirty minutes digestion of nuclear DNA of cells irradiated by 3 kJ m^{-2} with T4EndoV diluted 1:500 at room temperature generated app. 95% DNA fragmentation (Fig. A.3). Without T4EndoV treatment, the fraction of fragmented DNA in comet tails increased after irradiation only to 10% from 1 to 2 % of background value.

Comets on slides were stained with SYBR Gold (Molecular Probes/Invitrogen), viewed in epifluorescence with a Nikon Eclipse 800 microscope and captured and evaluated by the LUCIA Comet cytogenetic software (LIM Inc., Czech Republic).

2.4. Analysis of comet assay data

The fraction of fragmented DNA in comet tails (% T DNA) was used as a measure of DNA damage, nevertheless for an easy comparison of SSBs and CPDs repair kinetics, comet data are rather expressed as % of remaining damage, where damage after UV irradiation at $t = 0$ is set 100% for both lesions (Eq. (A.1)).

Data in this study were obtained in at least three independent experiments. Measurements of blind-labelled comet slides included 25 evaluated comets of four independent gel replicas in each experiment that totalled at least 300 comets analysed per experimental point. Time-course data were analysed for one-phase decay kinetics by Prism v.5 program (GrafPad Software Inc., USA).

2.5. Isolation and analysis of APT mutants

The dose 500 J m^{-2} was used to induce mutations in *APT*. After irradiation, samples were kept in darkness for 24 h to block light repair and generate mutations. Mutation rates were measured as the number of *APT* mutants that appeared as green foci of regenerating clones resistant to 2-Fluoroadenine (2FA). Treated protonemata were cultivated on plates with 8 μM 2FA and emerging foci were allowed to form colonies. Stable clones were then counted. Randomly selected clones were further propagated and their *APT* locus was PCR amplified and sequenced to identify the mutation(s) responsible for resistance. Details of mutant analysis are in Appendix Figure A.4 and Table A.1.

3. Results and discussion

3.1. Repair of UVB induced lesions

Repair of CPDs and 6-4PPs by excision NER pathway proceeds in four steps: Recognition of distorted DNA double helix by a “bulky” lesion, incision of the DNA strand on both sides of a lesion, filling the gap by DNA repair synthesis and religation of a newly synthesized patch.

DNA breaks formed during the incision step of dimer repair can be followed as SSBs by the A/N comet assay because they lead to fragmentation of nuclear DNA. Kinetics of formation and removal of SSBs during repair is plotted in Fig. 1 (open circles). Data are expressed as % of remaining damage, with damage after UV irradiation at $t = 0$ set to 100%. An increased number of SSBs due to NER is observed during period of approximately 6 h, with a peak at 1 h, when the number of breaks nearly doubles and is then followed by a gradual decrease, indicating saturation of repair capacity after 1 h and steady-state progression of repair afterwards. After 6 h the level of SSBs is the same as immediately after UV irradiation.

Removal of CPDs from nuclear DNA was followed after their conversion to SSBs by digestion of nuclei already embedded on comet slides with the CPD specific endonuclease T4EndoV prior to DNA unwinding and electrophoresis. T4EndoV has two associated enzyme activities: pyrimidine dimer glycosylase cleaving the glycosyl bond of the 5′-pyrimidine of CPD and AP-endonuclease cleaving the phosphodiester bond at a glycosylase-generated AP site. The kinetics of CPD removal in *P. patens* follows first order kinetics with an estimated half-life $\tau = 4$ h 14 min (Fig. 1, closed circles). As reported in *Arabidopsis*, CPD dark repair is several times slower than light repair and this might be true also in *P. patens* (Britt et al., 1993).

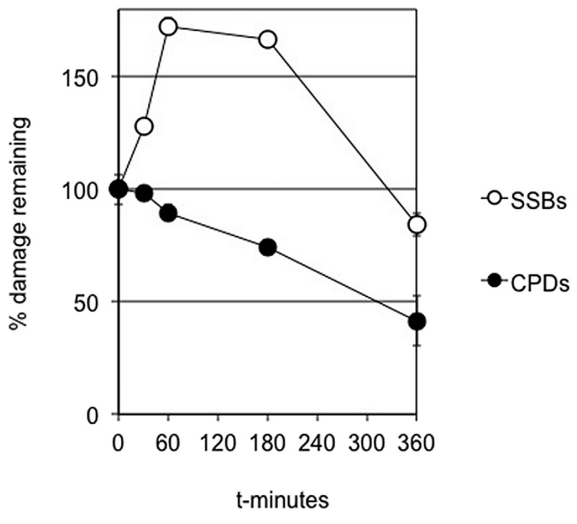

Fig. 1. The repair kinetics of SSBs and CPDs induced by 3 kJ m^{-2} UVB irradiation. Both SSBs and CPDs were determined in the same sample from which comet slides were prepared and processed either with or without the T4EndoV digestion step. Data are expressed as % of remaining damage, when damage after irradiation $t = 0$ is set to 100%. The number of SSBs first increases as a consequence of NER incisions, reaching a maximum after 1 h and then as NER proceeds their number decreases (opened circles). The number of induced CPDs gradually decreases from $t = 0$ following first order kinetics with a CPD half-life $\tau = 4$ h 14 min (closed circles).

3.2. UVB mutagenesis

Slow dark NER repair of UV dimers opens the possibility for their eventual, more rapid error-prone repair or any other error-prone tolerance mechanism that might underlie observed UV mutagenicity. For this reason we decided to detect mutagenesis endpoints as changes at the DNA sequence level.

Mutations in genes of nucleotide metabolism like *APT* confer resistance to halogenated bases like 2FA, which, when utilized by cell metabolism are toxic. This feature is used as a positive selection marker for identification of mutants. PCR amplification of the mutated gene DNA and its sequence analysis provides a description of the acquired mutations and gives an insight into how they occurred.

Firstly we examined the number of regenerating *APT* mutants in *P. patens* wild type and *pprad50*, *ppmre11*, *pplig4* and *ppku70* mutants, appearing spontaneously or induced by 500 J m^{-2} UVB, 1 mM MMS and 1 μg mL^{-1} Bleomycin and normalized the count to 1 g of dry tissue weight. Results are summarized in Fig. 2 (partial results of Bleomycin mutagenesis were previously published in (Hola et al., 2013; Kamisugi et al., 2012)). UV mutagenesis proved to be effective in the wt and repair mutants studied. Aside from an exceptional and enigmatic role of RAD50 in UV mutagenesis, we can speculate about the higher rate of UV mutagenesis in the *pplig4* background. Holá et al. (Hola et al., 2013) described a repair defect of oxidative damage by base excision repair (BER) and showed that LIG4, perhaps along with LIG1 (Waterworth et al., 2009) could substitute in plants that lacked LIG3 in this pathway. If we assume that absence of LIG4 abolishes the active error free BER pathway, then any error-prone repair or bypass of UV induced dimers becomes more relevant and could contribute to higher rates of mutagenesis. This is an interesting point, because BER repair of CPDs in plants was never previously seriously considered (Britt, 1995) regardless of the fact that this mechanism is active in bacteria (bacteriophage T4EndoV used in this study for detection of CPDs is an example) and perhaps also in other organisms.

3.3. Sequence analysis of UVB induced mutations

For detailed analysis of induced mutations we picked at random clones from mutation experiments and after their propagation isolated DNA and sequenced the *APT* locus. In all sequenced *APT* mutants we found mainly cytosine to thymine transitions (Table 1) that are typically formed after UV irradiation generating nearly exclusively pyrimidine dimers, but that rarely occur after other type of treatment like Bleomycin. UVB and Bleomycin induced mutations are summarized in Table B.1.

The production of mutations by agents that block DNA synthesis, such as CPDs, requires that there should be a mechanism to bypass the lesion so that the cell can remain viable even if the lesion is not removed. Trans-dimer bypass involves two steps: addition of a base opposite the damaged site and subsequent synthesis past the lesion. Error-prone bypass of CPD not compensated by removal of CPDs is responsible for a high mutation rate. The tendency to insert adenine opposite the first pyrimidine (and presumably also opposite the second) means that a large proportion of mutations will be "lost" because of insertion of the "correct" base, because thymine is the most frequent pyrimidine in dimers. Also, transitions should be more frequent than transversions because of the preference for purine insertions opposite pyrimidines (Rabkin et al., 1983). The high UV mutation rates indicate that in *P. patens* the error-prone bypass is very frequent and efficient on CPDs and on 6–4PPs that have not been removed by photolyases or by NER. This is also manifested by exclusive localization of UV induced transitions within exons 3, 4 and 5 in contrast to far more dispersed distribution of Bleomycin mutations (Fig. 3) that range from transitions/transversions, small (≧2 bp) deletions or insertions to large deletions up to 748 bp (Table B.1).

In our study we used artificial laboratory conditions (total dark or red light illumination) to dissect dark repair during dimer repair or 24-h fixation of mutations. Nevertheless in the real world under the daylight, when source of UV is sunshine, there is still approximately a 2-h window between induction of dimers by UV irradiation, before their elimination by light repair (Waterworth et al., 2002; Chen et al., 1994). NER is even slower than photoreactivation and thus cannot significantly contribute to offset consequences of quick error-prone repair. Moreover bypass is independent on the repair mechanism tested here by studying moss repair mutants and is solely dependent on ongoing DNA synthesis during irradiation. This is why we were able to follow the consequences of UV irradiation in a 1 day-subcultured protonemal culture with 50% of cells active in mitosis.

4. Summary

In actively dividing, apical plant cells, exposure to UVB induce

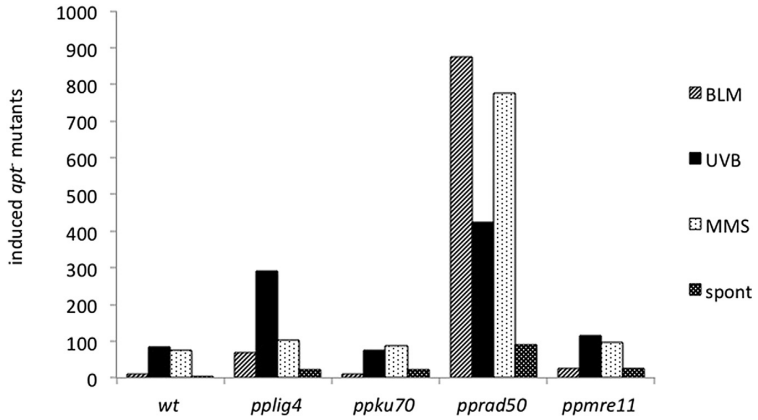

Fig. 2. Relative numbers of 2FA resistant mutants of *Physcomitrella patens* wild type and *pprad50*, *ppmre11*, *pplig4* and *ppku70*, differentiated into spontaneously appearing mutants and mutants induced by 500 J m^{-2} UVB, 1 mM MMS and 1 μg mL^{-1} Bleomycin respectively. The number of detected *APT* mutants is normalised to 1 g of dry tissue weight.

Table 1
Mutations induced by UVB irradiation within the *APT* locus of wt, *pprad50*, *ppmre11*, *pplig4* and *ppku70*. The observed spectra of induced mutations are principally base-substitution transitions.

Mutant line		Noncoding parts			Exons		
	APT mutant	Deletion	Insertion	Substitution	Deletion	Insertion	Substitution
WT	uvb3		**A**(3199)				T**C**A → **T**(1369)
	uvb25			AG**G**T → **T**(2351)			
pprad50	uvb2						TT**G**A → **A**(1809)
	uvb23						T**C**CA → **TT**(1894)
	uvb29						AC**C**A → **T**(1650)
ppmre11	uvb3						G**C**TC → **T**(1813)
	uvb5						T**C**CA → **T**(1893)
pplig4	uvb4						T**C**CA → **T**(1369) CC**G**A → **A**(1576)
	uvb5						G**C**CA → **T**(1834)
	uvb20		**A**(643)				
ppku70	uvb1						T**C**CA → **TT**(1368)

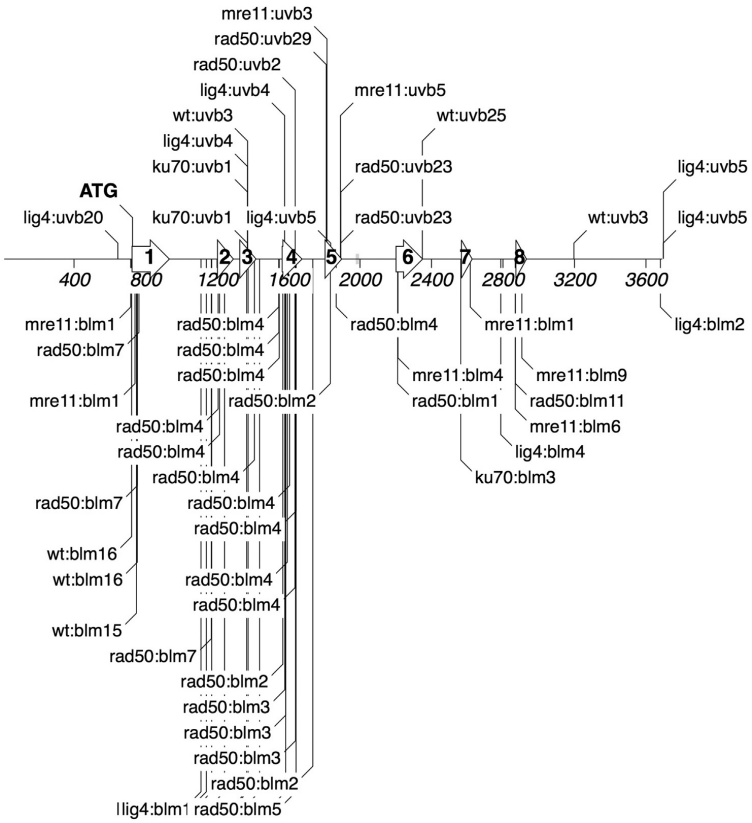

Fig. 3. Map of UVB and Bleomycin induced mutations within the *APT* locus. UVB induced mutations are depicted above and Bleomycin mutations below the schematic drawing of the *APT* locus, with ATG translation start indicated and exons represented as arrows. Mutation types for UVB are generally base substitutions, predominantly transitions in coding regions (Table 1 and Table B.1), whereas Bleomycin induces broad spectra of mutations from base substitutions of both types (transitions and transversions), insertions to deletions, in particular long deletions in *pprad50* and *ppmre11* (Table B.1).

robust mutagenesis via error-prone CPDs and 6-4PPs bypass DNA synthesis. Mutagenic activity is limited to DNA replication within dividing cells. When a mutated cell is not eliminated and mutation is tolerated during further plant development, then due to the clonal character of plant tissue it can initiate a change of phenotype (with or without external selection pressure). These changes do not need to be recognized as consequence of induced mutation, but rather considered as physiological effect of UVB. In this respect we proved the idea that induced mutagenesis due to unrepaired UV photoproducts could underlie the mechanism of UV impact on

plant phenotype.

