UNIVERSITY OF SILESIA
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Wydział Nauk Przyrodniczych

Instytut Biologii, Biotechnologii i Ochrony Środowiska

ADRIANNA HALAMA

Praca doktorska

Badania zależności metylacji DNA oraz uszkodzeń i naprawy DNA w komórkach *Brachypodium distachyon*

Promotor:

dr hab. Jolanta Kwaśniewska, prof. UŚ
Uniwersytet Śląski w Katowicach

Promotor pomocniczy:

dr Agnieszka Brąszewska
Uniwersytet Śląski w Katowicach

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SPIS TREŚCI

I. AUTOREFERAT

1. WSTĘP

Chromatyna w komórkach organizmów eukariotycznych występuje w dwóch formach upakowania, które mają związek z jej aktywnością transkrypcyjną: luźno upakowanej euchromatyny – transkrypcyjnie aktywnej oraz nieaktywnej transkrypcyjnie, silnie skondensowanej heterochromatyny (Saze i inni, 2012; Thiebaut i inni, 2019). Struktura chromatyny jest dynamiczną konfiguracją, na którą istotny wpływ mają modyfikacje epigenetyczne, w tym metylacja DNA. Proces ten polega na kowalencyjnym dodaniu grupy metylowej do piątego węgla pierścienia pirymidynowego cytozyny i jest katalizowany przez enzymy metylotransferazy, z użyciem S-adenozylometioniny, jako donora grupy metylowej (Van Holde i inni, 1995; Kapazoglou i Tsaftaris, 2011).

Organizmy roślinne są stale narażone na różne stresy środowiskowe, w tym suszę, temperaturę, metale ciężkie i wiele różnych czynników o działaniu mutagennym. Oddziaływanie stresów środowiskowych doprowadziło do wykształcenia odpowiedzi obronnych w genomie jądrowym, opartych na mechanizmach molekularnych (Boyko i Kovalchuk, 2008). Przykładem takich zmian są hipermetylacja lub hipometylacja DNA, które mogą prowadzić do zmian w ekspresji genów (Chinnusamy i Zhu, 2009). Analiza dynamicznego charakteru zmian jądrowych, jak organizacja chromatyny i modyfikacje epigenetyczne, jest nie tylko przedmiotem badań odpowiedzi komórek roślinnych na czynniki środowiskowe (Song i inni, 2021), ale również dynamicznie rozwijającą się dziedziną mutagenezy (Kim, 2019).

Działanie czynników mutagennych na komórki eukariotyczne można obserwować na różnych poziomach organizacji genomu: genów, chromosomów (efekt klastogenny), oraz całego organizmu (Jovtchev i inni, 2002). Do wykrywania klastogennych efektów działania mutagenów powszechnie wykorzystywane są testy cytogenetyczne (**Kwasniewska i Bara, 2022)[2]**. Wśród testów cytogenetycznych szczególnym zainteresowaniem cieszy się test mikrojąder, który umożliwia nie tylko wykrywanie, ale i ilościową ocenę efektów działania mutagenów. Po raz pierwszy

został on zastosowany w badaniach mutagennego wpływu promieniowania gamma na komórki merystemu korzeniowego *Vicia faba* (Evans i inni, 1959). Obecnie nadal jest on wykorzystywany w badaniach genotoksycznego wpływu czynników środowiskowych, takich jak pestycydy, węglowodory aromatyczne, metale ciężkie, promieniowanie jonizujące, czy chemikalia przemysłowe, na genom jądrowy roślin (Chen i inni, 2019; Oubane i inni, 2020; Klein i inni, 2021).

Mikrojądra (MN) to niewielkie, sferyczne struktury zlokalizowane w pobliżu jądra interfazowego. Mogą one powstać z acentrycznego fragmentu lub fragmentów jednego lub kilku chromosomów, powstałych w wyniku dwuniciowych pęknięć DNA, które nie zostały naprawione, bądź są naprawione błędnie. MN mogą również powstać z całych opóźnionych chromosomów, w wyniku depolimeryzującego działania różnych czynników na mikrotubule – efekt aneugeniczny (Terradas i inni, 2010; **Kwasniewska i Bara, 2022[2]**; Souguir i inni, 2022). Niektóre mikrojądra mogą również pochodzić z przerwania mostów anafazowych, powstałych z chromosomów dicentrycznych, połączonych chromosomów pierścieniowych lub chromatyd siostrzanych, oraz chromosomów, które połączyły się przez fuzję telomerów (Hintzsche i inni, 2017). Najczęściej w pojedynczej komórce występuje tylko jedno MN, ale obserwuje się również komórki o większej liczbie MN, w zależności od liczby fragmentów chromosomów lub chromosomów opóźnionych. Biorąc pod uwagę mechanizmy powstawania mikrojąder, częstość dzielących się komórek po traktowaniu mutagennym wpływa na częstość powstawania mikrojąder. Komórki muszą się podzielić, aby fragmenty chromosomów, lub całe chromosomy mogły zostać wyeliminowane poza nowo utworzone jądra potomne i utworzyć MN. Wyniki badań przeprowadzone w komórkach zwierząt i człowieka wskazują, że mikrojądra mogą zostać zarówno utracone z komórek, jak i włączone ponownie do jądra komórkowego (Hintzsche i inni, 2017). Brak jest natomiast wyników badań dotyczących losu mikrojąder w komórkach roślinnych.

Test mikrojąder jest prostszy i mniej czasochłonny, w porównaniu z badaniem genotoksycznych efektów czynników mutagennych z wykorzystaniem testów aberracji chromosomowych, przeprowadzanych w komórkach dzielących się podczas mitozy. Czynniki mutagenne często wpływają na obniżenie aktywności mitotycznej

komórek, co stanowi utrudnienie w wykonywaniu testu aberracji chromosomowych, a nie jest przeszkodą podczas wykrywania mikrojąder w komórkach niedzielących się (Jaskowiak i inni, 2020). Najczęściej test mikrojąder wykonuje się w komórkach merystemów korzeniowych *Vicia faba* i *Allium cepa* lub w komórkach mejotycznych (w stadium tetrady) gatunku *Tradescantia* (Mišík i inni, 2011). Wielkość mikrojąder może być różna – od dużych po niewielkie, trudne do wykrycia (‘dot-like’). Przypuszczano, że na podstawie wielkości mikrojąder można rozróżnić klastogenny i aneugeniczny efekt działania czynników mutagennych na genom *Allium cepa*, ze względu na symetryczny i jednorodny pod względem wielkości chromosomów kariotyp. Zatem duże mikrojądro mogłoby wskazywać na jego pochodzenie z całego chromosomu, a małe mikrojądro z fragmentu(ów) chromosomu(ów) (Leme i inni, 2008). Jednak wielkość mikrojądra może być związana z różnym stopniem kondensacji chromatyny fragmentu bądź całego chromosomu tworzącego mikrojądro. Wyjaśnienie pochodzenia mikrojąder wymaga zastosowania testu mikrojąder z zaawansowanymi technikami cytogenetycznymi jak fluorescencyjna hybrydyzacja *in situ* (FISH) (**Kwasniewska i Bara, 2022)[2].**

Badania dotyczące pochodzenia mikrojąder w komórkach ludzkich i zwierzęcych są podejmowane stosunkowo często, natomiast wiedza o mikrojądrach u roślin jest wciąż ograniczona. Praca **Kwasniewska i Bara (2022)[2]** stanowi podsumowanie dotychczasowej wiedzy o mikrojądrach w komórkach roślinnych. Do wykrywania mikrojąder początkowo wykorzystywano tylko klasyczne techniki barwienia chromosomów, jak metoda Feulgena, barwienia acetoorceiną i Giemsą (Weihao, 2017). Metody te wciąż cieszą się dużym zainteresowaniem w standardowej ocenie efektów działania czynników środowiskowych i mutagenów, polegającej na szacowaniu częstości jąder komórkowych z mikrojądrami (Gustavino i inni, 2016; Aguelmous i inni, 2020; Schiavo i inni, 2020). Obecnie coraz częściej stosuje się wykrywanie mikrojąder z zastosowaniem fluorescencyjnych metod barwienia chromosomów, takich jak barwienie DAPI (4’6-diamidino-2-phenylindole) czy oranżem akrydyny (Kus i inni, 2019; Klein i inni, 2021). Te szybkie metody barwienia chromosomów umożliwiają bardziej precyzyjne wykrywanie mikrojąder, a ponadto możliwa jest analiza mikrojąder o bardzo małej wielkości, w szczególności

u gatunków charakteryzujących się małym genomem jądrowym. Zastosowanie fluorochromów do wykrywania mikrojąder nie dostarcza informacji o ich pochodzeniu (Dias i inni, 2005).

Brachypodium distachyon jest gatunkiem modelowym dla zbóż klimatu umiarkowanego. Gatunek ten charakteryzuje się wieloma korzystnymi cechami, takimi jak mały, w pełni zsekwencjonowany genom, krótki cykl życiowy, niewielki rozmiar roślin, czy łatwość w uprawie. Posiada on także niewielką liczbę zróżnicowanych morfologicznie chromosomów (2n=10), o stosunkowo niewielkich rozmiarach - od 1,3 do 3,6 μm (Lusinska i inni, 2018), podczas gdy średni rozmiar chromosomów *Allium cepa* waha się w granicach 4,91 – 7,61 μm (Lima i inni, 2019). Pomimo, że niewielkie rozmiary chromosomów *B. distachyon* nie są dogodną cechą w badaniach aberracji chromosomowych i mikrojąder, to zastosowanie testu mikrojąder wraz z techniką FISH (fluorescence *in situ* hybridization) otworzyło nowe możliwości badania mikrojąder (Kus i inni, 2017). Ze względu na niewielki, w pełni zsekwencjonowany genom *B. distachyon* (~355 Mbp), możliwe było utworzenie bogatej biblioteki genomowego DNA w klonach BAC (ang. *bacterial artificial chromosome*) (Ozdemir i inni, 2008; Kus i inni, 2019). Stworzyło to możliwość wykorzystania określonych sekwencji DNA jako sond w technice malowania chromosomów (ang. *chromosome painting*, CP). Pozwoliło to na zidentyfikowanie określonych chromosomów lub ich fragmentów, wchodzących w skład mikrojąder, a tym samym zrozumienie ich pochodzenia i poznanie wrażliwości wybranych sekwencji DNA, poszczególnych chromosomów lub ich regionów na działanie mutagenów (Kus i inni, 2018). W badaniach komórek roślinnych szerokie zastosowanie w technice FISH jako sondy znalazły głównie wysoko powtarzalne sekwencje DNA, takie jak rDNA (rybosomalne DNA), sekwencje centromerowe i telomerowe. Badania pochodzenia mikrojąder z wykorzystaniem sond rDNA z zastosowaniem FISH przeprowadzono między innymi u takich gatunków roślin, jak *Hordeum vulgare* (Jovtchev i inni, 2002; Juchimiuk i inni, 2007; Juchimiuk-Kwasniewska i inni, 2011) czy *B. distachyon* (Kus i inni, 2017). Technika ‘multicolor FISH’ (mcFISH), poprzez użycie kilku sond skoniugowanych z różnymi fluorochromami, pozwala określić lokalizację kilku konkretnych sekwencji DNA