Contributions

MH and RV performed *APT* mutation experiments, sequencing and mutation analysis. KJA did comet assay experiments, data evaluation and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.12.013.

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BRIEF COMMUNICATION

Efficient biolistic transformation of the moss *Physcomitrella patens*

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Abstract

High rates of homologous recombination (HR) in comparison to other plants make the moss *Physcomitrella patens* an attractive model organism for genetic studies as well as biotechnological applications. We describe a simple protocol for the efficient biolistic transformation of protonemal tissue with minimum tissue handling steps. The transformation efficiency depends on the biolistic conditions. The bombardment of tissue with 1 μm gold particles yielded between 20 and 40 stable transformants per 1 μg of DNA. Transformation with circular plasmids generates higher frequencies of random transgene integration, whereas linear plasmids are more efficient in generating gene-targeted insertions.

Additional key words: *Helios* biolistic gun, moss protonemal tissue, particle size, random and targeted integration, regeneration.

The moss *Physcomitrella patens* is an attractive plant model (Cove 2005) to study gene function as well as a biotechnologically useful production plant (Frank *et al.* 2005). Particularly attractive features are its efficient somatic homologous recombination (HR) enabling targeted gene modification, not possible in higher plants (Schaefer 2002), and its dominant haploid phase during most of the life cycle, which make genetic changes directly evident.

Several methods have been successfully tested for delivery of DNA into *Physcomitrella* cells including even *Agrobacterium* T-DNA transfer. Currently, the method most commonly used to transform *Physcomitrella* is PEG-mediated delivery of dsDNA into protoplasts (Schaefer *et al.* 1991). This approach has several drawbacks, including the requirement for extensive tissue and protoplast manipulation, the relatively complicated procedure of PEG-mediated DNA uptake and the need to regenerate protoplasts with high efficiency under scrupulously sterile conditions. An alternative possibility is biolistic delivery of transforming DNA into intact tissue on metal microparticles (Sawahel *et al.* 1992, Cho *et al.* 1999, Bezanilla *et al.* 2003). We decided to evaluate biolistic approach in detail when we experienced only low efficiency of transformation (0.5 - 1 transformant per μg DNA) mediated by PEG.

There are two biolistic systems routinely used for acceleration of DNA-coated microparticles to penetrate tissue. The system firstly developed uses an accelerated plug (macrocarrier) carrying DNA-coated microparticles. Newer systems accelerate microparticles directly by a burst of helium from a rapidly opened valve (Gray *at al.* 1994). Gal-On *et al.* (1997) later simplified Gray's approach by showing that microparticles accelerated by a helium burst do not need a vacuum for efficient penetration of plant tissue and designed a simple hand held instrument for *in planta* transformation.

The potential of biolistic transformation has stimulated significant technical improvement and commercialization of delivery devices in recent years. The *PDS-1000/He* model represents the macrocarrier/ microcarrier system, whereas the *Helios* gene gun (both made by *BioRad*, Hercules, USA) is an acceleration device for direct bombardment of a target. The *Helios* gun uses DNA-coated gold particles, deposited on the inner wall of a plastic tube that are flushed off and accelerated by a burst of pressurized helium. However, the procedure developed by *BioRad* for coating of plasic tubing with microparticles is a time-consuming and error-prone procedure that requires a dedicated "Tubing Prep Station".

Here we describe a modified, low-cost biolistic

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Abbreviations: dsDNA - double stranded DNA; PEG - polyethylene glycol; T-DNA - transfer DNA of Ti-plasmid.
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application for delivery of the DNA-gold particle suspension to *Physcomitrella* protonemal tissue with the *Helios* gene gun and show high yields of both, random and targeted transformants. Our simple transformation protocol circumvents the technical complexity of the PEG-mediated protoplasts transformation.

For construction of the random integration vector we used plasmids and cassettes of the *pGreen* system (www.pgreen.ac.uk). To express enhanced yellow fluorescence protein (EYFP) we first modified the multiple cloning site (MCS) of the 2 × 35S cassette of the *pJIT60* vector to contain a *Nco*I site upstream of the *Eco*RI site. The whole expression cassette was then recloned between the *Sac*I and *Eco*RV of pGreenI 0029, which already contains an *NPTII* selection cassette. The *NcoI*/*Eco*RI fragment from pEYFP-C1Kana (kind gift of Dr. Y. Eshed, WIS, Rehovot, Israel) containing the EYFP coding region was then directly inserted into the cloning site to produce binary pKA127 vector (Fig. 1*A*). For delivery of linear DNA, *Apa*I site was used to cut the vector between reporter and selection cassette.

Gene-targeting vector was based on the sequence derived from the moss dehydrin gene *PpLEA2* (Phypa1_1:173331; GenBank Accession XM_001785041). Into pBS::Lea2 vector (kindly provided by Dr. A. Cuming, CPS, University of Leeds, UK) containing genomic coding region from amino acid 9 *Lea2A* and 3'-untranslated termination region *Lea2B* (Fig. 1*B*) , there was cloned in frame with 5´-Lea2 region *NPTII* amplified by PCR from pGreenI 0029 . The *NPTII* 5'-end PCR primer was designed to contain an *Nco*I site and the fragment was made blunt ended at the 5'-end by cutting with *Nco*I, and fill-in with the Klenow fragment of DNA PolI. Digestion at the 3'-end with *Eco*RI generated a fragment that could be cloned in frame with *Lea2A* between the *SmaI* and *EcoRI* sites generating the plasmid pKA133 (Fig. 1*B*). In this vector *NPTII* should only be expressed if a correct targeting event occurs, because there are two introns upstream of the *NPTII* fusion. If these are not spliced out, they will not permit *NPTII* expression, since there are 4 in-frame termination codons (3 in the first and 1 in the second intron). Vector was linearized for biolistic transformation by digestion with *Xba*I at the 3'-end of the targeting cassette.

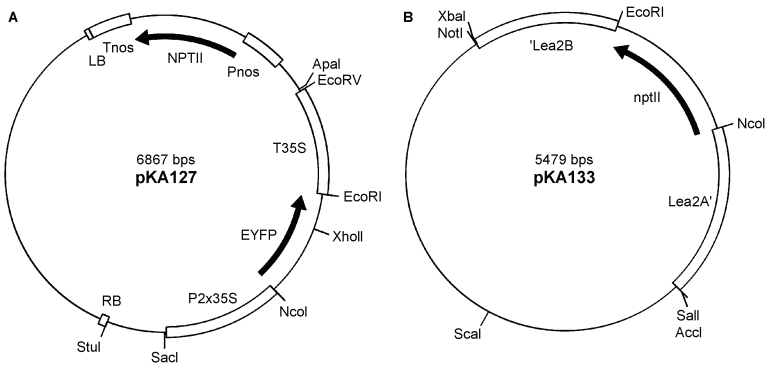

Fig. 1. Transformation vectors: *A* - pKA127 for random integration, B - pKA133 for targeted insertion into *Lea2* gene. For detail description see text.

The wild-type moss *Physcomitrella patens* Grandsen was grown on Petri plates with modified PPNH4 medium (Knight *et al.* 2002) with [g dm^{-3}] 0.8 $Ca(NO_3)_2 . 4 H_2O$, 0.25 $MgSO_4 . 7 H_2O$, 0.062 $FeSO_4 . 7 H_2O$, and 0.25 KH_2PO_4, pH 5.8 before autoclaving. Medium was enriched with 0.5 g dm^{-3} ammonium tartrate, *AltTES* macro- and microelements [μg dm^{-3}]: 55 $CuSO_4 . 5 H_2O$, 614 H_3BO_3, 55 $CoCl_2 . 6 H_2O$, 25 $Na_2MoO_4 . 2 H_2O$, 55 $ZnSO_4 . 7 H_2O$, 389 $MnCl_2 . 4 H_2O$, and 28 KI and solidified with 8 g dm^{-3} agar (*Duchefa*, Haarlem, The Netherlands) and overlaid with cellophane discs.

A *Helios* gun and microcarriers were used. The gun was operated according to the manufacturer's recommendation, although a new protocol for preparation of the DNA-coated microcarrier suspension and gun loading was developed. We tested four types of microcarriers, three gold particles (0.6, 1.0 and 1.6 μm), and *Tungsten M17*. The microcarriers (50 mg) were resuspended in 1 cm^3 of 100 % ethanol for 10 min to break particle clumps. Purified plasmid DNA was used either linearized or circular. The transforming DNA (2 μg in 2 mm^3) was added to the bottom of a 0.5 cm^3 Eppendorf tube and 5 mm^3 of 100 % ethanol were slowly pipetted dropwise down the side of the tube. Immediately 11 mm^3 of the particle/ethanol suspension were added to DNA/ethanol mixture, briefly vortexed and immediately used for delivery. A 5 mm^3 aliquot of DNA/ ethanol/microcarrier suspension was as a single droplet directly loaded into pieces of plastic tubing (cartridges) in the holder and the "charged" holder was then inserted into a gun. An armed *Helios* gun was then used for shooting moss tissue on Petri plates by 550 kPa helium bursts. One-week-old protonemal tissue on one plate

(equivalent of approximately 15 mg of moss dried tissue) was pooled into two "humps", approximately 3 cm in diameter and shot with particles from 3 cartridges (2 helium bursts through each cartridge). After shooting, tissue was roughly dispersed on the plate and allowed to recover on modified PPNH4 medium for 3 d without selection.

After 3 d of recovery, tissue was sheared by a homogenizer *T25* (*IKA*, Staufen, Germany) at 10 000 rpm in 8 cm^3 of distilled water and subcultured on four PPNH4 plates containing 50 μg cm^{-3} of G418 (*Gibco BRL*, Eggenstein, Germany) as needed for two or more weeks, when transformed foci were clearly visible. To select stable transformants individual foci were transferred for two-week release period onto medium without antibiotic and a second two-week selection period. Plants surviving the third round of selection were considered to be stable transformants. Transformants expressing EYFP were identified with *Leica* MZ16 fluorescence stereo-microscope equipped with a *Leica* YFP (excitation 510/20, barrier 560/40 nm) filter set.

DNA for analysis was isolated from 100 mg of 7-d-old protonema tissue using a *NucleoSpin* plant DNA kit (*Macherey-Nagel*, Düren, Germany). 7.5 μg of genomic DNA were digested with *Bam*HI, electrophoresed in a 0.7 % agarose gel and transferred to *Hybond-N*$^+$ nylon membrane (*GE-Healthcare*, Uppsala, Sweden) by alkali capillary transfer with 0.4 M NaOH and 0.6 M NaCl without UV fixation. Hybridization probe was an *Nco*I/*Xho*I fragment of pKA127 (Fig. 1*A*), labeled with digoxigenin-dUTP and used for hybridization at concentration 25 ng cm^{-3}. Labelling, hybridization and detection were carried out according to the manufacturer's instructions using a DIG DNA labeling and detection kit (*Roche*, Indianapolis, USA) and chemilumini-scence *CDP-Star* substrate (*Roche*). Chemiluminiscence signals were captured on *ChemiDoc* (*BioRad*).

To avoid damage of protonemal tissue we have tested the pressure range 400 - 800 kPa for direct shooting. The regular growth medium solidified with 8 g dm^{-3} agar shows severe signs of tissue damage when pressure over 550 kPa was used. To avoid a grouped impact pattern we found it necessary to use a diffusion screen (*BioRad*). With such a set up it was possible repeatedly to shoot plates from a distance of 5 cm and continue cultivation on the same plate. Two helium bursts were enough to remove all microcarriers from a single cartridge. During the first shot approximately 80 % of the microcarriers were flush out and remainder with the second shot.

The biolistic transformation was dependent on microcarrier size. The best yields of stable transformants per 1 μg of DNA were obtained with 1 μm spherical gold particles (20 transformants). *Tungsten M17* particles, a rod like crystals rated as 1 μm provided 9 transformants. Smaller particles 0.6 μm might not have sufficient momentum to effectively penetrate the tissue (3 transformants), whereas larger particles may disrupt tissue to an extent that prevents efficient recovery (8 transformants). In contrast to Cho *et al.* (1999) we have not detected any significant difference in transformation efficiency when using denatured DNA. In most transformants after the first round of G418 selection and in all stable transformants the yellow signal of expressed EYFP was positively detected in foci of 2 mm in diameter by fluorescence microscopy.

Construction of the targeting vector pKA133 does not allow selection without in frame intergration into the *Lea2* locus of the *Physcomitrella* genome. This is demonstrated by 5 - 10 time lower recovery of primary transformants in comparison to transformation with pKA127, where a substantial number of transient transformants expressing *NPTII* could be recovered (Table 1). The yield of transformants after the third round of G418 selection was only 30 % lower than the number of primary transformants for both, circular and linear form of the pKA133 vector.

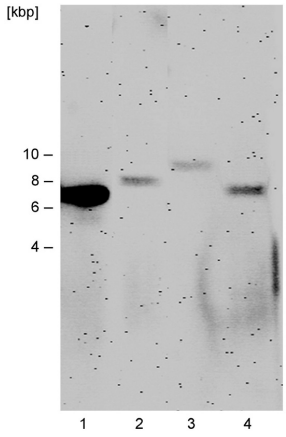

Fig. 2. Southern blot of *Physcomitrella* transgenic lines. Genomic DNA (7.5 μg) of two independent transformants was digested with BamHI (*lane* 2 and 3). *Eco*RI-linearized plasmid pKA127 at concentration 50 ng (*lane* 1) and 10× diluted in plant extract (*lane* 4) were positive controls. 502 bp digoxigenin-labeled *Nco*I/*Xho*II restriction fragment of pKA127 was used as hybridization probe.

To prove insertion into *Physcomitrella* genome several random transformants were analysed by Southern blot hybridization. For this purpose the moss genomic DNA was cut with *Bam*HI, which does not have a recognition site within transformation vector pKA127. Surprisingly in 10 transformants analyzed (Fig. 2) we found only single copy of the vector inserted in genome.

Biolistic transformation provides several advantages over PEG transformation mainly by reducing handling steps to a minimum and avoiding potential microbial contamination. Transformation is carried out on the same plate where *Physcomitrella* is grown until transfer to selective medium, and is completed within an hour. Optimized biolistic conditions, *e.g.*, the microcarrier size

and helium pressure, provide a high yield of stable transformants per μg of DNA. The limiting factor in the wider use of this approach is the need of a delivery device. We have adopted the *BioRad* hand-held *Helios* gene gun, instead of the *BioRad PDS-1000/He*, which is more widely used by the plant science community. The advantage of using the hand-held gun is the freedom of handling as well as a lower cost per shot. Direct loading of a microparticle suspension in the helium path is often used in custom-built systems (*e.g*. Gray *et al.* 1994, Gal-on *et al.* 1997) and also works well in the *Helios* gun, even though according to the manufacturer's protocol cartridges should be prepared in advance.

Table 1. Number of transformants during three cycles of G418 selection. Targeted transformation into *Lea2* locus was carried out with pKA133, whereas random transformation with pKA127.

	Circular			Linear		
	1	2	3	1	2	3
Integration to Lea 2	35	17	23	56	45	41
Random integration	322	55	40	236	46	22

The transformation efficiency depends on the form of the transforming DNA. Random integration is approximately twice as effective with a circular than with a linear vector. This suggests that random integration preferentially occurs as a single crossover event. In targeted transformation the situation is opposite and linearized pKA133 is nearly as twice as effective than circular, suggesting that linear form of transforming DNA promotes gene replacement by double crossover.

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MRE11 and RAD50, but not NBS1, are essential for gene targeting in the moss *Physcomitrella patens*

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MRE11 and RAD50, but not NBS1, are essential for gene targeting in the moss *Physcomitrella patens*

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ABSTRACT

The moss *Physcomitrella patens* is unique among plant models for the high frequency with which targeted transgene insertion occurs via homologous recombination. Transgene integration is believed to utilize existing machinery for the detection and repair of DNA double-strand breaks (DSBs). We undertook targeted knockout of the *Physcomitrella* genes encoding components of the principal sensor of DNA DSBs, the MRN complex. Loss of function of *PpMRE11* or *PpRAD50* strongly and specifically inhibited gene targeting, whilst rates of untargeted transgene integration were relatively unaffected. In contrast, disruption of the *PpNBS1* gene retained the wild-type capacity to integrate transforming DNA efficiently at homologous loci. Analysis of the kinetics of DNA-DSB repair in wild-type and mutant plants by single-nucleus agarose gel electrophoresis revealed that bleomycin-induced fragmentation of genomic DNA was repaired at approximately equal rates in each genotype, although both the *Ppmre11* and *Pprad50* mutants exhibited severely restricted growth and development and enhanced sensitivity to UV-B and bleomycin-induced DNA damage, compared with wild-type and *Ppnbs1* plants. This implies that while extensive DNA repair can occur in the absence of a functional MRN complex; this is unsupervised in nature and results in the accumulation of deleterious mutations incompatible with normal growth and development.

INTRODUCTION

DNA double-strand breaks (DSBs) represent one of the most cytotoxic forms of damage an organism can acquire (1). Such events occur with high frequency resulting from cellular metabolism (such as reactive radicals or stalled replication forks during S phase) and through the action of exogenous agents (such as ionizing radiation or chemical mutagens). Failure to repair such damage can lead to the irrecoverable loss of genetic material, with both immediate and long-term consequences: the onset of cancerous transformation in animal cells, or the failure to transmit genetic information in gametes (especially in plants, where there is no early developmental partitioning of germ-line and somatic cell lineages).

Unsurprisingly, all living organisms have evolved efficient mechanisms that can be deployed to sense DNA DSBs, activate DNA repair, cell-cycle arrest and sometimes apoptosis. Such is the importance of these mechanisms, that the genes encoding many of the essential components of the DNA repair machinery are highly conserved in evolution (2). In particular, this is true of the mechanism by which the broken ends of DNA molecules are recognized and recruited into DNA repair complexes. In eukaryotes, the MRN/MRX complex undertakes this task (3,4). This conserved complex is composed of three proteins, Meiotic recombination 11 (MRE11), Radiation sensitive 50 (RAD50), and

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Nijmegen Breakage Syndrome 1 (NBS1) (X-ray sensitive 2, XRS2 in the yeast, *Saccharomyces cerevisiae*). Together, the MRE11, RAD50 and NBS1 proteins form a multisubunit complex ($M_2R_2N_1$) that binds the ends of broken DNA molecules, and can tether the broken ends through dimerization between adjacent MRN complexes mediated by an association between the RAD50 components (3,5). The formation of MRN–DNA complexes also initiates a cell-cycle checkpoint through interaction with the phosphoinositide 3-kinase-related protein kinases (PIKKs) ATM and ATR (for 'Ataxia Telangiectasia Mutated' and 'Ataxia Telangiectasia mutated-like and Rad 3 related') and the DNA Protein Kinase catalytic subunit (DNA-PKcs) (6). These proteins phosphorylate multiple targets to initiate a cascade of downstream events leading to DNA DSB repair either by non-homologous end joining (NHEJ), a rapid but occasionally inaccurate mechanism, or through homologous recombination (HR), a conservative mechanism that uses an homologous sequence (e.g. a sister chromatid) as a template to restore the original sequence at the DSB site. In this latter pathway, an Mre11-specific nuclease activity is required (with other components) for the resection of DNA ends necessary for strand invasion (7).

Transgene integration into flowering plant genomes occurs through the agency of endogenous mechanisms that have evolved for the repair of DNA DSBs. In flowering plants, the integration of exogenous DNA whether directly delivered via microprojectile bombardment or protoplast transfection, or delivered by *Agrobacterium*-mediated transformation occurs predominantly at random positions throughout the genome, whereas gene targeting frequencies remain extremely low (8). Random integration of transgenes requires enzymes from the NHEJ pathway, and the inefficiency of GT probably reflects the prevalence of the NHEJ pathway in repairing DNA DSBs in angiosperms (9–11). In contrast with flowering plants, transformation of the moss, *Physcomitrella patens*, with DNA containing homology with genomic sequences results in preferential incorporation of the transforming DNA at these homologous sequences (12). This facility for 'gene targeting' is similar to that seen in *Saccharomyces* (13) and suggests a preference for the use of the HR-dependent pathway as the primary means of undertaking DSB repair, although molecular analyses of gene targeting events provide clear evidence for modification of the transforming DNA by both NHEJ and HR reactions upon integration (14,15). *Physcomitrella* thus represents an excellent model in which to analyse DNA-DSB repair pathways in plants, particularly in regard to its outstanding gene targeting efficiency (12). Previous studies have shown that PpRAD51, the protein at the core of the HR reaction, was required to preserve genome integrity and essential to achieve gene targeting (16,17). The mismatch repair *PpMSH2* gene was also shown to be essential to preserve genome integrity and to prevent homeologous gene targeting (18).

We have characterized the role of the *Physcomitrella* MRN complex in DNA DSB-repair and gene targeting. We find that in moss the major loss of function phenotypes of the MRN complex depends on PpRAD50 or PpMRE11, but not PpNBS1. Inactivation of either *PpRAD50* or *PpMRE11* reduced GT ~11-fold in both *Pprad50* and *Ppmre11* mutants, while illegitimate integration rates only slightly affected. Gene expression studies further show that *Ppmre11* and *Pprad50* strains display constitutively high expression of the DNA damage response, implying the activation of alternative pathways to minimize endogenous DNA damage in the mutant strains. The mutants exhibit a severe developmental phenotype, possibly associated with early senescence processes, and hypersensitivity to UV-B and bleomycin-induced DNA damage.

MATERIALS AND METHODS

Plant material

Physcomitrella patens (Hedw.) B.S.G. 'Gransden2004' was vegetatively propagated as previously described (19). Individual plants were cultured as 'spot inocula' on BCD agar medium supplemented with 1 mM $CaCl_2$ and 5 mM ammonium tartrate (BCDAT medium), or as lawns of protonemal filaments by subculture of homogenized tissue on BCDAT agar medium overlain with cellophane for the isolation of protoplasts. Transformation experiments were performed as previously described (20) using linear fragments of DNA generated either by digestion of transforming vectors with restriction enzymes (19) or by polymerase chain reaction (PCR) amplification (14). Growth conditions for the generation of deletion strains were as described previously (17).

Gene identification and isolation

Genomic DNA and total RNA were isolated from *Physcomitrella* as previously described (19). For verification of gene models, RNA was extracted from a polyribosome-enriched fraction: 7-day subcultured protonemal tissue (~5 g squeeze-dried chloronemal tissue) was homogenized in 30 ml extraction buffer [200 mM sucrose 40 mM Tris–HCl, pH 8.5, 60 mMKCl, 30 mM $MgCl_2$, 1% (v/v) Triton X-100, 2 mM dithiothreitol] and the extract was clarified at 25 000*g* for 20 min (Sorvall SS34 rotor). The supernatant was layered over a cushion comprising 1 M sucrose, 40 mM Tris–HCl, pH 8.5, 20 mM KCl, 10 mM $MgCl_2$ and centrifuged for 3 h at 141 000*g* (Beckman SW28 rotor). The pellet was drained and resuspended in 0.5 ml RNA extraction buffer for aqueous phenol extraction (19). RNA used for RT-PCR was first digested with RQ DNase I (Promega) to remove residual DNA. *Physcomitrella* genomic sequences encoding the *MRE11*, *RAD50* and *NBS1* genes were identified by BLAST search (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html). The available gene models were used for the design of PCR primers to amplify cognate genomic sequences, which were cloned in the plasmid pBluescript KS^+. PCR primers used are listed in Supplementary Table S1. In order to obtain a correct gene model for each sequence, full-length complementary DNA (cDNA) sequences were amplified from *Physcomitrella* polyribosome-derived RNA by RT-PCR. Total RNA (1 μg) was reverse

transcribed using an oligo-$dT_{12–15}$ primer and AMV reverse transcriptase as supplied in the Promega Reverse Transcription system in a 20-µl reaction. Following cDNA synthesis, the reaction mixture was diluted by the addition of 80 µl water, and 1 µl aliquots were used for PCR amplification using primers predicted to anneal with 5′- and 3′-untranslated region (UTR) sequences (Supplementary Table S1). PCR products were cloned by blunt-end ligation into the EcoRV site of pBluescript KS^+ for sequence analysis (ABI3130) in the DNA sequencing facility of the Leeds University Faculty of Biological Sciences. Predicted polypeptide sequences were aligned with the orthologous genes from *Arabidopsis thaliana*, *Homo sapiens* and *Saccharomyces cerevisiae* using CLUSTALW.

Targeted gene knockout

Gene disruption cassettes were constructed by ligating a selection cassette comprising a neomycin phosphotranferase gene driven by the Cauliflower Mosaic Virus 35S promoter and terminated with the CaMV gene 6 termination sequence (35S-*nptII*-g6ter) derived from the vector pMBL6 (14) into convenient restriction sites within the cloned *PpMRE11*, *PpRAD50* and *PpNBS1* genes to replace endogenous coding sequences. For the *PpMRE11* gene, the selection cassette was placed between residues 1763501 (in exon 4) and 1764332 (in exon 8) in JGI Phypa1_1/scaffold 18. For the *PpRAD50* gene the selection cassette was placed between residues 1431738 (in intron 13) and 1433260 (in intron 16) in JGI Phypa1_1/scaffold 51. For the *PpNBS1* gene, the selection cassette was placed between residues 276622 (in intron 4) and 277547 (in intron 7) in JGI Phypa1_1/scaffold 219. For targeted knockout of the moss genes, fragments of DNA containing these cassettes and flanked by ~1 kb of 5′- and 3′-flanking genomic sequence were PCR amplified. These linear fragments were used to transform *Physcomitrella* protoplasts, and stable transformants were selected following regeneration in medium containing 50 µg ml^{-1} G418 for 2 weeks, followed by subculture onto medium lacking antibiotic for 2 weeks, and a final subculture on selective medium. Targeted replacement of the native genes by the disruption cassette was confirmed by PCR reactions using external, gene-specific primers in combination with 'outward-pointing' selection cassette-specific primers (Supplementary Table S1). Single-copy allele replacements were identified by PCR using the external primer pairs, and the absence of additional transgene insertion in the genome was confirmed by Southern blot analysis. Conditions for PCR analysis were as previously described (14) and Southern blot analysis of genomic DNA was carried out as previously described (21), using the 35S-*nptII*-g6ter cassette as a hybridization probe.

For generation of deletion mutants *mre11Δ* and *rad50Δ*, the 5′-and 3′-targeting fragments were amplified from *P. patens* genomic DNA and cloned upstream (5′) and downstream (3′) of the loxP sites flanking the resistance cassette in plasmid pBNRF (17) to create the plasmids pMRE11delta and pRAD50delta, respectively. For the *PpMRE11* gene, a 1009-bp 5′-targeting fragment (coordinates 1763137-1764146 in JGI Phypa1_1/scaffold 18) and an 803-bp 3′-targeting fragment (coordinates 1765334–1766184) were PCR-amplified. For *PpRad50*, an 831-bp 5′ targeting fragment (coordinates 1426648–1427479 in JGI Phypa1_1/scaffold 51) and an 819-bp 3′-targeting fragment (coordinates 1435197–1436016) were PCR-amplified.

Moss protoplasts were transformed with pMRE11delta digested with BstXI and AseI, or with pRAD50delta digested with XbaI and NsiI. Stable disruptants were selected by successive subculture on selective and non-selective medium and PCR analysis as described above. Clean deletions in the *PpMRE11* (encompassing exons 7–10) and *PpRAD50* genes (exons 4–20) were obtained by transient Cre recombinase expression (18). Deletions in the recombinant loci were confirmed by PCR amplification using gene-specific external primers MRE11#1 and MRE11#2, and RAD50#1 and RAD50#2, respectively. Primers APT#14 and APT#19 were used as positive controls (Supplementary Table S1).

For gene targeting studies the vectors PpAPT-KO2 (17) and PpAPT-KO3 have been used. To obtain PpAPT-KO3, an internal 1631-bp SalI/BglII fragment containing the 35S:Hyg^R-LoxP marker was deleted in PpAPT-KO2 and replaced by an XhoI/BglII fragment from pBNRF (17) containing the 35S:Neo^R-LoxP marker.

Analysis of gene expression in mutants

Transcript abundance in selected knockout lines was determined by RT-PCR of cDNA. Total RNA was isolated from protonemal tissue (19) and 1 µg was reverse-transcribed using a Promega reverse transcription system. The 20-µl reaction mixture was diluted 25-fold and 5 µl aliquots were used for PCR. Detection of Mre11, Rad50 and Nbs1 mRNA in mutant lines was by RT-PCR using primers indicated in Supplementary Table 1.