w chromosomach, jądrach interfazowych, a także w mikrojądrach (Maierhofer i inni, 2002; Juchimiuk i inni, 2007). mcFISH jest często wykorzystywany w badaniach cytogenetycznych u ssaków, w tym człowieka (Marshall i inni, 1996; Natarajan i inni, 2003), ale ma również szerokie zastosowanie w badaniach komórek roślinnych (Hasterok i inni, 2002). Dzięki wykorzystaniu mcFISH z sondami telomerowymi, centromerowymi oraz 5S i 25S rDNA możliwe było poznanie pochodzenia mikrojąder u *Hordeum vulgare*, indukowanych hydrazydem kwasu maleinowego (MH) i N-nitrozo-N-metylomocznikiem (MNU) (Juchimiuk i inni, 2007). Technika ta umożliwiła również szczegółowe badania udziału poszczególnych chromosomów w tworzeniu mostów anafazowych u mutantów *Arabidopisis thaliana*, dzięki zastosowaniu klonów BAC specyficznych dla poszczególnych chromosomów (Puizina i inni, 2004). Wcześniejsze badania wykazały, że rozkład aberracji chromosomowych w jądrze komórkowym nie jest przypadkowy, a także że niektóre chromosomy bądź ich fragmenty preferencyjnie biorą udział w tworzeniu mikrojąder, powstałych w wyniku pęknięć DNA (Fimognari i inni, 1997; Leach i inni, 2001; Chung i inni, 2002; Norppa i Falck, 2003). Wykazano, że regiony NOR są często zaangażowane w tworzenie mikrojąder u niektórych gatunków roślin (Huang i inni, 2008), a także u człowieka (Tchurikov i inni, 2015). Zastosowanie sekwencji centromerowych, telomerowych oraz sekwencji rDNA jako sond do mcFISH u *B. distachyon* pozwoliło wykazać, że chromosomy Bd4 zawierające 5S rDNA są częściej zaangażowane w tworzenie mikrojąder, niż chromosomy Bd5 zawierające 35S rDNA. Ponadto wykazano, że mikrojądra są tworzone najczęściej z acentrycznych fragmentów chromosomów, oraz że dystalne części chromosomów są częściej zaangażowane w tworzenie mikrojąder niż części proksymalne (Kus i inni, 2017). W związku z wykazaniem, że regiony NOR są często zaangażowane w tworzenie mikrojąder przeprowadzono badania aktywności transkrypcyjnej genów 35S rRNA w komórkach *H. vulgare* z wykorzystaniem metody srebrzenia, a następnie przeprowadzono FISH z sondą 25S rDNA. Badania te pozwoliły określić, czy obecność genów rRNA w mikrojądrach jest sprzężona z ich aktywnością transkrypcyjną. Wyniki wykazały, że aktywność transkrypcyjna genów 35S rRNA jest zawsze utrzymywana w mikrojądrach, chociaż są one często eliminowane

podczas następnych cyklów komórkowych (Kwasniewska i Jaskowiak, 2016). Badania dotyczące aktywności transkrypcyjnej rDNA u *B. distachyon* wykazały, że aktywność transkrypcyjna genów 35S rRNA jest związana z metylacją DNA (Borowska-Żuchowska i Hasterok, 2017).

Badania chromatyny wchodzącej w skład mikrojąder dotyczyły również replikacji DNA. Cytologiczne badania replikacji DNA w jądrach komórkowych i mikrojądrach są możliwe dzięki zastosowaniu znakowania komórek bromodeoksyurydyną (BrdU, BrdUrd), a następnie jej immunocytochemicznej detekcji z wykorzystaniem przeciwciał anty-BrdU. Badania aktywności replikacji DNA w komórkach zwierzęcych, z wykorzystaniem tej techniki wykazały, że replikacja DNA w mikrojądrach zachodziła w 98% komórek z MN (Kramer i inni, 1990). Technika wykrywania miejsc replikacji DNA z wykorzystaniem znakowania BrdU ma jednak wiele wad, takich jak powiększenie wielkości sygnałów przez przeciwciała anty-BrdU oraz konieczność denaturacji DNA, w celu zwiększenia dostępności BrdU dla przeciwciał anty-BrdU (Buck i inni, 2008; Kwasniewska i inni, 2016). Technika BrdU została zastąpiona nowoczesną techniką znakowania o wyższej rozdzielczości — reakcji „Click-it", z wykorzystaniem 5-etynylo-2′-deoksyurydyny (EdU). Reakcja ta jest oparta na cykloaddycji między alkilem EdU, a azydkiem, połączonym z fluorochromem. Metoda ta nie wymaga denaturacji DNA, z racji na niewielki rozmiar azydku (0,6 kDa), a ponadto grupa alkilowa nie jest reaktywna w układach biologicznych (Buck i inni, 2008; Diermeier-Daucher i inni, 2009; Cavanagh i inni, 2011). Badania replikacji DNA w komórkach *H. vulgare*, z wykorzystaniem znakowania EdU wykazały, że replikacja DNA zachodzi w mikrojądrach, jednak tylko 1% mikrojąder charakteryzował się obecnością znakowania DNA w fazie S, niezależnie od rodzaju zastosowanego mutagenu (Kwasniewska i inni, 2016). Również badania z wykorzystaniem komórek nowotworowych człowieka wykazały, że w mikrojądrach zachodzi replikacja (Okamoto i inni 2012). Przeprowadzono również badania uszkodzeń DNA w postaci pęknięć pojedynczej i podwójnej nici DNA w mikrojądrach z wykorzystaniem testu TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) (Maluszynska i Juchimiuk, 2005; Terradas i inni, 2009, Kwasniewska i inni, 2016).

Test TUNEL oparty jest na polimeryzacji znakowanych nukleotydów do wolnych końców 3'-OH, powstałych na skutek pęknięć nici DNA, katalizowanej przez terminalną transferazę deoksynukleotydylową (TdT), a sygnały są wykrywane za pomocą mikroskopii fluorescencyjnej.

Analizy modyfikacji epigenetycznych DNA i histonów w chromosomach i jądrach komórkowych roślin dają nowe możliwości poznania roli dynamiki chromatyny w komórkach roślinnych w odpowiedzi na mutageny (Zhao i inni, 2019). Wykazano udział modyfikacji epigenetycznych histonów w adaptacji roślin pod wpływem stresu środowiskowego (Lämke i Bäurle, 2017). Istnieje wiele badań dotyczących modyfikacji epigenetycznych chromatyny mikrojąder u ssaków. Badania w komórkach człowieka wykazały, że tworzenie MN było indukowane epigenetycznie głównie poprzez utratę metylacji DNA. W szczególności hypometylacja DNA w obrębie heterochromatyny w regionach pericentromerowych chromosomów powodowała jej dekondensację, prowadząc do nieprawidłowej segregacji chromosomów i jej wykluczenia do MN (Luzhna i inni, 2013). U roślin przeprowadzono badania porównawcze wpływu działania czynników mutagennych: fizycznego – promieniowania gamma i chemicznego – MH, na globalne modyfikacje epigenetyczne chromatyny: H3K9me2, H4K5ac i 5mC, w komórkach *H. vulgare*. Wykazano, że charakter zmian modyfikacji epigenetycznych zależy od rodzaju zastosowanego mutagenu. Hydrazyd kwasu maleinowego (MH) wpływa istotnie na poziom metylacji i acetylacji histonów, natomiast promieniowanie gamma powoduje znaczące zmiany w poziomie metylacji DNA (Braszewska-Zalewska i inni, 2014). Dotychczas w komórkach roślinnych szczegółowo nie badano modyfikacji epigenetycznych w aspekcie powstawania mikrojąder indukowanych czynnikami mutagennymi.

Jednym z czynników mutagennych powszechnie wykorzystywanym w mutagenezie roślin jest hydrazyd kwasu maleinowego (MH). Dokładny mechanizm działania MH nie jest dobrze poznany. Jest on izomerem uracylu, mającym hamujące działanie na syntezę DNA. Jest on czynnikiem klastogennym, prowadzącym do pęknięć DNA, a w efekcie do powstawania aberracji chromosomowych i mikrojąder. Jest on powszechnie wykorzystywany w mutagenezie roślin, u gatunków takich jak

V. faba, *A. cepa*, *H. vulgare*, a także *B. distachyon* (De Marco i inni, 1995; Marcano i inni, 2004; Juchimiuk-Kwasniewska i inni, 2011; Kus i inni, 2018). Równie często wykorzystywanym mutagenem w badaniach z zakresu mutagenezy roślin jest N-nitrozo-N-metylomocznik (MNU). MNU jest czynnikiem alkilującym, który głównie indukuje mutacje punktowe (Kus i inni, 2019). W przeciwieństwie do MH działającego w fazie S cyklu komórkowego, MNU działa w fazie G2 (Maluszynska i Maluszynski, 1983). Potwierdzono, że MNU indukuje również powstawanie mikrojąder w komórkach roślinnych (Juchimiuk i inni, 2007).

2. HIPOTEZY I CELE PRACY

Dotychczasowe badania wskazują, że rozkład uszkodzeń DNA w roślinnym genomie jądrowym nie jest przypadkowy. Sekwencje DNA charakteryzują się zróżnicowaną wrażliwością na działanie mutagenów. Ważną rolę w powstawaniu uszkodzeń DNA oraz ich naprawie odgrywa stopień kondensacji chromatyny. Metylacja DNA jest najlepiej poznanym mechanizmem epigenetycznym zaangażowanym w regulację ekspresji genów i odpowiedź na stres. Wpływa ona na stopień kondensacji chromatyny, a zatem może decydować o jej wrażliwości na działanie różnych czynników stresowych, w tym mutagenów. Hypometylowane regiony genomu są bardziej wrażliwe na przemiany strukturalne. Ponadto w odpowiedzi na stres dochodzi do zmiany ekspresji genów, bez zmian w sekwencji DNA, co skorelowane jest ze zmianami poziomu metylacji DNA w loci tych genów. Indukowany stresem wzrost poziomu metylacji DNA może stanowić mechanizm chroniący genom jądrowy przed rearanżacjami.