For quantitative determination of the relative abundances of transcripts encoding DNA repair genes in wild-type and mutant strains, quantitative real-time PCR was carried out using a Qiagen Rotor-Gene Q instrument and Qiagen SYBR-Green PCR kit. RNA was isolated from 7-day subcultured protonemal tissue from each of three independent lines (wild-type, *Ppmre11KO*, *Pprad50KO* and *Ppnbs1KO*, respectively), with two replicates for each sample. Transcript abundance was estimated by reference to both internal and external reference sequences. As an external reference, *Physcomitrella* RNA samples were 'spiked' with tenfold serial dilutions (10^{-1}–10^{-4}) of an *in vitro* transcript from a full-length wheat 'Em' cDNA (22) prior to reverse transcription. These were used to test a number of candidate internal reference sequences, corresponding to *Physcomitrella* gene models Phypa1_1:227826 (SAND family endocytosis protein), Phypa1_1:209451 (Clathrin adapter complex subunit), Phypa1_1:224488 (Acyltransferase) and Phypa1_1:163153 (Ribosomal protein S4) for stability of expression in response to bleomycin treatment. Phypa1_1:227826 was subsequently selected as the

internal reference standard for the determination of the abundances of PpRad51-1 (Phypa1_1:206066), PpRad51-2 ((Phypa1_1:207856), PpPARP-1 (Phypa1_1:150949), PpPARP-2 (Phypa1_1:188096) PpKu70 (Phypa1_1: 60909), PpKu80 (Phypa1_6:23553) and PpCtIP (Phypa1_6:453490) transcripts. Relative transcript abundance was calculated using the $\Delta\Delta$Ct method and normalized to the wild-type value.

Bleomycin and UV-B sensitivity assays

Physcomitrella explants were inoculated as 'spot inocula' onto BCDAT-agar plates supplemented with bleomycin (Bleocin inj., Euro Nippon Kayaku GmbH, Germany) at concentrations indicated in the text, to determine sensitivity to chronic exposure to the drug. Plant growth was assessed by measurement of the surface area of each plant at intervals following inoculation by digital photography of the plates. The image analysis software 'ImageJ' (23) was used to convert the digital images to binary format and determine the colony area based on counting the number of pixels corresponding to each colony. Colony area determinations based on different photographs were normalized for each colony using the estimated area of the plate.

For acute toxicity testing, protoplast viability and protonemal growth were analysed. Viability was tested when protoplasts of wild-type and *mre11*, *rad50* and *nbs1* mutants in BCD liquid medium supplemented with mannitol were treated with bleomycin at concentrations indicated in the text for 1 h. Protoplasts were washed two times and then resuspended in liquid mannitol medium. After 20 h in the dark, the protoplasts were spread on BCD agar medium supplemented with mannitol ($\sim 10^5$ protoplasts per Petri dish). After 6 days regeneration, the number of survivors was counted. We repeated these experiments three times.

Protoplasts of wild-type, *mre11* and *rad50* mutants were spread ($\sim$50 000/plate) on protoplast agar medium (PpNH4 + 0.5% glucose + 8.5% mannitol). Plates were immediately irradiated with UV-B light (308 nm) in a Stratagene Stratalinker. The intensity of the irradiation was controlled using the internal probe of the Stratalinker and one plate of each strain was treated simultaneously. The experiment was repeated three times. Plates were immediately transferred to darkness for 24 h after treatment then to standard growth conditions for protoplast regeneration. Survival was determined after 1 week by microscopic observation.

Protonemal growth was tested by incubating 7-day-old protonemal tissue in BCDAT liquid medium containing bleomycin at concentrations indicated in the text for 1 h. The tissue was washed three times with medium lacking bleomycin and homogenized. Explants were inoculated onto BCDAT agar medium and recovery following treatment was determined by measuring the increase in plant surface area over a 3-week period.

Evaluation of spontaneous mutation frequency

Mutations in the *PpAPT* gene confer resistance to 2-Fluoroadenine (2-FA), a toxic compound for cells. The number of 2-FA resistant colonies that grow following protoplast regeneration reflects the frequency of spontaneous mutations. Protoplasts of wild-type, *mre11*, *rad50*, *nbs1* and *msh2* (18) mutants were regenerated for 6 days on BCD agar medium supplemented with mannitol ($\sim 10^5$ protoplasts per Petri dish) and then transferred on to BCD agar medium supplemented with 5 µM 2-FA (Fluorochem). After 2 weeks, the number of resistant clones was counted. Experiments were repeated three times and statistically analysed using Fisher's exact test.

Isolation of *apt* mutants after bleomycin treatments

One-day-old protonemata prepared from 10 plates of 7-day-old tissue (around 25.10^6 dividing cells) of wild-type and the *Pprad50KO* mutants were exposed to sublethal acute doses of bleomycin: 50 µg/ml for 2 h for wild-type and 0.1 µg/ml for 1 h for the *Pprad50KO* mutant, before being transferred onto cellophane-overlaid BCDAT agar medium supplemented with 2–3 µM 2-FA. After 3 weeks, resistant foci were clearly visible. Cellophane discs bearing resistant colonies were transferred to plates without 2-FA. This process was repeated three times until stable *Ppapt* clones were established. The results of selection are summarized in Supplementary Table S2. Genomic DNA was isolated as previously described (19) and the mutant *Ppapt* genes were amplified by PCR and sequenced using the primers listed in Supplementary Table S1 and indicated in Supplementary Figure S4.

Gene targeting assays

Transformation efficiency and *APT* targeting frequency were measured as previously described (17). Moss protoplasts (4.8×10^5) were transformed with the nonhomologous pBHRF or pBNRF plasmids (17) digested respectively with HindIII or XmaJI to produce a linear fragment containing the 35S::hygR or 35S::neoR markers, or with PpAPT-KO2 or PpAPT-KO3 plasmids digested respectively with BsaAI/HindIII or PvuI/BsrGI to produce the targeting *APT* fragment containing the 35S::hygR cassette (from pBHRF) or the35S::neoR cassette (from pBNRF) flanked by genomic *PpAPT* sequences. Targeted integration of PpAPT-KO2 or PpAPT-KO3 at the *APT* genomic locus confers resistance to 2-FA. We selected primary transformants (unstable + stable) with 25 mgl^{-1} hygromycin B (Duchefa) for PpAPT-KO2 or with 50 mgl^{-1} G418 (Duchefa) for PpAPT-KO3. Integrative transformants were isolated following a second round of selection. Protonemal explants from these transformants were then transferred onto medium containing 5 µM of 2-FA to detect *APT* gene targeting events. Experiments were repeated three times.

DNA-DSB repair assays

Protonemal lawns of wild-type and mutant strains subcultured for 1 week were used to generate protonemal tissue for DNA repair assays by shearing tissue collected from single 9-cm plates with an IKA T2T Digital Ultra Turrax homogenizer at maximum speed (24 krpm) for

1 min in 5 ml of liquid BCD medium. This was spread on BCD-agar medium overlaid with cellophane and grown for 1, 7 or 14 days prior to harvesting for bleomycin treatment.

Protonemata were gently transferred from cellophane to liquid BCD medium in 4-cm wells of a six-well microtitre plate to avoid drying. DSBs were induced by addition of bleomycin to 10, 20, 30 and 50 μg ml^{-1} for 1 h. Following treatment, the tissue was thoroughly rinsed in H_2O in disposable 22-μm mesh funnels (Partec GmbH, Germany), blotted on filter paper and either flash-frozen in liquid N_2 ($t = 0$) or left to recover on BCD-agar plates overlaid with cellophane for the indicated repair times, before being frozen in liquid N_2. All handling and transfer of protonemata was with tweezers.

DNA-DSBs were detected by a neutral comet assay (24) as described previously (25,26). Approximately 100 mg of frozen tissue was cut with a razor blade in 300 μl phosphate-buffered saline (PBS)+10 mM ethylenediaminetetraacetic acid (EDTA) on ice and the tissue debris removed by filtration through 50-μm mesh funnels (Partec GmbH, Germany) into Eppendorf tubes on ice. Fifty microlitres of nuclear suspension was dispersed in 200 μl of melted 0.7% LMT agarose (15510-027, GibcoBRL, Gaithersburg, USA) at 40° C and four 80-μl aliquots were immediately pipetted onto each of two agarose coated microscope slides (two duplicates per slide), covered with a 22 × 22-mm cover slip and then chilled on ice for 1 min to solidify the agarose. After removal of cover slips, slides were dipped in lysis solution (2.5 M NaCl, 10 mM Tris–HCl, 0.1 M EDTA, 1% N-lauroyl sarcosinate, pH 7.6) on ice for at least 1 h to dissolve cellular membranes and remove attached proteins. The whole procedure from chopping tissue to dipping into lysis solution takes ~3 min. After lysis, slides were twice equilibrated for 5 min in Tris–borate–EDTA (TBE) electrophoresis buffer to remove salts and detergents. Comet slides were then subjected to electrophoresis at 1 V/cm (~12 mA) for 5 min. After electrophoresis, slides were dipped for 5 min in 70 % EtOH, 5 min in 96% EtOH and air-dried.

DNA 'comets' were viewed in epifluorescence with a Nikon Eclipse 800 microscope after staining with SybrGold stain (Molecular-Probes Invitrogen, USA) and evaluated by the Comet module of the LUCIA cytogenetics software suite (LIM, Praha, Czech Republic).

Comet assay data analysis

The fraction of DNA in comet tails (% tail-DNA) was used as a measure of DNA damage. Data for the wild-type strain and the three mutant lines (*Pprad50*, *Ppmre11* and *Ppnbs1*) analysed in this study were obtained in at least three independent experiments. In each experiment, the % tail-DNA was measured at seven time points: 0, 5, 10, 20, 60, 180 and 360 min after treatment and in control tissue without treatment. Measurements included four independent gel replicas of 25 evaluated comets totalling at least 300 comets analysed per experimental point.

The percentage of damage remaining as plotted on figures after given repair time (t_x) is defined as:

$$\%\ \text{damage remaining}\,(t_x) = \frac{\left\{\begin{array}{l}\text{mean \%T DNA damage}\,(t_x)\\ -\ \text{mean \%T DNA damage (control)}\end{array}\right\}}{\left\{\begin{array}{l}\text{mean \%T DNA damage}\,(t_0)\\ -\ \text{mean \%T DNA damage (control)}\end{array}\right\}} \times 100$$

Repair kinetics following two-phase decay kinetics defined as:

SpanFast = (Y0-Plateau)*PercentFast*0.01
SpanSlow = (Y0-Plateau)*(100-PercentFast)*0.01
Y = Plateau + SpanFast*exp(-KFast*X) + SpanSlow* exp(-KSlow*X)

was analysed by linear regression of experimental data with the Prism v.5 program (GrafPad Software Inc., USA). Goodness of fit characterized by R-squared was better than 0.99.

RESULTS

Identification of MRN complex genes

Sequence homology searches of the draft *Physcomitrella* genome identified single putative homologues of the *MRE11*, *RAD50*, and *NBS1* genes on sequence scaffolds 18, 51 and 219, respectively. Whilst EST sequences were available to provide partial support for predicted gene models for the *PpMRE11* and *PpNBS1* genes, no corroborative evidence was available for the *PpRAD50* gene, and the automated gene prediction software had not generated a gene model. We therefore generated gene models for all three genes based on BLASTX similarity to flowering plant proteins (Arabidopsis, rice and maize) to identify putative full-length protein coding sequences, and used these models to design PCR primers for the amplification of full-length protein coding sequences by reverse transcription-PCR of moss polyribosome-derived RNA. The resulting cDNA sequences and genomic models have been deposited in GenBank (Accession Nos: JF820817 and JF820820 for *PpMRE11*; JF82018 and JF82021 for *PpRAD50*; JF82019 and JF82022 for *PpNBS1*) and the curated and structurally annotated gene models entered in the JGI *Physcomitrella* genome browser in which they were assigned the Protein ID numbers Phypa1_1:235701 (*PpMRE11*), Phypa1_1:235526 (*PpRAD50*) and Phypa1_1:235702 (*PpNBS1*).

The deduced polypeptide sequences were compared with the corresponding human, yeast and flowering plant sequences (Supplementary Figure S1). Like both the *Arabidopsis* and human genes, the *PpMRE11* gene comprises 22 protein-coding exons. There is extensive similarity among all the MRE11 polypeptides (Supplementary Figure S1a) especially within the N-terminal two-thirds of the protein. The *Physcomitrella* MRE11 protein contains the characteristic phosphoesterase motifs within the nuclease domain, the capping domain and amino acids

(N123 and W225) shown to be essential for the MRE11–NBS1 interaction (4).

The RAD50 sequences (Supplementary Figure S1b) are also well conserved at the amino-acid sequence level, and show good conservation of functionally important domains. The *Physcomitrella* protein contains the characteristic Walker A and Walker B adenosine triphosphatase (ATPase) motifs at either end of the sequence that associate to form a crucially important ATP-binding cassette (27) and that typify the RAD50 protein. These are separated by a long coiled-coil domain with a central CXXC zinc-hook (CPCC in both *Physcomitrella* and *Arabidopsis*) by which pairs of RAD50 proteins interact in the tethering of broken chromosome ends by the MRN/MRX complex (28).

The *Physcomitrella* NBS1 protein (Supplementary Figure S1c) has an N-terminal fork-head associated domain, a partial BRCT domain, and putative SQ-dipeptide phosphorylation sites and conserved MRE11-interacting motifs in the C-terminal region, as identified in all previously identified NBS1 orthologs (4).

Generation of targeted knockouts of the MRN complex genes

We used gene targeting to generate mutant alleles of the *PpMRE11*, *PpRAD50* and *PpNBS1* genes. For the *PpMRE11* and *PpRAD50* genes, two types of mutant were generated: disruption mutants, designated *mre11KO* and *rad50KO*, in which several exons were replaced by an antibiotic selection cassette and deletion mutants, designated *mre11Δ* and *rad50Δ*, in which a number of exons were replaced by a selection cassette that was subsequently removed by *cre-lox* recombination (Figure 1A). For the *PpNBS1* gene, we generated a disruption mutant (*nbs1KO*) and a deletion mutant in which the complete coding sequence was deleted (*nbs1Δ*). Gene targeting events were identified by PCR and Southern blot analyses to identify lines in which precise modification of the target genes had occurred without additional insertion of the targeting constructs at adventitious loci. For the deletion mutants we confirmed by PCR that a portion of the coding regions was removed using primers that flanked the deletion (Supplementary Figure S2). RT-PCR analysis established that the full-length transcripts were no longer produced in the mutants (Figure 1B). For all further experiments, we used two independent disruption or two independent deletion strains which displayed similar phenotypes.

Gene targeting is strongly decreased in mre11 and rad50 mutants

The MRN complex is one of the earliest respondents to DNA-DSBs and plays a central role in controlling repair pathway choice between NHEJ and HR (5). The importance of the MRN complex for DSB repair by HR has been shown in mre11-deficient chicken DT40 cells in which gene targeting efficiency is strongly reduced (29). In contrast, a somatic hyper-recombination phenotype has been described in the *Arabidopsis rad50* mutant (30). In order to examine the involvement of the MRN complex in genetic transformation of *Physcomitrella* we determined transformation and gene targeting rates in wild-type, *mre11Δ*, *rad50Δ* and *nbs1KO* cells after transformation with either an homologous vector designed to inactivate the *PpAPT* gene (PpAPT-KO2 or PpAPT-KO3) or a vector sharing no homology with the moss genome (pBHRF or pBNRF) to determine the rate of untargeted transgene integration. Relative transformation frequency (RTF) was reduced to approximately one-third of the wild-type level in *mre11* and *rad50* mutants, but gene targeting (GT) was reduced by at least an order of magnitude in both *Ppmre11Δ* and *Pprad50Δ* strains compared to WT, while untargeted integration frequencies were approximately double that observed in WT (Table 1). These data demonstrate that an active MRN complex is required to achieve high GT efficiencies in *Physcomitrella*, but that a low level of GT is possible in its absence. They also indicate that the untargeted integration of DNA is still supported following the loss of MRN function but that this pathway is not significantly up-regulated as has been observed to occur in *Pprad51* mutants (17). Noticeably, RTF, GT and untargeted integration rates were unaffected in the *nbs1KO* mutant. These observations suggest that both PpRAD50 and PpMRE11, but not PpNBS1, are directly involved in DNA DSB recognition and the targeted integration of transgenes following transformation.

PpMRE11 and PpRAD50 but not PpNBS1 are essential for normal growth and development

All the plants containing disruptions or deletions in the *PpMRE11* and *PpRAD50* genes exhibited a severe developmental phenotype (Figure 2). On minimal BCD medium, protonemal growth was strongly reduced and eventually ceased after a month (Figure 2A, C and G). At this stage, colonies comprised both chloronemal and caulonemal cells and carried only a few abortive gametophore initials, whereas WT colonies carried numerous fully differentiated leafy shoots (Figure 2A, C and G). In both mutants, the proportion of chloronemata was enhanced on ammonium tartrate-supplemented medium (BCDAT), which improved protonemal growth (Figure 2B, D and H) and enabled the isolation of numerous protoplasts. The rate of protoplast regeneration was approximately half that of WT (data not shown). Gametophore differentiation was also slightly improved on BCDAT medium, with numerous leafy shoot initials observed in 2-month-old colonies of both mutants (Figure 2E, F, I and J). However, further development into fully expanded leafy shoots was arrested in both strains, although at an earlier stage in *rad50* mutants than in *mre11* mutants (compare Figure 2F and J) and both mutants were thus unable to differentiate reproductive organs. In contrast, all of the *Ppnbs1KO* disruptant lines were indistinguishable from wild-type in both growth rate and developmental progression, producing normal gametophores and viable spores, demonstrating that the disruption of *PpNBS1* was neither detrimental to development nor to meiosis. These data show that a functional MRN complex is essential for normal

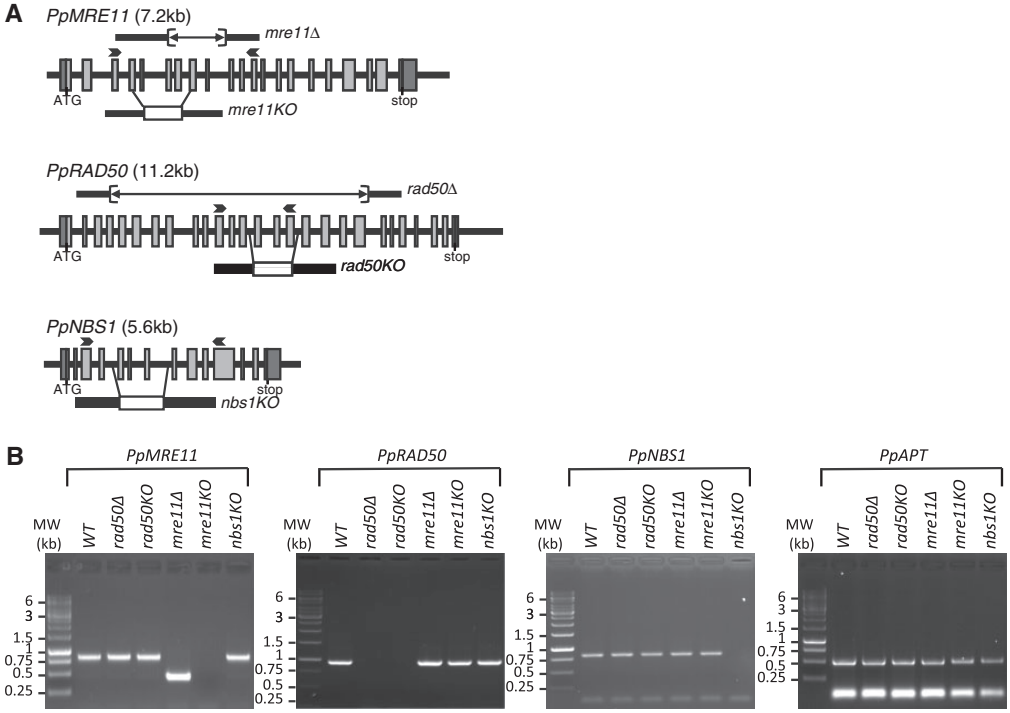

Figure 1. Targeted disruption of *Physcomitrella* MRN genes. (**A**) Structure of the *PpMRE11*, *PpRAD50* and *PpNBS1* genes. Exons are represented by shaded boxes, with 5′- and 3′-UTR sequences in darker grey. The region deleted by cre-lox excision of a selection cassette is shown as a line above each gene. For the replacement constructs (below each gene) the extent of targeting sequence homology is indicated by the line, and the P35S-*nptII-g6ter* selection cassette is shown as a white box, replacing the genomic region indicated by the lines joining the gene structure diagram and the replacement cassette. Arrows indicate position of primers used for RT-PCR analysis. (**B**) RT-PCR analysis of MRN transcripts in wild-type and mutant plants. RNA was isolated from protonemal tissue of wild-type and mutant lines for cDNA synthesis and PCR amplification using gene-specific primers (PpMRE11#1 + PpMRE11#2 for *MRE11*, PpRAD50#1 + PpRAD50#2 for *RAD50*, PpNBS1#1 + PpNBS1#2 for *NBS1*). The *PpAPT* transcript has been used as control (primers: PpAPT#14 + PpAPT#19). Primers are listed in Supplementary Figure S4.

Table 1. Comparison of transformation and gene targeting efficiencies

Genotypes	PpAPT-KO				pBHRF or pBNRF	
	RTF[a]	Antib[R]	2FA[R]	GT[b]	RTF[a]	Antib[R]
Wild type	1 ± 0.1[c]	287 (95.7 ± 10.8[c])	212 (70.7 ± 11.2[c])	73.9 ± 3.3[c]	0.09 ± 0.04[d]	14 (7 ± 2.8[c])
mre11Δ	0.37 ± 0.1[c]	81 (27 ± 4[c])	6 (2 ± 0.5[c])	7.4 ± 1[c]	0.2 ± 0.01[d]	34 (17.7 ± 2.8[c])
rad50Δ	0.35 ± 0.1[c]	76 (25.3 ± 4.5[c])	4 (1,3 ± 0.6[c])	5.3 ± 2.1[c]	0.17 ± 0.01[d]	31 (15.5 ± 2.5[c])
nbs1Δ	0.94 ± 0.1[c]	261 (87 ± 5.6[c])	178 (59.3 ± 4.5[c])	68.2 ± 1.4[c]	0.17 ± 0.03[d]	24 (12 ± 1.4[c])

[a]Relative transformation frequencies (RTF in $^{0}/_{00}$) express the frequency of antibiotic-resistant transgenic strains in the whole regenerated population.
[b]GT efficiencies (in percentage) express the frequency of 2-FA resistant among the population of antibiotic-resistant transgenic strains.
[c]Average and standard deviation was determined from three independent experiments, each of them performed in duplicates.
[d]Average and standard deviation was determined from two independent experiments, each of them performed in duplicates.

completion of processes involved in development. Noticeably, *PpNBS1* is not required to complete these processes. The similar phenotype displayed by both *rad50* and *mre11* mutants argues for the involvement of the whole MRN complex in these processes. Our data indicate that this complex is involved in the coordination between developmental programme and DNA damage repair and/or cell-cycle control, and future experiments will assess the molecular mechanisms underlying these MRN functions.

The mre11 and rad50 mutants display increased sensitivity to DNA damage but no significant mutator phenotype

Wild-type and *mre11* and *rad50* mutant plants were also analysed for their sensitivity to DNA damaging agents.

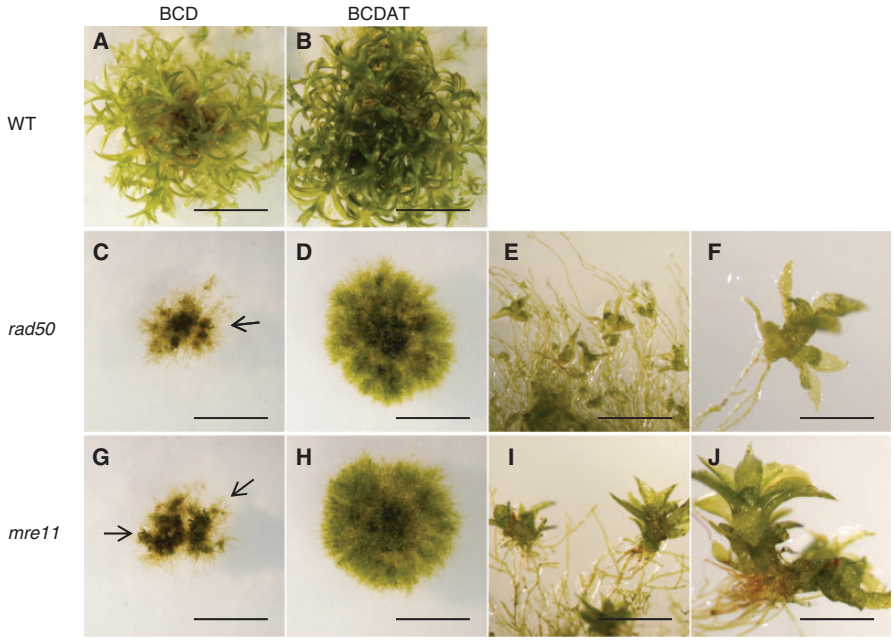

Figure 2. Vegetative developmental phenotypes of *mre11* and *rad50* mutants. WT (**A** and **B**), Rad50 7-20 (**C–F**) and Mre11 1-195 (**G–J**) 30-day-old colonies grown on BCD (A, C and G) or BCDAT (B, D and H) medium, scale 1 cm. (E, F, I and J) aborted gametophores observed at the edge of 2-month-old colonies grown on BCDAT, scale bar 500 mm in E and I, 200 mm in F and J.

Sensitivity of mutants and WT strains to UV-B (308 nm) was investigated using a protoplast survival assay (17). Both strains displayed increased sensitivity to UV-B compared to the WT (Figure 3). We further investigated sensitivity of the mutants to the DSB inducing agent bleomycin. We first monitored the growth of WT and mutant explants submitted to chronic exposure to different concentrations of bleomycin over a 3-week period. In WT and *nbs1KO* strains, growth was impaired at low doses (1–40 ng/ml), whilst higher concentrations (200 ng/ml–1 μg/ml) were lethal (Figure 4A and Supplementary Figure S3). In contrast, *Ppmre11* and *Pprad50* disruption and deletion mutants displayed hypersensitivity to bleomycin. At concentrations below 8 ng/ml, little or no growth took place, although the tissue remained green. At or above this concentration, all *mre11* and *rad50* mutant lines were killed (Figure 4A, Supplementary Figure S3A and B).

We tested the acute toxicity of bleomycin in wild type and of the different mutants at the cellular level. Following incubation for 1 h with increasing concentrations of bleomycin, the ability of protoplasts to divide and regenerate into colonies was assessed by subculture on drug-free medium. Survival was calculated as the ratio of protoplasts surviving after 15 days regeneration following treatment to the number of protoplasts undergoing normal regeneration without treatment. The LD50 for the wild type and *nbs1* mutant was about 500 ng/ml bleomycin, whereas the *mre11* and *rad50* cells were more sensitive, with an LD50 of ~50 ng/ml (Figure 4B). The *mre11* and *rad50* cells were even more sensitive than the *rad51-1-2* double mutant, already described as hypersensitive to bleomycin (16).

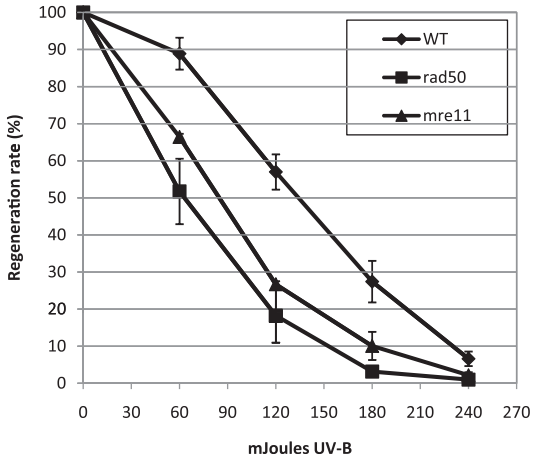

Figure 3. Hypersensitivity of the *rad50* and *mre11* mutants to UV-B treatment. Survival curves of wild type and *rad50* and *mre11* mutant protoplasts regenerating after exposure to UV-B treatment. Wild-type survival is represented with diamonds, *rad50* mutant survival is represented with squares and *mre11* mutant survival is represented with triangles. Error bars indicate SDs based on at least two independent experiments in all cases.

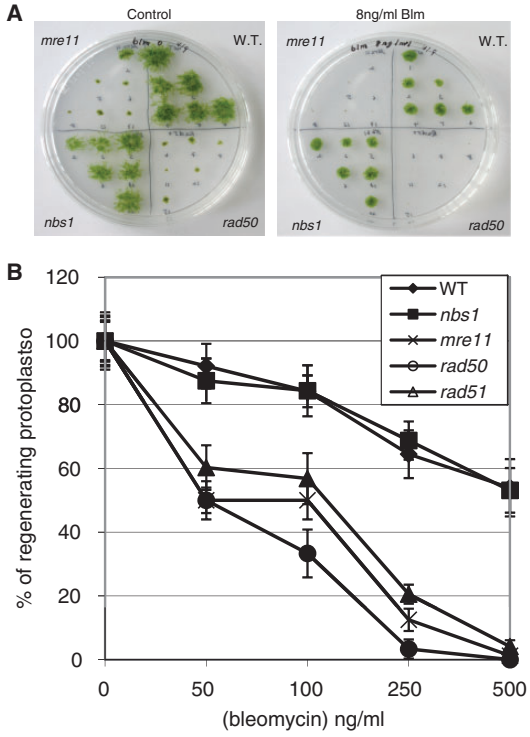

Figure 4. Hypersensitivity of the *rad50* and *mre11* mutants to bleomycin. (**A**) Wild-type and mutant plants were inoculated as six explants into quadrants of plates containing standard growth medium with or without bleomycin at 8 ngml^{-1}. For the mutant strains, each inoculum represents an independent disruption line. The photograph illustrates the extent of growth 10 days following inoculation of the explants. (**B**) Survival curves of wild type and *nbs1*, *mre11*, *rad50* and *rad51* mutant protoplasts regenerating after exposure to bleomycin treatment. Error bars indicate SD based on at least two independent experiments in all cases.