Hipotezy badawcze:

1. Poziom metylacji DNA zmienia się w odpowiedzi genomu jądrowego *Brachypodium distachyon* na działanie wybranych mutagenów chemicznych, a tym samym może odgrywać rolę w powstawaniu i naprawie uszkodzeń DNA
2. Metylacja /demetylacja DNA prowadząca do zmian w kondensacji chromatyny może stanowić czynnik decydujący o odpowiedzi genomu jądrowego *B. distachyon* na traktowanie mutagenami
3. Zmiany w poziomie metylacji DNA zależą od rodzaju zastosowanego mutagenu i mechanizmu jego działania
4. Loci rDNA w poszczególnych mikrojądrach, indukowanych działaniem mutagenów różnią się poziomem metylacji DNA
5. Udział loci rDNA w tworzeniu mikrojąder zależy od obecności zmetylowanego DNA w ich obrębie

Głównym celem badań pracy doktorskiej była próba zrozumienia roli metylacji DNA w odpowiedzi komórek roślinnych na stres, jaki stanowi traktowanie mutagenami. Odpowiedź komórek na działanie wybranych mutagenów chemicznych, charakteryzujących się różnymi mechanizmami działania: hydrazydu kwasu maleinowego (MH) i N-nitrozo-N-metylomocznika (MNU) obserwowano na poziomie cytologicznym jako powstawanie mikrojąder. Analizowano obecność fluorescencji przeciwciała Alexa Fluor 488 skoniugowanego pośrednio z 5- metylocytozyną (5mC) oraz jej intensywność w jądrach i mikrojądrach komórek *B. distachyon* kontrolnych oraz po traktowaniu MH lub MNU. Badania obejmowały porównawczą analizę zmian metylacji DNA w jądrach komórkowych po traktowaniu wybranymi mutagenami oraz udziału metylowanego DNA w powstałych mikrojądrach.

Podjęto również próbę poznania udziału i porównania metylacji DNA w loci 5S i 35S rDNA w mikrojądrach *Brachypodium distachyon*, po traktowaniu MH, z sekwencyjnym wykorzystaniem immunocytochemicznego wykrywania 5mC oraz techniki FISH, z sondami 5S i 25S rDNA.

3. MATERIAŁY I METODY

3.1. Materiał roślinny i traktowanie mutagenne

Materiałem wykorzystanym w niniejszych badaniach były nasiona *Brachypodium distachyon* (2n=10), genotyp Bd21. Nasiona pochodziły z banku nasion USDA National Plant Germplasm System w USA (USDA-NPGS). Nasiona wysiewano na szalki Petriego wyłożone wilgotną bibułą. Po 72 h kiełkowania nasion, otrzymane siewki traktowano 4 mM hydrazydem kwasu maleinowego (MH, Sigma) lub 3 mM N-nitrozo-N-metylomocznikiem (MNU, Sigma) przez 3 h, w ciemności, w temperaturze pokojowej. W trakcie traktowania wodne roztwory mutagenów napowietrzano. Po traktowaniu siewki płukano trzykrotnie po 5 minut w wodzie destylowanej, a następnie ponownie umieszczano na szalkach Petriego, wyłożonych wilgotną bibułą. Grupę kontrolną stanowiły siewki po 72 h kiełkowania, moczone w wodzie destylowanej przez 3 h, w tych samych warunkach jak dla siewek traktowanych mutagenami. Korzenie utrwalono w acetoalkoholu (metanol – kwas octowy, 3:1) w trzech czasach postinkubacji: 0 h, 10 h i 20 h, i przechowywano w -20 ˚C. Doświadczenie z mutagennym traktowaniem z użyciem mutagenów MH i MNU powtórzono dwukrotnie.

3.2. Maceracja enzymatyczna i przygotowanie preparatów cytogenetycznych

Preparaty cytogenetyczne wykonywano według procedury opisanej przez Jenkinsa i Hasteroka (2007). Korzenie siewek *B. distachyon* płukano w 0,01 M buforze cytrynianowym przez 30 min. w celu usunięcia utrwalacza. Następnie odcinano merystemy korzeniowe i przeprowadzano macerację enzymatyczną przy użyciu mieszaniny enzymów maceracyjnych – 1% pektynazy (Sigma) oraz 2% celulazy (Sigma), w temp. 37 ˚C przez 1,5 h. Zmacerowany materiał płukano ponownie w 0,01 M buforze cytrynianowym przez 30 minut. Merystem korzeniowy umieszczano w 45% kwasie octowym, rozdrabniano go igłami, a następnie przenoszono na szkiełko podstawowe w kropli kwasu, przykrywano szkiełkiem nakrywkowym i rozgniatano. W celu usunięcia szkiełka nakrywkowego preparaty umieszczono na suchym lodzie. Preparaty przechowywano w 4 ˚C. Preparaty

wykonane metodą maceracji enzymatycznej wykorzystano do immunocytochemicznego wykrywania 5mC, a następnie fluorescencyjnej hybrydyzacji *in situ* (FISH) z sondami 5S i 25S rDNA.

3.3. Immunocytochemiczne wykrywanie 5mC

Denaturację chemiczną DNA na preparatach enzymatycznych przeprowadzono poprzez umieszczenie preparatów w roztworze 0,25 M wodorotlenku sodu (NaOH, Merck) i 1 M chlorku sodu (NaCl, Merck) na 30 min. w 4 ˚C. Preparaty płukano trzykrotnie po 5 minut w wodzie destylowanej i umieszczono w roztworze 1M Tris-HCl (VWR) na 30 minut. Po tym przeprowadzono dehydratację w szeregu alkoholowym: 70%, 90% i 100%, poprzez umieszczenie preparatów w każdym roztworze na 5 minut. Na preparaty nakropiono 5% BSA (Bovine Serum Albumin, Sigma) w 1xPBS (Phosphate Buffered Saline), przykryto folią i umieszczono w wilgotnej komorze, w temperaturze pokojowej na 1 h. Następnie usunięto folię, a na preparaty nakropiono przeciwciało I-rzędowe 'antibody- mouse anti-5-methylcytosine' (rozcieńczone 1:100 w 1xBSA, Abcam) i inkubowano 24 h, w 4˚C. Preparaty płukano trzykrotnie po 5 minut w 1xPBS, w temperaturze pokojowej. Następnie nakropiono przeciwciało II-rzędowe 'antibody goat anti-mouse IgG, skoniugowane z Alexa Fluor 488' (rozcieńczone 1:100 w 1xBSA, Invitrogen, Molecular Probes), przykryto folią i inkubowano w wilgotnej komorze, w ciemności, w temperaturze 37˚C przez 1 h. Preparaty płukano trzykrotnie po 5 minut w 1xPBS w temperaturze pokojowej, a następnie zamykano w Vectashield (Vector Laboratories) z DAPI (2,5 µg/ml, Serva).

3.4. Analiza preparatów cytogenetycznych po immunocytochemicznym wykrywaniu 5mC, z wykorzystaniem mikroskopii fluorescencyjnej oraz systemu rejestracji obrazu

Preparaty analizowano w mikroskopie fluorescencyjnym Carl Zeiss Imager Z2, z oświetleniem fluorescencyjnym HxP, o mocy 120W. Obrazy rejestrowano z użyciem kamery cyfrowej AxioCam ICc5 i obiektywu immersyjnego o powiększeniu x100. Analizę metylacji DNA poprzedzono oceną częstości komórek

korzeni *B. distachyon* z mikrojądrami**.** Częstość mikrojąder oszacowano dla 3000 komórek z każdej grupy: kontrolnej oraz po traktowaniu MH lub MNU.

Następnie przeprowadzono obserwacje obecności lub braku sygnałów przeciwciała ‘goat anti-mouse IgG’, skoniugowanego z Alexa Fluor 488, w jądrach komórkowych i/lub mikrojądrach. Średnią intensywność fluorescencji Alexa Fluor 488 w jądrach komórkowych bez mikrojąder oraz w jądrach matecznych i ich mikrojądrach przeprowadzono z wykorzystaniem programu ImageJ. Intensywność fluorescencji Alexa Fluor 488 zmierzono jako średnią wartość parametru Integrated Density (IntDen). Zintegrowany parametr gęstości jest sumą wszystkich pikseli w obszarze zainteresowania (region of interest, ROI). Ośmiobitowe obrazy z fluorescencją Alexa Fluor 488 poddano segmentacji za pomocą parametru wartości progowej, a intensywność fluorescencji mierzono jako wartości średnie z parametru gęstości. Wyniki tych pomiarów oszacowano w jednostkach względnych. Obecność sygnałów 5mC i średniej intensywności fluorescencji Alexa Fluor 488, w komórkach po traktowaniu MH i MNU analizowano dla 100 mikrojąder. Analizę statystyczną wykonano z użyciem testu ANOVA oraz testu Tukey, $p < 0,05$.

Podczas analizy obecności i poziomu 5mC, przed przeprowadzeniem procedury FISH, rejestrowano specyficzną lokalizację jąder komórkowych i mikrojąder.

3.5. Znakowanie sond i procedura FISH

Preparaty po immunocytochemicznym wykrywaniu 5mC przygotowano do procedury FISH poprzez usunięcie pozostałości olejku immersyjnego ze szkiełka nakrywkowego z użyciem acetonu, a następnie płukanie pod bieżącą wodą, w celu usunięcia szkiełka nakrywkowego. Procedurę FISH przeprowadzono na preparatach po wcześniejszej akwizycji obrazów. FISH przeprowadzano według procedury opisanej przez Jenkinsa i Hasteroka (2007) z nielicznymi modyfikacjami.

Jako sondy do FISH wykorzystano 5S i 25S rDNA (Roche Diagnostics, Basel, Switzerland), znakowane odpowiednio digoksygeniną-11-dUTP i tetrametylorodaminą-5-dUTP (Roche Diagnostics) poprzez nick-translację z zastosowaniem Nick-Translation mix (Roche Diagnostics). Procedurę FISH rozpoczęto nakropieniem RNazy na preparaty i inkubacją w wilgotnej komorze,

w temperaturze 37˚C przez 1 h. Następnie preparaty płukano trzykrotnie po 5 minut w 2xSSC, po czym przeniesiono do 1% formaldehydu w 1xPBS na 10 minut w temperaturze pokojowej. Preparaty ponownie płukano trzykrotnie po 5 minut w 2xSSC. Do przeprowadzenia denaturacji i hybrydyzacji DNA wykorzystano mieszaninę hybrydyzacyjną złożoną z 50% formamidu, 10% siarczanu dekstranu, 2xSSC, 0,5% SDS, 200 µl sond 5S i 25S rDNA oraz wody. Mieszaninę hybrydyzacyjną nakropiono na preparaty, przykryto folią i inkubowano w 70˚C przez 4,5 minuty. Po tym czasie temperaturę obniżono do 37 ˚C, preparaty przeniesiono do cieplarki i inkubowano przez 24 h, w temperaturze 37 ˚C. Pohybrydyzacyjne płukania preparatów przeprowadzono trzykrotnie po 5 minut w 20% formamidzie w 0,1xSSC i dwukrotnie po 5 minut w 2xSSC, w temperaturze 42˚C, a następnie trzykrotnie po 5 minut w 2xSSC w temperaturze pokojowej. Po tym czasie preparaty płukano przez 10 minut w Tween20/4xSSC w temperaturze pokojowej. Następnie na preparaty nakropiono 180 µl buforu blokującego (5% odtłuszczone mleko w proszku w 4xSSC), przykryto folią i inkubowano w wilgotnej komorze, w temperaturze pokojowej przez 30 minut. W celu detekcji sondy 25S rDNA, skoniugowanej z digoksygeniną-11-dUTP, na preparaty nakropiono przeciwciało pierwszorzędowe, skoniugowane z FITC (Roche Diagnostics), zmieszane z buforem blokującym (5% odtłuszczone mleko w proszku w 4xSSC) w stosunku 1:11, następnie preparaty przykryto folią i inkubowano 1 h w wilgotnej komorze, w temperaturze 37˚C. Po tym czasie preparaty płukano 3 razy po 8 minut w Tween20/4xSSC, w temperaturze 42˚C, a następnie preparaty odwadniano w szeregu alkoholowym: 70%, 90% i 100% i pozostawiono do wyschnięcia. Preparaty zamykano w Vectashield (Vector Laboratories) z DAPI (2,5 µg/ml, Serva).

3.6. Analiza preparatów cytogenetycznych po FISH, z wykorzystaniem mikroskopii fluorescencyjnej oraz rejestracji obrazu

Analiza obecności sekwencji 5S i 35S rDNA, w jądrach komórkowych i mikrojądrach *B. distachyon* po traktowaniu MH była możliwa dzięki zapisaniu ich konkretnej lokalizacji na preparatach po immunocytochemicznym wykrywaniu 5mC.

Te same komórki po przeprowadzonej procedurze FISH analizowano z wykorzystaniem mikroskopu fluorescencyjnego Carl Zeiss Imager Z2, z oświetleniem fluorescencyjnym HxP, o mocy 120W. Obraz zarejestrowano z użyciem kamery cyfrowej AxioCam ICc5 i obiektywu immersyjnego o powiększeniu x100. Analizowano częstość mikrojąder z sygnałami 5S lub 35S rDNA, lub 5S i 35S rDNA, lub bez sygnałów. Analizę przeprowadzono dla 350 jąder komórkowych z mikrojądrami po traktowaniu MH. Sekwencyjne zastosowanie immunocytochemicznego wykrywania 5mC, a następnie fluorescencyjnej hybrydyzacji *in situ* (FISH) z sondami 5S i 25S rDNA umożliwiło jednoczesną analizę występowania sygnałów 5S i 35S rDNA, oraz sygnałów 5mC w tych loci w mikrojądrach.

4. WYNIKI I DYSKUSJA

4.1. Ocena klastogennego działania MH i MNU w postaci mikrojąder w komórkach *B. distachyon*

Ocenę klastogennego działania MH i MNU poprzez oszacowanie częstości mikrojąder przeprowadzono z wykorzystaniem barwienia DAPI jąder komórkowych merystemów korzeniowych *B. distachyon.* Analizę przeprowadzono w trzech czasach po zakończeniu traktowania mutagenami (czasach postinkubacji): 0, 10 i 20 h. Analizie poddano również komórki korzeni kontrolnych, nie traktowanych. Ocena częstości mikrojąder po traktowaniu mutagennym jest powszechnie wykorzystywana do oceny klastogennego działania różnych związków (Leme i inni, 2008). Częstość występowania mikrojąder w komórkach po traktowaniu MH wynosiła od 0,33% do 0,53%, a po traktowaniu MNU od 0,63% do 3,03%. Komórki z mikrojądrami z najwyższą częstością obserwowano w 0 h postinkubacji, a następnie częstość ta obniżała się wraz z wydłużaniem czasu postinkubacji. Mikrojądra wciąż obserwowano w komórkach korzeni w 20 h po zakończeniu traktowania MH lub MNU. Nie obserwowano mikrojąder w komórkach kontrolnych. Klastogenny efekt MH u *B. distachyon* był już wcześniej badany (Kus i inni, 2017; 2018; 2019), natomiast brak wcześniejszych doniesień dotyczących indukowania mikrojąder przez MNU u tego gatunku. Obniżenie częstości występowania mikrojąder indukowanych przez MH i MNU, wraz z czasem postinkubacji może wskazywać, że mikrojądra są eliminowane z komórek (Morales-Ramirez i inni, 1999). Mikrojądra mogą zostać ponownie włączone do jądra komórkowego (Ege i inni, 1974) lub ulec degradacji (Rao i inni, 2008, Krupina i inni, 2021). Najprawdopodobniejsza teoria wskazuje, że mikrojądra są eliminowane z komórek w czasie kolejnych cyklów komórkowych (Morales-Ramirez i inni, 1999).

4.2. Analiza metylacji DNA w komórkach *B. distachyon,* kontrolnych oraz traktowanych MH i MNU, w oparciu o immunocytochemiczne wykrywanie 5mC

Analizę metylacji DNA w komórkach korzeni *Brachypodium distachyon,* kontrolnych oraz traktowanych hydrazydem kwasy maleinowego (MH) lub N-nitrozo-N-metylomocznikiem (MNU), w oparciu o immunocytochemiczne wykrywanie 5mC przeprowadzono w jądrach komórkowych, a dla materiału traktowanego mutagenem również w mikrojądrach.

W celu oszacowania częstości mikrojąder z obecnymi sygnałami 5mC w komórkach *B. distachyon* po traktowaniu MH i MNU, przeprowadzono immunocytochemiczne wykrywanie 5mC z wykorzystaniem przeciwciała I i II-rzędowego, skoniugowanego z fluorochromem Alexa Fluor 488. Analizowano komórki korzeni *B. distachyon* po traktowaniu MH i MNU, w trzech czasach postinkubacji: 0 h, 10 h i 20 h. Sygnały 5mC były obecne we wszystkich analizowanych jądrach komórkowych, zarówno w komórkach kontrolnych, jak i traktowanych, podczas gdy mikrojądra charakteryzowały się obecnością sygnałów lub ich brakiem. Wykazano, że częstość mikrojąder z sygnałami 5mC różniła się w komórkach traktowanych MH i MNU. Była ona wyższa po traktowaniu MNU i wynosiła w zależności od czasu postinkubacji od 75% do 86%, natomiast po traktowaniu MH - od 58,5% do 68%. Wraz z czasem postinkubacji po traktowaniu MH i MNU częstość mikrojąder posiadających sygnały 5mC wzrastała, i w 20 h postinkubacji była o około 10% wyższa niż w 0 h postinkubacji. Obecność sygnałów 5mC w mikrojądrach wskazuje na hipermetylację DNA, zaangażowanego w tworzenie mikrojąder. U roślin metylacja DNA jest ograniczona do określonych regionów genomu, np. u Arabidopsis większość metylowanego DNA występuje w lokalnych powtórzeniach tandemowych lub odwróconych, transpozonach i innych rozproszonych powtórzeniach wokół centromerów i w euchromatynie (Arabidopsis Genome Initiative, 2000). Podobną lokalizację metylowanego DNA w regionach pericentromerowych wykazano dla *B. distachyon* (Borowska i inni, 2011). Obecność sygnałów 5mC w większości mikrojąder może świadczyć o ich prawdopodobnym pochodzeniu z fragmentu(ów) chromosomu(ów) pericentromerowego(ych), które są

hipermetylowane już w komórkach kontrolnych. Niewykluczone, że w efekcie działania mutagenu nastąpiła metylacja DNA w innych regionach chromosomu(ów), które później brały udział w tworzeniu mikrojądra. Zwiększenie częstości mikrojąder zawierających zmetylowane DNA i jednoczesne obniżenie częstości mikrojąder wraz z wydłużeniem czasu postinkubacji, zarówno po traktowaniu MH jak i MNU, może wskazywać, że obecność metylacji DNA w mikrojądrach może być skorelowane z ich eliminacją z komórek. Wraz z obecnością 5mC w mikrojądrach indukowanych działaniem MH i MNU analizowano również poziom 5mC. Średnia intensywność fluorescencji Alexa Fluor 488 w mikrojądrach różniła się w zależności od mutagenu zastosowanego do ich indukcji. Średnia intensywność fluorescencji Alexa Fluor 488 była wyższa w mikrojądrach indukowanych MH niż indukowanych MNU, we wszystkich trzech zastosowanych czasach postinkubacji. Ponadto obserwowano, że w trakcie zastosowanych czasów postinkubacji średnia intensywność fluorescencji Alexa Fluor 488 w mikrojądrach, indukowanych MH, jak i MNU obniżała się. Ten wynik może wskazywać na aktywne procesy demetylacji DNA w mikrojądrach zachodzące wraz z czasem postinkubacji. Demetylacja DNA w mikrojądrach może być związana z aktywnymi procesami replikacji DNA (Kwasniewska i inni, 2018). Obecność sygnałów EdU w MN wykazana przez Kwasniewską i innych (2018) może również wskazywać na inkorporowanie jej w jądrze komórkowym w fazie S, a następnie utworzenie z tej chromatyny MN. Uzyskane wyniki dotyczące poziomu metylacji DNA w jądrach i mikrojądrach sugerują możliwy udział mechanizmów epigenetycznych w (nie)stabilności genomu *B. distachyon* poddanego działaniu czynników mutagennych. Powyższe wyniki badań zostały opisane w pracy **Bara i inni (2021)[1]**.

Analizę intensywności fluorescencji Alexa Fluor 488 przeprowadzono również w jądrach komórkowych nietworzących mikrojąder, zarówno w kontroli, jak i po traktowaniu MH i MNU. Wykazano, że poziom 5mC różnił się w jądrach kontrolnych i jądrach po zastosowaniu mutagenów. Porównanie intensywności fluorescencji Alexa Fluor 488 w 0 h postinkubacji wykazało, że była ona najniższa w komórkach kontrolnych, wyższa po traktowaniu MH, a najwyższe wartości osiągnęła po traktowaniu MNU. Intensywność fluorescencji Alexa Fluor 488 zależała również od

czasu postinkubacji i była różna dla różnych grup eksperymentalnych. W komórkach kontrolnych średnia intensywność fluorescencji Alexa Fluor 488 była podobna w 0 i 10 h postinkubacji, a w 20 h zaobserwowano jej nieznaczny wzrost. W komórkach po traktowaniu MH obserwowano obniżenie intensywności fluorescencji Alexa Fluor 488 w 10 h postinkubacji, natomiast w 20 h intensywność fluorescencji wzrosła do poziomu obserwowanego w 0 h postinkubacji. Wpływ czasu postinkubacji na poziom 5mC zaobserwowano również w przypadku jąder komórkowych traktowanych MNU. Średnia intensywność fluorescencji Alexa Fluor 488 wzrosła w 10 h postinkubacji, a kolejny wzrost obserwowano w 20 h postinkubacji. Interesujące wydaje się, że średnia intensywność fluorescencji Alexa Fluor 488 nieznacznie zmieniła się w kontroli wraz z czasem postinkubacji, pomimo zapewnienia stałych warunków wzrostu. Zmiany te, związane prawdopodobnie z procesami zachodzącymi podczas rozwoju korzeni, były obserwowane już wcześniej (Chen i inni, 2018). Rodzaj zmian intensywności fluorescencji Alexa Fluor 488 (wzrost lub obniżenie) w jądrach komórkowych po traktowaniu mutagenami były inne niż w jądrach komórek kontrolnych. Zmiany średniej intensywności fluorescencji Alexa Fluor 488 różne po traktowaniu mutagenami o różnych mechanizmach działania mogą być związane z procesami naprawy specyficznych uszkodzeń DNA. MH jest mutagenem klastogennym, działającym w fazie S cyklu komórkowego, natomiast MNU powoduje głównie mutacje punktowe, ale również wpływa na uszkodzenia chromosomów (Marcano i inni, 2004; Juchimiuk i inni, 2007). Dalsze badania z wykorzystaniem innych związków chemicznych i fizycznych, które charakteryzują się różnymi mechanizmami działania mogą dostarczyć nowych, interesujących odpowiedzi dotyczących różnic w metylacji DNA w odpowiedzi na działanie mutagenów.