Acute treatment of intact protonemal tissue also adversely affected the subsequent growth rate of the recovering protonemata, with the *mre11* and *rad50* mutants being more sensitive to bleomycin than the wild type (Supplementary Figure S3C).

Mutator phenotypes in the absence of proteins essential for HR have been described in *S. cerevisiae* or *A. nidulans* (31,32). We therefore evaluated the mutator phenotype of the moss MRN mutants to assess their ability to repair endogenous DNA damage, using as reporter loss of function of the adenine phosphoribosyl transferase gene (*PpAPT*) as previously described (18). The frequencies of *apt* mutations were lower than 3×10^{-7} in wild type, *rad50* and *nbs1* mutants, was 4×10^{-7} for *mre11* but was ~100-fold higher (3.3×10^{-5}) in *msh2* mutants (Supplementary Table S3), which is in good accordance with previous results obtained with this mutant (18). These results indicate that loss of proteins of the MRN complex does not lead to a significant mutator phenotype in *P. patens*. It is most likely that DNA-DSB repair defects in the *mre11* and *rad50* mutants cause genomic damage so much more severe than the point mutations seen in the *msh2* mutant that cell death results.

DNA-DSB repair is not affected in mre11 and rad50 mutants

Gene targeting in the *mre11* and *rad50* mutants was severely impaired, while untargeted integration frequencies were 2-fold higher than those observed in WT. Since the *rad50* and *mre11* mutants were clearly impaired in growth and hypersensitive to DNA damage, we reasoned that the mutants remained capable of ligating broken ends of DNA molecules, but in an 'unsupervised', and therefore inaccurate manner. We tested this by directly estimating the ability of wild-type and mutant strains to repair DNA damage following acute exposure to bleomycin using single nucleus gel electrophoresis (the 'comet assay'). Treatment with bleomycin for 1 h resulted in a linear, dose-dependent fragmentation of genomic DNA in both wild-type and mutant lines, with the *rad50* and *mre11* lines exhibiting a greater susceptibility to DNA damage than the wild-type and *Ppnbs1* lines, respectively (Figure 5A). The rate of repair of DSBs was determined by measuring the proportion of fragmented DNA at intervals during a recovery period.

Both wild-type and mutant lines exhibited similarly high rates of DNA repair with a characteristic biphasic profile: an initial rapid phase ($t_{1/2}$ 1–4 min) accounting for ~60% of the fragmented DNA, followed by a slower phase ($t_{1/2}$ 7–90 min) accounting for the remainder (Figure 5B, Table 2). The rate of DNA repair was closely correlated with the age of the protonemal tissue following subculture. Tissue that was homogenized and subcultured for only 1 day comprised largely short protonemal fragments, four to seven cells in length. This tissue exhibited the most rapid repair kinetics (Figure 5B, Table 2). Tissue that was subcultured for 1 week comprised longer filaments 15–20 cells in length, whilst after subculture for 2 weeks, the filaments were over 30 cells long. These tissues were progressively slower in their DNA repair kinetics (Figure 5C, Table 2) with an increasing proportion of the DNA-DSBs being repaired with slow-phase kinetics. We ascribe these age-related differences to the relative representation of apical cells within the protonemal population. *Physcomitrella* protonemata grow by serial division of the apical cells, so that in a 1d-subcultured homogenate, we estimate the proportion of mitotically active apical cells to comprise 30–50% of the total cell population. This proportion will be 10–15% in 7d-subcultured tissue, and ~3% in 14d-sucbultured protonemata. Thus, the initial rapid phase of DNA repair can be accounted for by processes undertaken in the mitotically competent apical cells, whilst the slow-phase repair kinetics is likely due to processes carried out in mitotically inactive subapical cells.

Although differences can be seen in the rates of DNA repair between mutant and wild-type strains, these are not dramatic. Clearly, the extensive fragmentation of DNA that occurs during the initial bleomycin treatment is being rapidly reversed, even in mutants in which

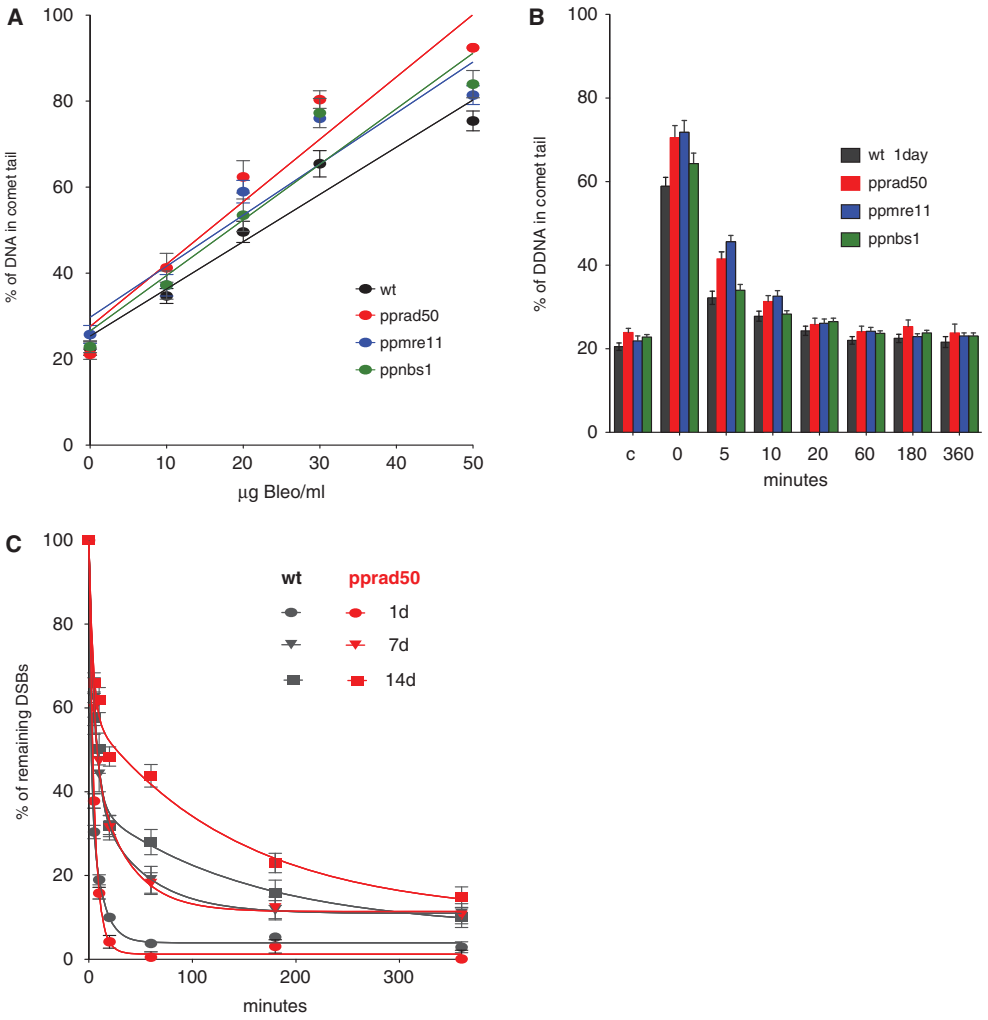

Figure 5. Kinetics of DNA repair in wild-type and mutant plants. (**A**) Bleomycin dose-response. Protonemal tissue from wild-type and mutant lines was treated with bleomycin for 1 h at the indicated concentrations, prior to nuclear extraction and the analysis of DNA damage by single-cell electrophoresis (the 'comet assay'). The extent of DNA damage is indicated by the proportion of DNA detected in the fragmented fraction (the 'comet tail'). The background level of genomic DNA damage in all lines is similar, at between 20 and 30%, indicating that the mutations have no significant effect on natural levels of DNA fragmentation. (**B**) Repair kinetics in 1-day regenerated protonemata. In both wild-type and mutant lines, the fragmentation of DNA induced by bleomycin is repaired with rapid kinetics ($t_{1/2}$ between 1 and 4 min). (**C**) Repair kinetics in relation to protonemal age. As protonemata are regenerated for longer periods (resulting in a concomitant reduction in the proportion of mitotically active apical cells), so the proportion of the rapid phase DNA repair declines. This occurs in both the wild-type and the *rad50KO* mutant lines.

Table 2. Kinetics of DNA repair in wild-type and mutant strains

Genotypes	Tissue age	$t_{1/2}$fast (min)	% fast	$t_{1/2}$ slow (min)
wild-type	1d	1.2	61.4	7.6
wild-type	7d	4.0	67.6	32.9
wild-type	14d	3.8	67.7	103.4
mre11KO	1d	4.1	96.5	84.1
nbs1KO	1d	1.9	84.1	17.0
rad50KO	1d	2.9	71.4	5.6
rad50KO	7d	2.9	52.6	18.2
rad50KO	14d	2.7	46.8	100.0

components of the principal DNA surveillance and repair system for both HR and NHEJ-mediated repair (PpMRE11 and PpRAD50) have been eliminated.

MRN mutants exhibit enhanced repair gene expression

One possibility is that in the absence of a viable MRN complex, DNA-DSBs are repaired, but in an 'unsupervised' manner. In the absence of the tethering function to hold broken ends in close proximity, repair may be

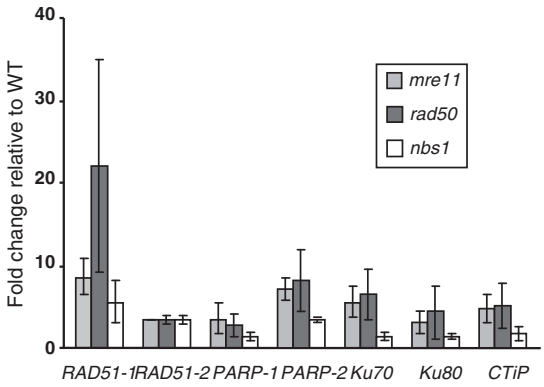

Figure 6. Gene expression analysis of DNA repair genes in the MRN mutants. Quantitative determination of the relative abundances of transcripts encoding DNA repair genes (*PpRad51-1*, *PpRad51-2*, *PpPARP-1*, *PpPARP-2*, *PpKu70*, *PpKu80* and *PpCtIP*) in 7-day-old wild-type, *Ppmre11KO*, *PpRad50KO* and *Ppnbs1KO* strains was done by quantitative real-time PCR. Relative transcript abundance was calculated using the ΔΔCt method and normalized to the wild-type value. Error bars indicate SD based on at least three independent experiments with two replicates for each sample in all cases.

inaccurate, generating increased numbers of deletions and promoting end joining between inappropriate ends, resulting in increased disruption of essential genes and consequent loss of viability and cell death. Analysis of the expression of a selection of DNA repair genes, implicated in HR, NHEJ and alternative end-joining processes, showed a significantly enhanced accumulation of repair gene transcripts in MRN mutants relative to wild-type, and this was most marked in the *Ppmre11KO* and *Pprad50KO* mutants (Figure 6). This suggests that in the absence of a functional MRN complex, the cell initiates an emergency response by the rest of the DNA repair machinery. The levels of induction of the repair genes observed in the mutant lines are comparable to those seen in wild-type protonemal tissue in response to DNA-DSB induction by bleomycin (Whitaker,J., personal communication).

We then directly tested the nature of DNA repair in an MRN mutant line, by screening a series of *apt* mutants generated by bleomycin treatment of both wild-type and the *Pprad50KO* mutant. Sublethal doses of bleomycin, determined by growth tests of bleomycin-treated protonemal tissue (Supplementary Figure S3C), were used to generate mutants selected on the basis of resistance to 2-FA. The mutability of the *PpAPT* gene in the *Pprad50KO* line was observed to be at least an order of magnitude greater than that in the wild type (Supplementary Table S2). The nature of the induced mutations was examined by PCR-amplification and sequencing of the *APT* gene from a number of lines. For wild-type, each mutant analysed contained only point mutations within the *APT* coding sequence and introns (Table 3). In contrast, three of the seven mutants analysed in the *Pprad50KO* background contained deletions, varying in length between 10 and 747 bp (Table 3). This supports our working hypothesis that MRN-unsupervised repair generates more severe forms of genomic damage.

DISCUSSION

GT Efficiency is reduced in the *Physcomitrella rad50* and *mre11* mutants

In *Physcomitrella* the protein at the heart of the HR pathway, PpRAD51, is required simultaneously to enable targeted integration by HR, and to repress untargeted insertion by an as yet unidentified molecular mechanism (17). The unique GT efficiency of *Physcomitrella* suggests that DNA DSBs are predominantly repaired by HR in moss cells. Our analysis of mutants in the principal sensor of DNA DSBs, the MRN complex, further shows that although a fully active MRN complex clearly appears to be necessary for high-efficiency targeted transgene integration, a background level of HR with gene targeting reduced to ~8.6% of wt is still maintained in the absence of either a functional PpMRE11 or PpRAD50 protein. This contrasts with the complete abolition of gene targeting seen in *rad51* null mutants, a component specific to the HR pathway (17). Noticeably the overall frequency of untargeted transgene integration is not reduced in the *mre11* and *rad50* mutants relative to wild type (Table 1), implying that whatever mechanisms undertake random transgene integration, these are relatively unimpaired in the absence of PpMRE11 or PpRAD50. Together with the observation that a number of DNA repair genes show enhanced expression levels in *mre11* and *rad50* mutants, our results suggest that while some HR-mediated repair may still operate in *mre11* and *rad50* mutants, the HR pathway is unlikely to account for the majority of the DNA-DSBs that are rapidly religated in these mutants.