W kolejnych badaniach porównano średnią intensywność fluorescencji Alexa Fluor 488 w jądrach komórkowych *B. distachyon* po traktowaniu MH i MNU, które nie tworzyły mikrojąder oraz tych, które były mateczne dla mikrojąder. Wyższą intensywnością fluorescencji Alexa Fluor 488 charakteryzowały się jądra nietworzące mikrojąder, w porównaniu z jądrami matecznymi dla mikrojąder, zarówno po traktowaniu MH, jak i MNU. Dla komórek po traktowaniu MH, taką zależność

obserwowano we wszystkich zastosowanych czasach postinkubacji, a dla komórek traktowanych MNU w 10 i 20 h postinkubacji. Niższy poziom 5mC w jądrach matecznych dla mikrojąder w porównaniu z jądrami nietworzącymi mikrojąder, zarówno po traktowaniu MH i MNU może sugerować, że proces tworzenia i eliminacji mikrojąder może być skorelowany z metylacją DNA. Ponadto, zaobserwowano generalną regułę, że wszystkie jądra komórkowe *B. distachyon* po traktowaniu MNU charakteryzowały się wyższym poziomem 5mC niż po traktowaniu MH.

4.3. Analiza metylacji DNA w loci 5S i 35S rDNA w komórkach *Brachypodium distachyon* traktowanych MH

Analizę metylacji DNA w loci 5S i 35S rDNA w mikrojądrach *B. distachyon* indukowanych MH, przeprowadzono z sekwencyjnym zastosowaniem immunocytochemicznego wykrywania 5mC, a następnie FISH z sondami 5S i 25S rDNA (**Bara-Halama i inni, 2022)[3]**.

W eksperymencie kontrolnym wykazano, że w jądrach komórkowych, nie traktowanych MH występują dwa sygnały 5S rDNA i dwa sygnały 35S rDNA, co odpowiada liczbie loci rDNA w genomie jądrowym *B. distachyon* (Kus i inni, 2017). Analiza obecności lub braku sygnałów 5S i/lub 35S rDNA w mikrojądrach, indukowanych działaniem MH wykazała ich występowanie w 4 wariantach: brak sygnałów rDNA w mikrojądrze, obecność jednego sygnału 5S rDNA, obecność jednego sygnału 35S rDNA oraz obecność zarówno 5S rDNA, jak i 35S rDNA. Czas postinkubacji (0h, 10 h, 20 h) nie wpływał na częstości mikrojąder z sygnałami rDNA. Spośród mikrojąder z sygnałami rDNA z najwyższą częstością - 39% obserwowano mikrojądra z sygnałami 5S rDNA oraz mikrojądra z sygnałami 5S i 35S rDNA - 35%. Mikrojądra z sygnałami 35S rDNA obserwowano z najniższą częstością, wynoszącą 26%. Kus i inni (2017) również wykazali, że chromosomy Bd4 z 5S rDNA były częściej zaangażowane w tworzenie mikrojąder w komórkach *B. distachyon*, niż chromosomy Bd5 z 35S rDNA. Podobne różnice we wrażliwości regionów 5S i 35S rDNA na traktowanie MH obserwowano wcześniej w komórkach *Hordeum vulgare* (Kwasniewska i Jaśkowiak, 2016)

i *Crepis capillaris* (Kwasniewska i inni, 2012; Kwasniewska i Mikołajczyk, 2014). W komórkach *C. capillaris* dzięki połączeniu testu kometowego z FISH wykazano, że sygnały 5S rDNA były obecne w ogonie komety częściej niż 35S rDNA. Zjawiska te można wytłumaczyć powiązaniem transkrypcyjnie aktywnego 35S rDNA z jądrem komórkowym (Rapp i inni, 2000; Kwasniewska i inni, 2012). To może tłumaczyć mniejszą podatność 35S rDNA, reprezentującego 'housekeeping genes', na fragmentację i migrację do ogona komety. Połączenie testu mikrojąder z analizą aktywności transkrypcyjnej genów 35S rRNA w komórkach *H. vulgare*, po traktowaniu MH pozwoliło wykazać, że ekspresja 35S rDNA jest zawsze utrzymywana w mikrojądrach, mimo że są one eliminowane podczas następnego cyklu komórkowego (Kwasniewska i Jaskowiak, 2016).

Badania przeprowadzone w ramach pracy doktorskiej wykazały, że mikrojądra posiadające sygnały rDNA stanowiły średnio 15% wszystkich mikrojąder. Tylko 6% posiadało sygnały 5mC w loci rDNA, a w 9% nie obserwowano sygnałów 5mC w tym regionie. 85% stanowiły mikrojądra bez sygnałów rDNA, z czego 24% mikrojąder posiadało sygnały 5mC, a 61% mikrojąder nie posiadało ani sygnałów rDNA ani sygnałów 5mC. Czas postinkubacji (0h, 10 h, 20 h) nie wpływał znacznie na częstości mikrojąder z sygnałami rDNA i 5mC.

Metylacja DNA jest procesem charakterystycznym dla heterochromatyny. Istnieją dowody sugerujące, że zwiększona kondensacja chromatyny może odgrywać rolę w eliminacji DNA z jądra komórkowego. W krzyżówce pszenicy z prosem perłowym, jednorodzicielska eliminacja genomu prosa perłowego występuje we wczesnych stadiach rozwoju zarodka. Chromosomy prosa perłowego tworzą mikrojądra, które są na ogół bardziej skondensowane, niż jądra i zawierają heterochromatynę oraz euchromatynę lub wyłącznie heterochromatynę (Gernand i inni, 2005). Podobnie sytuacja ma się w przypadku owadów z rodziny Sciaridae, u których zestaw chromosomów ojca ulegający eliminacji wykazuje niski poziom acetylacji histonu H4/H3, który jest zwykle wskaźnikiem heterochromatynizacji (Goday i Ruiz, 2002). W przeprowadzonych w ramach pracy doktorskiej badaniach sygnały 5mC odnotowano w 30% wszystkich analizowanych mikrojąder (w tym 6% mikrojąder z sygnałami rDNA i 24% mikrojąder bez sygnałów rDNA), co sugeruje,

że heterochromatynizacja, związana z metylacją DNA może nie odgrywać tak ważnej roli w eliminacji DNA z jądra komórkowego w formie mikrojądra, spowodowanej działaniem czynników mutagennych. Jednak nie tylko metylacja DNA jest charakterystyczna dla heterochromatyny, ale także inne markery, takie jak poziom metylacji histonów H3K9 i H3K27, które mogą zostać wykorzystane w przyszłych badaniach dotyczących mechanizmów tworzenia mikrojąder, indukowanych działaniem mutagenów.

Analizowano także szczegółowo częstość mikrojąder z sygnałem 5S lub 35S rDNA, albo sygnałami 5S i 35S rDNA z jednoczesną obecnością sygnałów 5mC w tych loci. Wyniki wykazały, że częstość mikrojąder niosących 5S rDNA, bez występujących sygnałów 5mC w tym regionie była dwukrotnie wyższa, niż częstość mikrojąder niosących 5S rDNA z sygnałami 5mC i wynosiła odpowiednio 26%. Ponieważ demetylacja DNA jest związana z dekondensacją chromatyny, taki wynik może wskazywać, że niemetylowane, mniej skondensowane miejsca 5S rDNA są bardziej narażone na powstawanie pęknięć podwójnej nici DNA (DSB - double strand breaks), wywołanego działaniem mutagenu. Hipotezę tę potwierdzają badania ludzkich limfocytów, które wykazały, że dekondensacja regionów heterochromatycznych w 1 i 9 chromosomie, jest skorelowana z tworzeniem mikrojąder przez te chromosomy, po traktowaniu idoksyurydyną (Fauth i Zankl, 1999). Nie zaobserwowano różnic w obecności sygnałów 5mC lub ich braku w mikrojądrach z sygnałami 35S rDNA – częstość takich mikrojąder wynosiła po 13%. Częstość mikrojąder z sygnałami 5S i 35S rDNA, z jednoczesnymi sygnałami 5mC wynosiła 15%, a bez sygnałów 5mC - 20%. Wykazano, że spośród wszystkich mikrojąder z sygnałami rDNA, 41% z nich miało również sygnały 5mC. Wyniki te można interpretować w odniesieniu do wzorców metylacji DNA w submetacentrycznych chromosomach *B. distachyon* Bd4 i Bd5, które posiadają odpowiednio geny 5S i 35S rRNA. Poszczególne chromosomy *B. distachyon* posiadają charakterystyczny wzór metylacji DNA. Występują różnice w lokalizacji metylacji DNA pomiędzy chromosomami homologicznymi oraz pomiędzy ramionami danego chromosomu (Borowska i inni, 2011). Jednak niektóre regiony chromosomów, np. pericentromerowe, są niezmiennie silnie zmetylowane we

wszystkich chromosomach. Chromosomy Bd4 i Bd5 charakteryzują się charakterystyczną lokalizacją 5mC. W przypadku chromosomu Bd4 obserwuje się dwa piki o dużej gęstości ognisk 5mC, z niewielkim zmniejszeniem metylacji w regionie niosącym locus 5S rDNA. Wysoki poziom metylacji DNA jest często skorelowany z hamowaniem transkrypcji. 5S rDNA jest wyjątkiem od tej reguły, ponieważ pozostaje aktywnym regionem genetycznym, mimo że jest wysoce metylowany (Mathieu i inni, 2002). W wyniku działania czynników mutagennych może ulegać demetylacji i być bardziej podatnym na tworzenie MN.

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II. PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY

II.1.

Bara A.W., Braszewska A., Kwasniewska J.

DNA methylation – an epigenetic mark in mutagen-treated *Brachypodium distachyon* cells.

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DNA Methylation—An Epigenetic Mark in Mutagen-Treated *Brachypodium distachyon* Cells

Adrianna Wiktoria Bara, Agnieszka Braszewska and Jolanta Kwasniewska *

Plant Cytogenetics and Molecular Biology Group, Faculty of Natural Sciences, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland; adriannabara@gmail.com (A.W.B.); agnieszka.braszewska@us.edu.pl (A.B.)

* Correspondence: jolanta.kwasniewska@us.edu.pl

Abstract: The chromatin structure is significantly influenced by some epigenetic modifications including DNA methylation. The nuclear organization plays an essential role in the cell response to external stresses including mutagens. We present an analysis of the correlation between epigenetic modifications and the instability of the *Brachypodium distachyon* genome, which are observed as micronuclei, following maleic hydrazide (MH) and nitroso-N-methylurea (MNU) treatments. We compared the level of DNA methylation in the control (untreated) and mutagen-treated *B. distachyon* nuclei. An immunostaining method using specific antibodies against modified DNA anti-5-methylcytosine was used for the evaluation of DNA methylation in a single nucleus and micronucleus. Interestingly, we showed an alteration of DNA methylation in cells after mutagenic treatments. The results indicate that DNA methylation might be involved in the response of the *B. distachyon* genome to mutagenic treatments. This demonstrates that analyses of the epigenetic modifications should be integrated into current plant genetic toxicology in order to explain the mechanisms of DNA damage and repair in plants.

Keywords: *Brachypodium*; DNA methylation; maleic hydrazide; micronuclei; N-nitroso-N-methylurea

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1. Introduction

The nuclear organization plays a crucial role in the cell response to external stresses. The chromatin structure, including the euchromatin–heterochromatin ratio, is one of the nuclear genome features that determine its sensitivity to environmental mutagenic factors [1]. It is known that condensed chromatin can even play a protective role for transcriptionally active euchromatin [2]. The chromatin structure represents a highly dynamic configuration, and it is significantly influenced by some epigenetic modifications, including DNA methylation. This process involves the covalent addition of a methyl group to the fifth position of cytosine in the pyrimidine ring at the chemical level, which is catalyzed by the methyltransferase enzymes using S-adenosyl methionine as the methyl group donor. The spatial distribution and organization of chromatin as well as dynamic changes, including epigenetic chromatin modifications, have frequently been evaluated to study the plant cell response to environmental factors [3,4]. Stresses such as treatment with aluminum, salt, cold or drought can induce changes in the gene expression via the hypomethylation or hypermethylation of DNA [5]. An analysis of the dynamic nature of nuclear changes also seems to be a promising field in mutagenesis today. It has become clear that the nuclear architecture, including epigenetic chromatin modifications, has a broad functional role in the response of mutagen-treated cells [6].

Changes in the epigenetic modifications strongly depend on the type of mutagen, e.g., gamma rays cause significant changes in the DNA methylation level. In contrast, maleic hydrazide (MH) induces changes in the histone methylation and acetylation levels [7]. Moreover, it was suggested that the global DNA methylation in mutagen-treated cells might be involved in specific aspects of DNA repair [8]. The role of DNA methylation was

proven to play a role in human carcinogenesis and cancer therapies [9]. More data on the role of the epigenetic modifications in DNA damage and repair are needed.

Many plant genotoxicity tests have been developed to investigate the cytogenetic effects of various agents that potentially induce DNA damage. Among them, the micronuclei (MN) test, which was proposed by Evans et al. [10], is widely used to analyze the genotoxic effect of a large number of chemical and physical agents [11–16]. There is also an example of using the MN test in *Brachypodium distachyon* (L.) *P. Beauv.* cells [17–19]. Micronuclei are defined as small, extranuclear bodies that are formed from chromosome fragments or entire chromosomes as a result of the chromosome breakage, kinetochore damage or disturbances of the cell cycle. The unrepaired DNA double-strand breaks that result from the misrepair of these strand breaks may lead to MN formation [20]. The molecular cytogenetic approaches, especially fluorescence in situ hybridization (FISH), are very useful in detecting micronuclei and identifying specific chromosomes or chromosome fragments in the micronuclei to determine their origin [21–24], especially in species with a small genome [17–19]. However, an understanding of the mechanisms of MN formation is still incomplete. *Brachypodium distachyon* is a widely accepted model grass with numerous favorable features and resources, such as a small, simple and fully sequenced nuclear genome, small plant stature and rapid life cycle [25,26]. *B. distachyon* is a therophytic species, widespread in Europe where it characterizes annual meadows protected by Habitat Directive 92/43 EEC as a priority habitat called a Pseudo-steppe with grasses and annuals of the Thero-Brachypodietea (6620* code) [27] plant community that forms a mosaic with many other types of perennial vegetation [28]. Vogel and Hill [29] demonstrated that *B. distachyon* is a promising plant for mutagenesis purposes. Protocols have been established for the mutagenesis of *B. distachyon* with sodium azide [30,31], fast neutron radiation [32] and gamma radiation [33]. Our previous studies have shown a positive response of *B. distachyon* to mutagenic treatments using MH and x-rays [17–19]. *B. distachyon* was proven to be well suited for analyzing the plant nuclear genome stability. There are cyto-molecular resources, including the FISH-based and chromosome-specific approaches such as chromosome barcoding and chromosome painting, that enable a detailed insight to be obtained into the micronuclei structure that is induced by mutagens. The involvement of specific DNA sequences in micronuclei formation has shown that the distribution of the DNA break points is not random. Chromosome size, gene density and other aspects of the chromatin organization are the factors that influence the preferential origin of the MN from specific chromosomes in animals and humans [34–37].

At present, the methodological possibilities enable some essential questions regarding the correlation between chromatin structure, epigenetic modifications and the instability of the plant genome in mutagenesis to be addressed. In this study, we analyzed the level of DNA methylation, which is a well-known heterochromatin marker in the nuclei and micronuclei in *B. distachyon* after chemical mutagenesis. Mutagens that are characterized by different mechanisms of action: maleic hydrazide (MH), which is a clastogenic agent that can lead to chromosome breaks and also cause spindle fiber defects [38], and nitroso-N-methyl-urea (MNU), which is an alkylating agent that mainly induces gene mutations but also leads to chromosomal aberrations, were used to induce micronuclei in *B. distachyon* root meristematic cells [19]. The mutagens used in this study act in the different cell cycle phases: MH in the S phase and MNU in the G2 phase [39]. The aim of this study was to analyze and compare the level of DNA methylation in the control (untreated) and mutagen-treated *B. distachyon* nuclei. An immunostaining method using specific antibodies against modified DNA anti-5-methylcytosine was used to determine the presence and level of DNA methylation that were eliminated from the nucleus as micronuclei.

2. Results

2.1. Micronuclei in the B. distachyon Cells

Before detecting 5 mC, the slides with the nuclei were stained with DAPI. The frequencies of *B. distachyon* root meristematic cells that formed micronuclei (control and treated) were estimated. The number of micronuclei in a single cell was not higher than one. The frequency of micronuclei after treatment with MH or MNU varied from 0.53% to 3.03% (Figure 1). The highest frequency of cells with micronuclei was observed at 0 h after treatment after which it decreased with the posttreatment time. However, at 20 h after treatment with MH or MNU, cells with micronuclei were still observed. No MN were observed in the control cells.

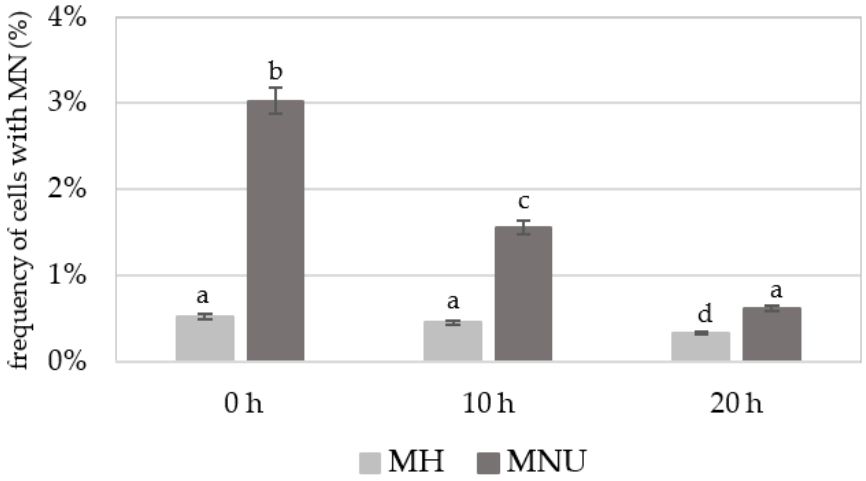

Figure 1. Frequency of *B. distachyon* cells with micronuclei (MN) after MH and MNU treatment. No micronuclei were observed in the control cells. ANOVA followed by Tukey HSD test, $p < 0.05$; mean ± SD, statistically significant differences are indicated by different letters.

2.2. The Presence of 5 mC Signals in the Control and Mutagen-Treated B. distachyon Cells

We performed the procedure of the immunocytochemical detection of **5**-methylcytosine (5 mC) with the secondary antibody conjugated with Alexa Fluor 488 and without the primary antibody. No unspecific binding of the secondary antibody to these samples was detected. The 5 mC signals were always observed in the nuclei in both the control and treated nuclei, whereas the micronuclei that were induced by MH or MNU were either labeled by 5 mC or not. The examples of control nuclei and nuclei with and without micronuclei after MH treatment are shown in Figure 2. We analyzed the frequencies of the micronuclei (MN) with and without 5 mC signals after MH (Figure 3) and MNU treatments (Figure 4) in the *B. distachyon* cells, followed by different posttreatment times: 0 h, 10 h, 20 h. The frequency of MN with 5 mC signals differed in the MH- and MNU-treated cells. It was higher after the MNU treatment and ranged from 75% to 86%, whereas after MH treatment, it was 58.49–68%. The use of posttreatment times after mutagenic treatment with MH and MNU caused an increase in the frequency of MN with 5 mC signals. The frequency of the MN with 5 mC signals increased during the subsequent postincubation times by up to 10% after both MH and MNU treatments.