NBS1 is not required for growth and development or for HR in *Physcomitrella*

Phenotypic analyses of mutants in the MRN complex in moss failed to identify a detectable difference between wild-type and *Ppnbs1* knock-outs. In eukaryotes, the MRN-complex proteins act as the 'gatekeepers' of the DNA-DSB response, directing the repair of DSBs into either the NHEJ or HR pathways through the activation of the ATM or ATR kinases that (in mammalian cells) are recruited to sites of DNA damage through analogous mechanisms involving conserved interaction motifs (6). The NBS1 protein is involved in the recruitment of ATM to DNA-DSBs and ATRIP is involved in the recruitment of ATR to single-stranded DNA (ssDNA). The recruitment of ATM is mediated by its direct interaction with NBS1 which becomes phosphorylated at residues conserved between the *Arabidopsis* and *Physcomitrella* NBS1 sequences (6,33). In *A. thaliana*, *nbs1*/*atm* double mutants appear additive in their negative consequences for growth and fertility relative to the wild-type and single mutants (34). ATM is necessary for the imposition of a cell-cycle checkpoint, and for the induction of DNA-damage-responsive gene expression in *Arabidopsis*, in which the principal DNA repair pathway is through NHEJ (33,35). DNA repair in *Physcomitrella* is believed

Table 3. Mutations identified in the *APT* genomic sequence in wild-type and *Pprad50-KO* 2-FA resistant clones

Clone#	Genotype	Mutations in CDS		Mutations in introns	
		Point mutations	Deletions[a]	Point mutations	Deletions[a]
1	*rad50/apt*	+ T (2095)[a]		+ T (1683)[a] Δ T (2517)[a]	–
2	*rad50/apt*	Δ T (1706)[a]	1450–1455	+ T (1683)[a]	–
3	*rad50/apt*	A to T (1461)[a] G to C (1534)[a]	1466–1521	+ T (1683)[a]	–
4	*rad50/apt*	T to C (1291)[a] C to G (1752)[a] Δ T (1730)[a]		Δ G (1524)[a] + T (1683)[a] + T (2517)[a]	–
5	*rad50/apt*	Δ G (1524)[a]	–	+ T (1683)[a]	–
7	*rad50/apt*	–	1050–1797	–	–
11	*RAD50/apt*	A to G (1491)[a] A to C (1498)[a]	–	Δ A (1052)[a] T to C (2330)[a] G to C (2327)[a] + GT (2328)[a]	–
12	*RAD50/apt*	–	–	Δ A (1052)[a] T to G (1376)[a] C to A (1569)[a] + G (2328)[a]	–
13	*RAD50/apt*	A to C (2475)[a] Δ T (2499)[a]	–	A to T (1591)[a]	–

[a]Position 1 corresponds to the first nucleotide in the genomic *PpAPT* sequence DQ117987.

to operate primarily via the HR pathway, which in mammalian cells, at least, depends principally on the activity of the ATR kinase. Thus, impairment of ATM-related signalling in the *Ppnbs1KO* mutant may have relatively little impact on growth and fertility, if NHEJ is subordinate to HR. This conclusion is also supported by the observation that HR-dependent gene targeting is unaffected in the *Ppnbs1KO* mutant. In contrast, NBS1 has been shown to be essential to HR in chicken DT40 cells, possibly by processing recombination intermediates (36) and in human cells recruitment of ATR to sites of DNA damage is dependent on ATM (37). This implies that in *Physcomitrella* NBS1 may not be involved in the production of single-stranded tails that are the substrates for HR and that induction of the HR pathway, potentially by the ATR signalling, is independent of ATM. In this respect *Physcomitrella* would more resemble budding yeast than mammals, as Tel1, the yeast equivalent of ATM, has only minor effects on end-processing and is not required for focus formation by Mec1, the yeast homolog of ATR (38,39). Alternatively, despite the conservation of the ATM interaction domain in the PpNBS1 protein, ATM activation might be independent of NBS1 in *Physcomitrella*. In this context, it would be of interest to study the exact roles of ATM and ATR in *Physcomitrella*.

RAD50 and MRE11 are essential for growth and development in *Physcomitrella*

Null mutants in any components of the MRN complex are lethal in vertebrates (5) and are severely compromised in both budding (40) and fission yeast (41). This is not the case in plants: in *Arabidopsis*, *AtRad50* and *AtMre11* mutants are impaired in growth, fertility and in their ability to recover from genotoxic stress (42,43), whereas *Atnbs1* mutant plants grow normally and are fully fertile but are sensitive to the DNA cross-linking agent, mitomycin C (34).

Our analyses show that defects in the MRN complex can adversely affect moss development. While the *Ppnbs1KO* mutant completed its life cycle normally and displayed wild-type levels of susceptibility to DNA damage, the *Ppmre11* and *Pprad50* strains displayed a strong and similar developmental phenotype. This included defects in cell viability (reduced protoplast regeneration rates), in cell-cycle progression and cell growth (reduced colony growth) and in the completion of a complex developmental programme (abortive leafy shoot development). Precocious arrest of colony growth was also observed on minimal medium, which most likely reflects early senescence. This pleiotropic phenotype is much stronger than that observed in the HR-deficient *Pprad51* mutants (16,17) and implies that genome integrity is more severely impaired by loss of function of the MRN complex than by the inactivation of the HR pathway. The phenotype of *Ppmre11* and *Pprad50* mutants also differs from that previously reported for *Ppmsh2* mutants which do not display a strong juvenile phenotype but accumulate mutations and phenotypic alterations during development (18). Noticeably both *Pprad51* and *Ppmsh2* mutants also displayed a detectable mutator phenotype that is absent in *Ppmre11* and *Pprad50*, probably because MRN mutants accumulate a more extensive and harmful type of DNA damage that accelerates senescence.

Induced DSBs in *Physcomitrella* can be repaired via a mechanism independent of the MRN complex

Direct analysis of DNA-DSB repair by single-cell electrophoresis showed little difference in the rate of repair of DNA-DSBs in the *mre11* and *rad50* mutants compared to

wild type. Whilst some HR-mediated transgene integration still occurs in *mre11* and *rad50* mutants, it is unlikely that single-strand annealing (SSA) or homologous strand exchange (HR), which require end-processing, account for this rapid religation. Therefore, the repair of DSBs in the *mre11* and *rad50* mutants probably occurs via a pathway related to NHEJ. However, the reduced growth and survival of these mutants indicate that such a pathway reduces the genetic stability characteristic of MRN-supervised DNA repair.

Two different NHEJ pathways have been already described, the highly efficient canonical Ku- and DNA ligase IV-mediated NHEJ pathway (C-NHEJ) in which most ends are successfully rejoined without alteration of DNA sequence information (44) and an evolutionarily conserved alternative end-joining pathway (A-NHEJ) (45) thought to proceed via microhomology-mediated end joining (MMEJ), even if the relationship between A-NHEJ and MMEJ is still unclear (45). A-NHEJ represents a major source of DSB-induced genome rearrangements (translocations, deletions and inversions) (46–48) and appears to utilize binding of DNA ends by PARP-1 (polyADP ribose polymerase) and ligation by DNA Ligase III in a Ku-independent process (49,50) and involve the interaction between the MRN complex and DNA ligase IIIα/XRCC1 (51). The function of DNA ligase III is absent in plants, being substituted by DNA ligase I in base-excision repair (52). It may therefore be significant that the *Ppmre11* and *Pprad50* mutants show substantially elevated levels of expression of PARP and other DNA-repair associated genes, relative to the wild type, and this elevated gene expression may be responsible for the activity of an A-NHEJ repair pathway in the absence of an active MRN complex. Existence of A-NHEJ in plants has been inferred from observations that although the frequency of transgene insertion was reduced in mutants deficient in NHEJ components such as *Atku80* and *AtligIV*, it was not abolished (53–55), from the observation of illegitimate fusions between chromosome arms in telomerase-deficient *Arabidopsis*, even in an *Atku80*/*Atmre11* mutant background (56), from the recent demonstration of rapid ligation of bleomycin-induced DNA-DSBs in the NHEJ-deficient *Atku80* and *Atlig4* mutants (57) and from kinetic measurements of assembly and processing of DSB-specific γ-H2AX complexes in *Arabidopsis* mutants deficient in core components of the C-NHEJ and A-NHEJ pathways (58). In budding yeast both C-NHEJ and A-NHEJ are MRX-dependent processes, with the exonuclease activity of Mre11 playing an important role (59–62), whilst in vertebrates varying roles for MRN complex components have been reported (63–66). Whatever the role of the MRN complex in C-NHEJ or A-NHEJ in plants, it is likely that an NHEJ-like pathway mediates the rapid DSB repair observed in *Physcomitrella mre11* and *rad50* mutants. However, because these mutants are clearly hypersensitive to DNA damage yet do not show a mutator phenotype, it would appear that whatever rejoining of DNA ends is occurring, it is 'unsupervised' and results in genomic perturbations so severe that cells suffering bleomycin-induced breakage soon die.

The rapid interaction of the MRN complex with DNA-DSBs is essential for their stabilization, through the tethering of the adjacent free ends by the Rad50 coiled-coil/zinc hook domains (5). By retaining broken ends in close proximity, the MRN complex thereby supervises the DNA repair process, ensuring that the correct ends are rejoined, and recruiting additional factors required for either NHEJ or HR-based repair. In the absence of such tethering, unsupervised end-joining by backup pathways might occur between unrelated DNA sequences, with the concomitant accumulation of cytotoxic mutations accounting for the reduced rates of growth and enhanced sensitivity to DNA-damaging agents observed in the *Ppmre11* and *Pprad50* mutants. Combinations of mutations affecting C- or A-NHEJ (58) with *mre11* or *rad50* mutations should give us insight into the mechanism behind this DSB DNA repair.

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SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3, Supplementary Figures 1–4.

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DNA repair in plants studied by comet assay

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EVENT ABSTRACT

Back to Event

DNA repair in plants studied by comet assay

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Comet assay in plants.

From the first description of the comet assay with isolated nuclei rather than whole cells it became evident that assay is well suited to be applied in plants (Koppen & Verschaeve, 1996). Disintegration of tissue by quick chopping with a razor blade, direct collection of released nuclei by patting and pipetting enables to process samples in time shorter than 2 minutes, the time prerequisite to study quick repair (Kozak et al, 2009).
Plants are due to their sessile nature permanently exposed to environmental stresses (drought, salinity), ionizing (IR) and UV radiation and genotoxins, which directly or indirectly via generation of reactive oxidative species (ROS) damage their DNA. Radiomimetic Bleomycin functions as a catalyst to produce ROS leading to clusters of oxidized DNA lesions, single (SSB) and double (DSBs) strand breaks similarly as IR (Steighner & Povirk, 1990). Incurred SSBs and DSBs are easily distinguished and measured by comet assay when varying conditions of mainly used protocol with electrophoresis in 0.3 M NaOH, pH>13 solution (A/A assay). DSBs are detected under "neutral" conditions by N/N assay in regular electrophoretic buffer (Kozak et al, 2009; Olive & Banath, 2006), whilst SSBs are revealed by A/N assay, with alkali-unwinding step in 0.3 M NaOH prior electrophoresis (Angelis et al, 1999; Menke et al, 2001). Better resolution in DSBs and SSBs assays is observed when "neutral" conditions are set between pH 9-10, still well bellow DNA denaturing pH<11.6 (Bradley & Kohn, 1979).

Repair of DSBs.

DSBs are one of the most cytotoxic forms of DNA damage that must be repaired by recombination, predominantly via non-homologous joining of DNA ends (NHEJ) in higher eukaryotes. However, analysis of DSB repair kinetics of Arabidopsis NHEJ mutants atlig4 and atku80 with the N/N assay showed that repair of Bleomycin induced DSB is biphasic and rapid alternative repair pathways is active (Fig. 1). Surprisingly, kinetic measurements showed that rapid DSB repair was faster in the NHEJ mutant lines (t1/2 5.5 min.) than in wild-type Arabidopsis (t1/2 7.9 min.). Kozák et al. (Kozak et al, 2009) provided the first characterization of this alternate KU/LIG4-independent repair pathway that rapidly removes the majority of DSBs present in nuclear DNA and found its dependence on components of structural maintenance of chromosomes (SMC) complexes, namely SMC6b (MIM) of SMC5/6 complex, kleisin AtRAD21.1 and 3 of cohesin SMC1/3 complex (da Costa-Nunes et al, 2014) and structurally related SMCHD protein GMI1 (Bohmdorfer et al, 2011).
Moreno-Romero et al. (Moreno-Romero et al, 2012) studied CK2 protein required for maintenance and control of genomic stability and used N/N assay to show that DSBs were more rapidly repaired in atck2 mutant than in control plants. Their results suggest that atck2 plants are more proficient in repairing DSBs and other lesions produced by IR or Bleomycin, despite their hypersensitivity to these agents.
The moss Physcomitrella patens is unique besides the high frequency of homologous recombination for haploid state and filamentous growth during early stages of the vegetative growth. Sheared filaments enables to establish protonemal cultures with up to 50% dividing cells and makes Physcomitrella an excellent model plant to study DNA damage responses.
Kamisugi et al. (Kamisugi et al, 2012) used N/N assay to study Physcomitrella mutants of pivotal DSB repair MRN complex (MRE11, RAD50, NBS1). Kinetics of DNA-DSB repair in wild-type and mutant plants revealed that Bleomycin-induced fragmentation of genomic DNA was quickly repaired at approximately equal rates (t1/2 3 min) in each genotype, although both, the ppmre11 and pprad50 mutants exhibited severely restricted growth and development and enhanced sensitivity to UV-B and Bleomycin-induced DNA damage. This implies that while extensive DNA repair can occur in the absence of a functional MRN complex; it is unsupervised in nature and results in the accumulation of deleterious mutations incompatible with normal growth and development, the similar phenomenon as observed by Moreno-Romero et al. (Moreno-Romero et al, 2012) in atck2 plants. When MRN complex and CK2, both assumed to be associated with error-free homologous recombination are disabled, then NHEJ error-prone repair prevails at a cost of induced mutations in continuous DNA. And indeed this was proved by sequencing of mutated APT locus in which we identified deletions of various lengths as could be expected as an outcome of NHEJ repair mechanism.
Combination of comet and mutation assays explained contradictory observation of rapid DSB repair in mutants with phenotype sensitive to induction of DSBs. Evidently plants evolved this attitude of rapid reconstitution of genome integrity from the early evolution stages (mosses were one of the first plants to colonize land) to higher eukaryote seed plants, where during every plant cycle embryos undergo severe DNA damage during seed maturation (generated by ROS released upon desiccation) and efficient recovery during seed germination.

Repair of SSBs.