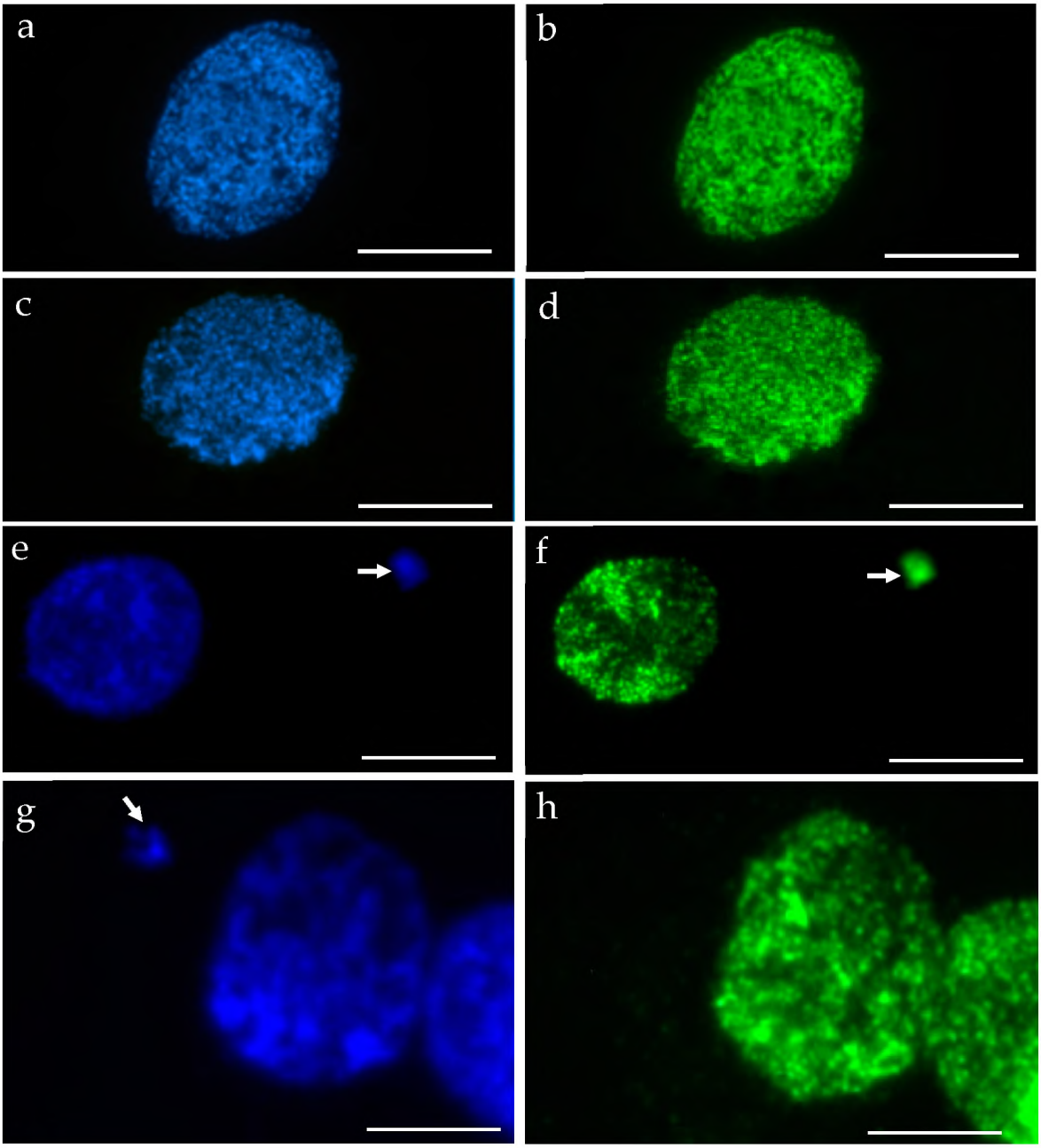

Figure 2. *B. distachyon* interphase nuclei: DAPI stained, blue (**a**,**c**,**e**,**g**) that were used for the immunocytochemical detection of 5 mC, green (**b**,**d**,**f**,**h**): (**a**,**b**) control, (**c**–**h**) treated with MH. Nuclei without micronuclei (**a**–**d**) and with micronuclei (**e**–**h**) were observed. The arrows indicate the micronuclei. The 5 mC signals were always present in the nuclei. Two different kinds of micronuclei were distinguished: those with 5 mC signals (**e**,**f**) and those without signals (**g**,**h**). Scale bars = 10 µm.

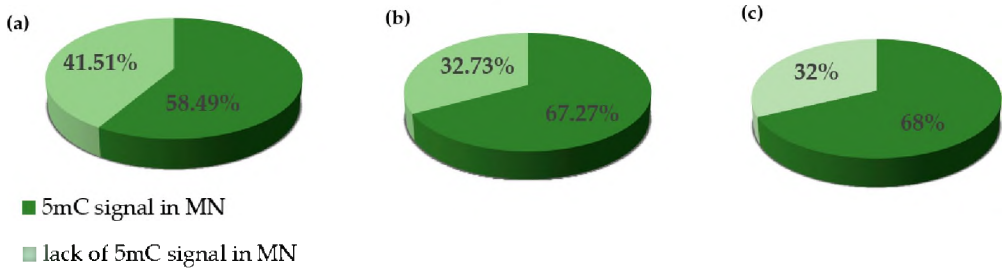

Figure 3. The frequencies of micronuclei (MN) with and without 5 mC signals after **MH treatment** at different posttreatment times: (**a**) 0 h, (**b**) 10 h, (**c**) 20 h.

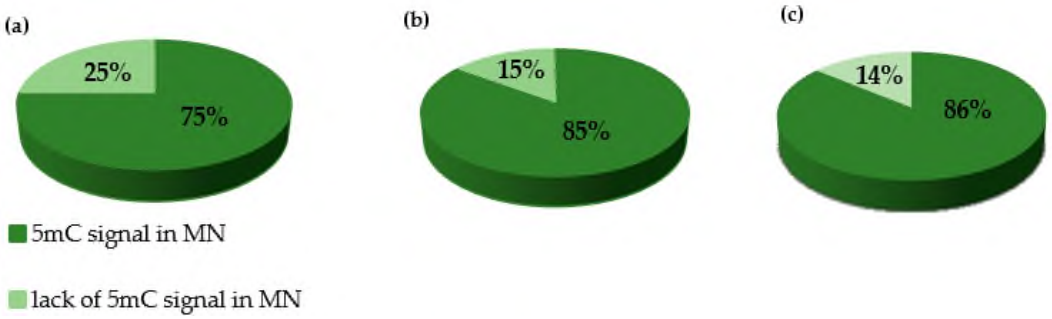

Figure 4. The frequencies of micronuclei (MN) with and without 5 mC signals after **MNU treatment** at different posttreatments times: (**a**) 0 h, (**b**) 10 h, (**c**) 20 h.

2.3. The Level of 5 mC in the Control and Mutagen-Treated B. distachyon Cells

An analysis of the average fluorescence intensity of Alexa 488 in the *B. distachyon* nuclei without micronuclei revealed differences between the control and the MH- and MNU-treated cells (Figure 5). At 0 h, the lowest fluorescence intensity of Alexa 488 was observed in the control cells, this significantly increased after the MH treatment and especially after the MNU treatment. The highest fluorescence intensity of Alexa 488 was observed after the MNU treatment. In the control cells, the 5 mC level was very similar at 0 h and 10 h of the posttreatment time and significantly increased at 20 h. After the treatment with MH, there was a significant decrease in the fluorescence intensity at 10 h of posttreatment, and at 20 h it increased to the same value as at 0 h. The effect of the posttreatment time on the 5 mC level was also observed after the treatment with MNU. The intensity of Alexa 488 increased by 13% at 10 h and 25% at 20 h compared to 0 h.

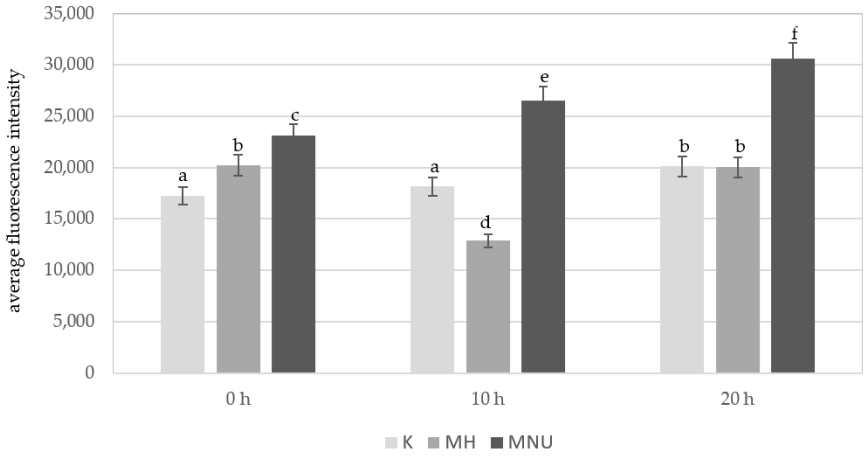

Figure 5. Comparison of the average fluorescence intensity of Alexa 488 that was used to detect 5 mC in the *B. distachyon* nuclei, without MN: in the control and MH- or MNU-treated cells at different posttreatment times. Fluorescence of Alexa 488 was measured in relative units. ANOVA followed by Tukey HSD test, $p < 0.05$; mean ± SD, statistically significant differences are indicated by different letters.

A comparison of the average fluorescence intensity of Alexa 488 in the nuclei with and without micronuclei in MH-treated cells at different posttreatment times showed that the nuclei without micronuclei were characterized by a higher fluorescence intensity than in the parental nuclei for the micronuclei (Figure 6).

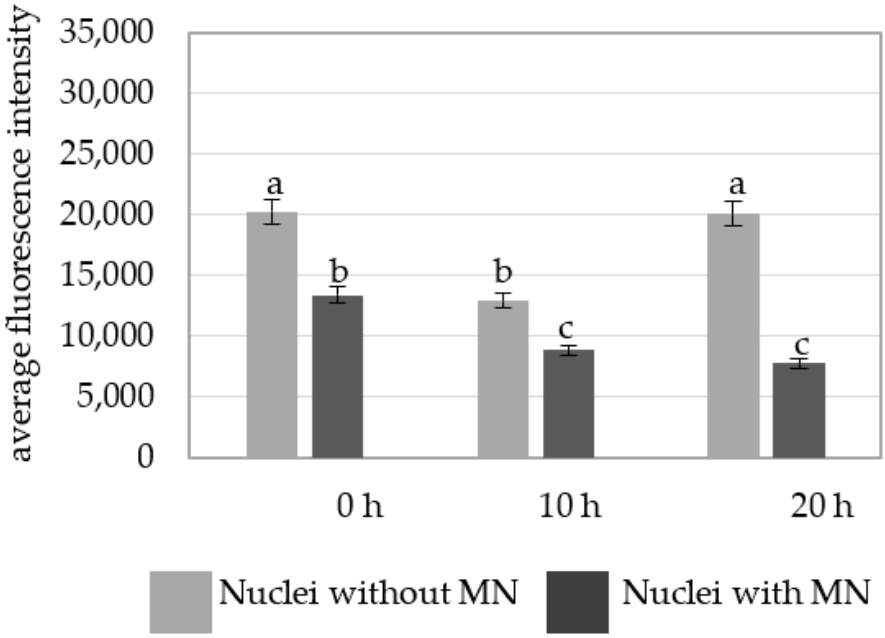

Figure 6. A comparison of the average fluorescence intensity of Alexa 488 that was used to detect 5 mC in the *B. distachyon* nuclei with and without micronuclei in the MH-treated cells at different posttreatments times. Fluorescence of Alexa 488 was measured in relative units. ANOVA followed by Tukey HSD test, $p < 0.05$; mean ± SD, statistically significant differences are indicated by different letters.

The intensity of Alexa 488 fluorescence in the MNU-treated cells was also higher in the cells without micronuclei at 10 h and 20 h (Figure 7).