Induction of DSB in not an isolated event and depends on inducing agent. Often DSB is a result of clustered damage induced e.g. by impact of radiation beam on DNA or by interaction of agents like Bleomycin with DNA.
Holá et al. (Hola et al, 2013) used N/N and A/N assays to find out whether the repair kinetics of Physcomitrella wt and ppliga4 differ in response to Bleomycin-induced DSBs and SSBs. In both lines, the Bleomycin induced DSBs are rapidly repaired with a biphasic kinetic (Fig. 2A) and the half-lives of DSBs survival are similar to half-lives of other Physcomitrella mutants mentioned above. Contrary to DSBs, SSBs are repaired far less efficiently. Slow SSB repair might be common feature of plants since Donà et al. (Donà et al, 2013) using N/N and A/A assays described similar phenomenon in Medicago truncata cells irradiated with □□-ray. The SSB repair kinetic in wild type Physcomitrella is clearly biphasic and in this respect parallels repair of MMS induced SSBs in wild type Arabidopsis (Waterworth et al, 2009). In contrast to DSBs, substantially smaller fraction of SSBs is repaired with fast kinetics; the defect even more manifested in ppliga4. It suggests an important role for LIG4 in the repair of DNA lesions like modified basis, AP sites that are usually detected as SSBs and are repaired via base excision repair (BER). It is noteworthy that LIG3 is not represented in plants and as showed earlier (Waterworth et al, 2009) principal substitute for LIG3 in BER is LIG1. Because LIG1 is essential for cell viability, atlig1 was generated as an RNAi line with 40% of remaining LIG1 activity. SSBs repair course in atlig1 is a consequence of unbalanced BER due to attenuated ligation step leading to accumulation of breaks during early stages of recovery followed by their later gradual removal. Evidently the knockout mutation in ppliga4 does not have such severe effect on repair of SSBs; nevertheless, the SSB repair defect of ppliga4 clearly manifests that LIG4 is also involved in the repair of SSBs in plants. Effect of break accumulation during early stages recovery is not unique to LIG mutants. Similar effect is depicted on Fig. 2B in Arabidopsis plants with PARP1 impaired either by knockout mutation leading to atparp1 or by two inhibitors, the selective PARP1 inhibitor AG14361 devised by Pfizer for sensitization of cancer cells prior irradiation treatment or unspecific PARP inhibitor 3-aminobenzamide (3-ABA). It is interesting to point out the conservation of PARP system between kingdoms, since selective AG14361 inhibitor of HsPARP1 is also effective in Arabidopsis and provides same repair phenotype as knockout AtPARP1 mutation.

Conclusions

Plant comet assay proved to be a reliable method for studies of damage and the repair of genomic DNA, like in mammals and humans also in plants. Rapid isolation of nuclei from virtually any plant tissue enables to study rapid response of plant cells to DNA damage at virtually any conditions. Comet assay is now successfully applied for basic and applied research from lower (algae, moss) to higher eukaryotic crop plants (rice, wheat). However, even in time of contemporary boom of indirect methods to study repair, direct, unbiased measurement of the integrity of genomic DNA remains beloved domain of the comet assay over counting of γH2AX foci, whose formation depends anyway on phosphorylation signaling cascade.

Fig. 1. Kinetic of DSB repair in Arabidopsis. Fractions of remaining DSBs were calculated for 0, 5, 10, 20 and 60 min repair time points after the treatment with 30 μg/mL Bleomycin. Maximum damage is normalized as 100% at t = 0 for all lines. Wild type Col0 and atlig4 rapidly repair induced DSBs during the first 10 min, while atlig4 has even faster repair rates than wild type. Contrary to wild type, atlig1 and atsmc6b have clearly slower initial DSB repair, with a striking repair defect in SMC6B mutant. Adopted from (Kozak et al, 2009).

Fig. 2A. SSB and DSB repair kinetics in Physcomitrella. SSBs were induced in Physcomitrella protonemata with 50% of dividing cells by 1 hr treatment with 2 □□g/mL Bleomycin; bright blue: wild type, dark blue: ppliga4, and determined by A/N assay. DSBs were induced in the same tissue by 1 hr treatment with 30 □□g/mL Bleomycin, green: wild type, orange: ppliga4, and determined by N/N assay. Repair kinetics is plotted as % of DSBs remaining over the 0, 5, 10, 20, 60, 180, and 360 min recovery period. Maximum damage is normalized as 100% at □□ = 0 for all lines. Adopted from (Hola et al, 2013).
Fig. 2B. Effect of mutation and of inhibitors of PARP1 on SSB repair kinetics. SSBs induced by 1 hr treatment with 2 mM MMS in atparp1 (red) and in Arabidopsis wt in presence of 3 mM 3-aminobenzamide (3-ABA, turquoise) and 10 μM HsPARP1 specific AG14361 (green) inhibitors. (Angelis and Kozák, unpublished data)

Figure 1

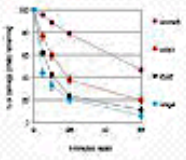
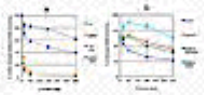


Figure 2



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7. Diskuse

O důležitosti reparačních mechanismů svědčí fakt, že u řady organismů jsou mutace v genech reparačních drah letální, nebo vedou ke vzniku závažných defektů ve vývoji, fertilitě a ke snížené vitalitě. U živočichů často vede inaktivace genů pro reparační proteiny k letalitě již během embryogeneze, což stěžuje charakterizaci reparačního mechanismu.

Oproti tomu mutanti v reparačních genech u rostlin bývají většinou životaschopní a fertilní. To může souviset se specifickými vlastnostmi rostlin, která je nutí udržovat vysokou míru genomové stability. Mezi tyto vlastnosti patří mj. absence zárodečné linie a vznik gamet diferenciací somatických buněk, nebo jejich způsob života vázaný na jedno místo, během něhož jsou vystaveny různým a často extrémním stresům životního prostředí jako např. zvýšená radiace, sucho, opakovaná dehydratace/hydratace, zvýšená koncentrace soli včetně těžkých kovů ap. Proto rostliny potřebují účinné a robustní reparační mechanismy, které zajistí stabilitu a integritu DNA i v případě rozsáhlého poškození genomu. Potřeba zachování stability a integrity genomové DNA může převážit nad nutností zachování přesnosti genetické informace. Důsledkem toho je, že použité reparační dráhy mohou být náchylné k tvorbě chyb (error-prone) a vnášet do genomu mutace.

Možnost přípravy životaschopných mutant reparačních genů usnadňuje studium jejich účasti a způsobu zapojení do reparace. Cenným nástrojem pro získání konkrétního mutanta je cílené vyřazení genu – tzv. genový knockout. U vyšších eukaryot včetně rostlin se transformovaná DNA integruje převážně nehomologním způsoben, nezávisle na sekvenci. Přestože bylo vynaloženo značné úsilí, všechny pokusy o vytvoření efektivní techniky cíleného vyřazování genů u kvetoucích rostlin se ukázaly jako málo účinné (Puchta, 2002). Oproti tomu u *Physcomitrelly* se díky vysoké frekvenci homologní rekombinace integruje vnášená DNA převážně homologním způsobem a účinnost cíleného vyřazování genů je tedy vysoká (Kamisugi et al. 2006).

Transformace *Physcomitrelly* je rutinně prováděna PEG zprostředkovanou transformací protoplastů (Hohe et al. 2004). Tento způsob transformace je vysoce účinný, nicméně je časově a navíc i technicky náročný a vyžaduje značnou manipulaci s rostlinným materiálem což zvyšuje riziko kontaminace. Alternativou je transformace biolistickou metodou, která je rychlejší, snazší a riziko

kontaminace transformovaného materiálu je elimininováno díky minimální manipulaci s rostlinným pletivem (Šmídková et al. 2010). Limitujícím faktorem širšího používání této metody je pořízení speciálního vybavení tzv. genové zbraně (Gene gun).

Biolisticky je transformována sedmidenní kultura protonemy, která je po transformaci homogenizována na krátké fragmenty filament a vysazena na médium se selekcí. Úspěšně transformované buňky se dělí a rostou jako protonemální filamenta bez podpory růstu rostlinnými hormony či indukce kalogeneze.

Díky haploidnímu stádiu protonemy, lze snadno identifikovat mutanty vznikající cílenou i náhodnou mutagenezí, protože změna nebo vyřazení genu se projeví ihned ve fenotypu.

U mutant reparačních genů se obvykle zjišťuje citlivost vůči genotoxinům. U linií *Physcomitrelly* mutantních v genech pro proteiny reparace DSB bylo srovnání citlivosti fenotypu stanoveno jejich schopností přežívat na médiu se vzrůstající koncentrací Bleomycinu (Kamisugi et al. 2012; Holá et al. 2013). Hypersenzitivita vůči Bleomycinu byla pozorována u mutant *pprad50* a *ppmre11* komplexu MRN (Kamisugi et al. 2012). Jelikož MRN komplex je klíčovou složkou reparace DSB, předpokládalo se, že citlivost vůči Bleomycinu je způsobena sníženou schopností mutant opravovat DSB.

Proto byl studován vliv mutace na kinetiku reparace přímým stanovením přítomných DSB metodou kometového testu. Kometový test na rozdíl např. od často používaného nepřímého stanovení DSB v savčích buňkách, založeném na imunofluorescenční detekci γH2AX, monitoruje přímé fyzické poškození DNA. Rychlá izolace buněčných jader prakticky z jakéhokoli rostlinného materiálu po působení genotoxinů umožňuje zachycení a studium rychlé reparace (Kozak et al. 2009). Kometový test je volbou podmínek testu vhodný nejen pro studium reparace DSB, ale po úpravě experimentálních podmínek i pro studium kinetiky opravy SSB a specifických lézí jako CPD (Angelis et al. 2015; Holá et al. 2013; Holá et al. 2015).

Studium kinetiky reparace DSB u mutant *pprad50* a *ppmre11* ukázalo, že přes jejich citlivost k Bleomycinu, u nich reparace DSB probíhá stejně rychle a efektivně jako u wt (Kamisugi et al. 2012). Na základě tohoto zjištění byla zformulována hypotéza, že citlivost mutant není důsledkem absence reparace DSB,

ale naopak přítomností účinné reparační dráhy, která je podle výsledku kometového testu schopná rychle a účinně DSB odstranit, nicméně za cenu vzniku závažných mutací. Důsledkem vzniku takových mutací, zejména vznikají-li v životně důležitých genech, je pozorovaný citlivý fenotyp.

Pro potvrzení této hypotézy bylo zjišťováno, jaký druh mutací vzniká u wt, *pprad50* a *ppmre11* po působení Bleomycinu v přirozeně se vyskytujícím *APT* genu. Mutace v *APT* vede ke vzniku rezistence k 2–FA (Gaillard et al. 1998), což umožňuje snadnou selekci *apt* mutantů a charakterizaci mutací sekvenováním *APT* lokusu.

Zatímco u wt 2–FA rezistentních mutant byly nalezeny převážně bodové mutace – substituce a 1 bázové inzerce, u *pprad50* a *ppmre11* 2–FA rezistentních mutantů byly kromě bodových mutací objeveny i rozsáhlé delece několika desítek až stovek bází (Kamisugi et al. 2012; Holá et al. 2013). Kumulace těchto závažných mutací v celém genomu je příčinou hypersenzitivního fenotypu *pprad50* a *ppmre11* vůči Bleomycinu.

Studium bylo později rozšířeno na linii mechu mutantní v genu pro LIG4, klíčový protein reparace dvouvláknových zlomů DNA C-NHEJ mechanismem. U *pplig4* 2–FA rezistentních mutantů nebyly nalezeny žádné rozsáhlé delece, většina nalezených mutací byly inzerce 1 báze (Holá et al. 2013). Kinetika opravy DSB neukázala, stejně jako u *pprad50* a *ppmre11,* výraznou odchylku od wt. Nicméně Bleomycin indukuje vznik nejen DSB, ale i SSB, oxidativního poškození bází a vzniku AP míst. Tato poškození jsou v kometovém testu detekována jako SSB. Kinetika reparace SSB ukázala, že oprava probíhá u *pplig4* výrazně pomaleji než u wt (Holá et al. 2013). Tento typ poškození je přednostně opravován drahou excisní reparace bází. V savčích buňkách tato dráha zahrnuje LIG3, která se ale u rostlin nevyskytuje a u *Arabidopsis* je principiálně zastoupena LIG1 (Waterworth et al. 2009). Defekt v reparaci jednovláknových zlomů u *pplig4* ukazuje, že na opravě mechanismem BER se u *Physcomitrelly* významně podílí i LIG4.

BER odstraňuje přednostně poškození, která nezpůsobují výraznou deformaci dvoušroubovice. Objemné léze, způsobující distorzi dvoušroubovice jsou odstraňovány zejména nukleotidovou excisní reparací. Typickými objemnými lézemi jsou cyklobutan pyrimidinové dimery a 6‘-4’ pyrimidin-pyrimidon fotoprodukty vznikající působením UV záření. Pro zjištění, zda by se některý ze studovaných proteinů mohl nějakým způsobem podílet na odstraňování tohoto

typu poškození, byl u jednotlivých linií *Physcomitrella* indukován vznik poškození působením UV záření na jednodenní kulturu mechu. Jednodenní protonema obsahuje až 50% dělících se apikálních buněk, a představuje tak dělící se pletivo, které se u vyšších rostlin nachází pouze ve velmi omezeném množství ve vrcholových meristémech.

U rostlin jsou fotoprodukty odstraňovány světlem aktivovanými fotolyázami a v nepřítomnosti aktivních fotolyáz mechanismem NER. Obě tyto dráhy jsou bezchybné a nevedou ke vzniku mutací. UV záření je ale obecně velmi mutagenní. Podle frekvence vzniku *apt* mutantů se zdá, že mutagenita UV záření je dokonce vyšší než mutagenita BLM indukujícího vznik DSB. Ozáření jednodenní protonemy indukuje u většiny linií mechu vznik mnohem většího počtu *apt* mutant než působení Bleomycinu (Holá et al. 2015).

Kinetika opravy CPD ukazuje, že pyrimidinové dimery jsou v nepřítomnosti světla odstraňovány poměrně pomalu. Polovina indukovaných dimerů je odstraněna po více než 4 hodinách (Holá et al. 2015). Ve všech UV indukovaných *apt* mutantech byly nalezeny převážně tranzice cytosinu na tymin. Pomalá reparace umožňuje uplatnění dalších, potencionálně mutagenních, mechanismů opravy či tolerance poškození. CPD blokují replikační komplex a mohou způsobit kolaps replikační vidličky a vznik dvouvláknového zlomu (Kaufmann a Cleaver 1981; Petermann et al. 2010). Aby se této situaci vyhnuly, využívají buňky mechanismus syntézy přes poškození, který jim dovoluje poškození DNA překonat. Jedná se ale o nepřesný mechanismu, který vede často ke vzniku mutací, čímž je dána vysoká mutagenita UV záření.

8. Závěr

Disertační práce se zabývá zavedením nového modelového organismu mechu *Physcomitrella patens*, který se díky kombinaci unikátních vlastností *Physcomitrelly* ukázal jako velmi vhodný model pro studium reparace DNA. Bylo možné ukázat, že:

- Po vyřazení „error-free“ HR je výsledný, k indukci DSB hypersenzitivní fenotyp mutantů *ppmre11* a *pprad50*, důsledkem rychlé a efektivní opravy DSB, která ale vede ke vzniku závažných mutací v celém genomu.
- LIG4 - klíčový protein NHEJ reparační dráhy, je u *Physcomitrelly* významný také pro reparaci objemově malých poškození mechanismem BER.
- Vysoká mutagenita UVB záření je dána zejména vznikem mutací „error-prone“ syntézou DNA přes neodstraněné CPD a 6-4PP během replikace DNA v dělících se buňkách.

Možnost využití a kombinace různých metod u mechu *Physcomitrella patens* potvrzuje, že *Physcomitrella* je skutečně jedním z nejvhodnějších rostlinných modelů pro studium reparace DNA.

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