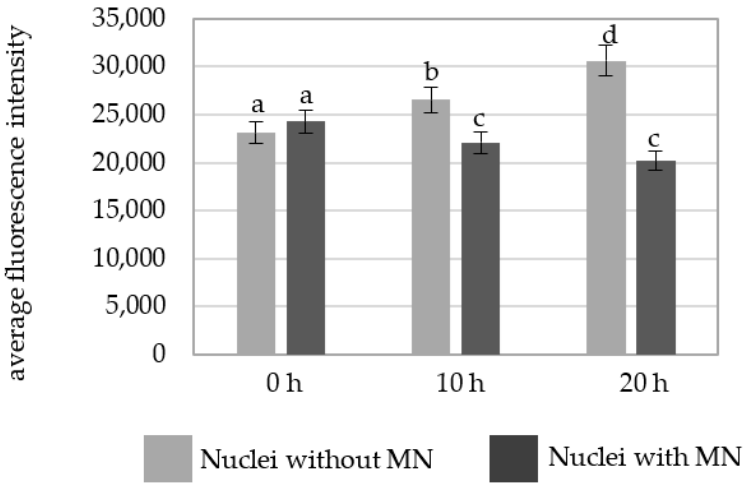

Figure 7. A comparison of the average fluorescence intensity of Alexa 488 that was used to detect 5 mC in the *B. distachyon* nuclei with and nuclei without micronuclei in the MNU-treated cells at different posttreatment times. Fluorescence of Alexa 488 was measured in relative units. ANOVA followed by Tukey HSD test, $p < 0.05$; mean ± SD, statistically significant differences are indicated by different letters.

There were significant differences in the fluorescence intensity of Alexa 488 in the parental nuclei for micronuclei between the MH and MNU treatments (Figure 8). The MNU-treated parental nuclei were characterized by a higher fluorescence intensity than the MH-treated parental nuclei.

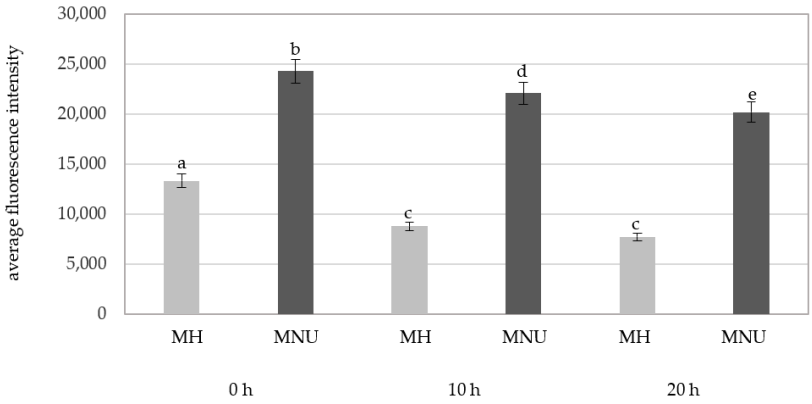

Figure 8. A comparison of the average fluorescence intensity of Alexa 488 that was used to detect 5mC in the *B. distachyon* parental nuclei with micronuclei in MH- and MNU-treated cells at different posttreatments times. Fluorescence of Alexa 488 was measured in relative units. ANOVA followed by Tukey HSD test, $p < 0.05$; mean ± SD, statistically significant differences are indicated by different letters.

We also analyzed the fluorescence intensity of Alexa 488 in the micronuclei that were induced by MH and MNU (Figure 9). The Alexa 488 fluorescence intensity differed for the MH- and MNU-induced micronuclei; it was significantly higher in the micronuclei that formed as a result of the MH treatment at all of the posttreatment times. A decrease in the Alexa 488 fluorescence intensity in MH- and MNU-induced micronuclei at 20 h compared with 0 h was observed with the posttreatment time by 55% after the MH treatment and 75% after the MNU treatment.

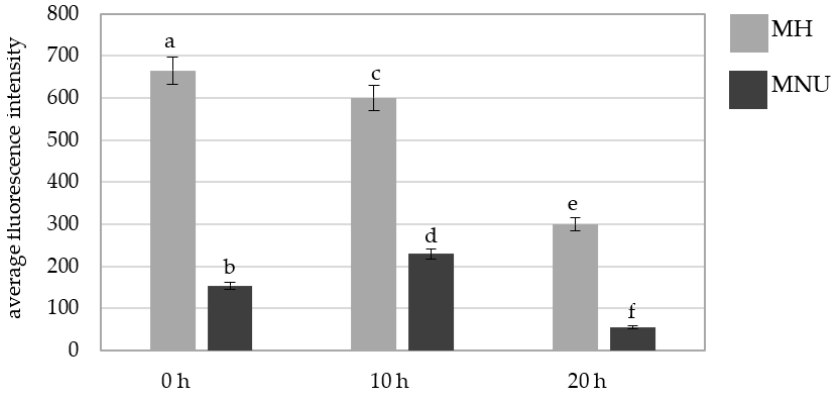

Figure 9. A comparison of the average fluorescence intensity of Alexa 488 that was used to detect 5 mC in the *B. distachyon* micronuclei in the MH- or MNU-treated cells at different posttreatment times. Fluorescence of Alexa 488 was measured in relative units. ANOVA followed by Tukey HSD test, $p < 0.05$; mean ± SD, statistically significant differences are indicated by different letters.

3. Discussion

Environmental epigenetics is a rapidly expanding area of research. Different environmental chemicals that can modify epigenetic marks are known in humans and animals [40]. Hypermethylation is the default epigenetic state and assists in maintaining genome integrity. Several genetic studies have indicated that global DNA hypomethylation is associated with increased genome instability such as changes in ploidy and chromosomal abnormalities [41]. The connection between DNA hypomethylation and genome instability is especially well documented in the context of cancer [42]. While there are numerous molecular studies on the role of various epigenetic modifications in DNA damage and repair in plants [43–49], there is still a lack of analyses of these changes in single nuclei in respect to chromosome aberrations.

We analyzed the DNA methylation in *B. distachyon* single nuclei and micronuclei after mutagenic treatments with two chemical mutagens—MH and MNU. The evaluation of the micronuclei (MN) formation after mutagenic treatment, which was used in this study, is commonly used to measure a compound's clastogenic effect [11]. DNA damage, together with DNA repair, can be evaluated using the MN test, because the double-strand breaks (DSBs) that are caused by mutagens, if not repaired, can lead to micronuclei formation. We showed that MH and MNU induced micronuclei in the *B. distachyon* root meristematic cells. The clastogenic effect of MH in *B. distachyon* was previously reported by our group [17–19], whereas MNU was proven to induce micronuclei in this species for the first time. Whether micronuclei can be re-engulfed by the cell nucleus [50] and whether the micronuclear content can be degraded independently of further cell division are still unknowns [51]. Most probably, micronuclei are considered to be genetic material that is lost from a cell. A decrease in the frequency of micronuclei with the posttreatment times was observed after the MH and MNU treatments, which proves that the micronuclei are eliminated from the cells [12]. This decrease was accompanied by an increase in the number of methylated MN, which suggests that DNA methylation may be correlated to the elimination of chromatin from the nucleus as MN.

We analyzed the presence and the level of **5**-methylcytosine using the fluorescence intensity of Alexa 488 in *B. distachyon* nuclei and micronuclei after mutagenic treatment. To date, different molecular approaches have been developed to map genome-wide DNA methylation. The bisulfite sequencing approach would be helpful to map single-cell genome-wide DNA methylation in animals [52] and plants [53,54]; nevertheless, this does not enable the analysis of the DNA methylation in relation to chromosomes aberrations. The bisulfite sequencing approach will technically not be feasible in single nuclei and micronuclei due to the extremely small size of Brachypodium nuclei, and especially micronuclei, which prevents their isolation. The cytogenetic approach is time-consuming; however, it enables researchers to perform an in situ analyses within a single micronucleus together with its parental nucleus. Unfortunately, this method is much more time-consuming than the molecular methods. Flow cytometry offers an automated high-throughput platform that is reproducible; however, it needs to be standardized for each plant species and the fluorochrome that is to be used and does not provide data on the in situ localization of DNA methylation. A similar methodological approach using manual scoring with a microscope and the appropriate software for analyzing DNA methylation was previously used in human cells [55].

Our data on the level of DNA methylation in nuclei and micronuclei suggest the possible involvement of epigenetic mechanisms in the (in)stability of the *B. distachyon* genome when subjected to mutagens. 5 mC fluorescence intensity showed the level of DNA methylation slightly changed in the control during the applied posttreatments time, although we provided the controlled growth conditions. Such changes, which take place across time during root development, have been observed previously [56]. The fluctuations of fluorescence intensity corresponding to 5 mC across time in mutagen-treated cells was completely different. Therefore, we can cautiously conclude that the fluctuation of fluorescence intensity after mutagen treatment is not only related to the level of 5 mC in the control but also to mutagen action and specific DNA repair processes. This is also supported by the fact that different types of fluctuation were shown for MH and MNU treatment, which were characterized by different mechanisms of action. Unexpectedly, the average fluorescence intensity of Alexa 488 that was analyzed in the nuclei that did not form micronuclei increased after MNU treatment compared to the control, and it also increased with posttreatment times, thereby indicating that DNA hypermethylation processes occurred. The different response that was observed after the MH treatment could have resulted from a different mechanism of action of the applied mutagens. Simultaneously, the significantly lower level of 5 mC in the parental nuclei for MN compared with the nuclei without MN after both the MH and MNU treatments, as well as the decreasing level of 5 mC in the micronuclei during the posttreatments, could indicate that the formation and elimination of MN was

somehow correlated epigenetically via the loss of DNA methylation. The decreased level of 5 mC with the posttreatment times indicates that there are active processes of DNA demethylation in the MN. This scenario is possible if DNA replication takes place in the MN [57], which can be proven by using the "click" reaction with **5**-ethynyl-**2′**-deoxyuridine (EdU) [58]. However, it is also possible that the decrease in the level of 5 mC in the parental nuclei could be the result of the loss of DNA into the MN and as the consequence of DNA elimination from nuclei. Surprisingly, the number of micronuclei with 5 mC signals increased with postincubation times. The hypomethylation of heterochromatin in the pericentromeric region is associated with chromatin decondensation, which leads to improper chromosome segregation and exclusion into the MN in humans [59]. The presence of Alexa 488 signals in the micronuclei indicates the hypermethylated DNA localization. In plants, DNA methylation is restricted to specific genomic regions, e.g., in Arabidopsis, and most of the methylated DNA is composed of local tandem or inverted repeats, transposons and other dispersed repeats around the centromeres and in the euchromatin [60]. A similar localization in the pericentromeric regions has been shown for *B. distachyon* [61]. Thus, because most of the micronuclei contain the signals of Alexa 488, they probably originated from the chromosome fragment(s) of the pericentromeric region(s), which are hypermethylated as a result of chromosome breakage or whole chromosomes as a result of kinetochore disturbances. It was previously shown that the localization of chromosome aberrations within the *B. distachyon* genome is not random [17–19]. On the other hand, methylation of the coding regions is common among eucaryotes and, therefore, could be a preventive mechanism for micronuclei formation [62]. According to Fenech et al. [20], the main mechanism of MN formation that originates from chromosome missegregation is the hypomethylation of the centromeric and paracentromeric regions—the satellite repeats. The epigenetic mechanisms of MN formation in animals were revealed using folate, which is a B group vitamin that is crucial for DNA methylation [63]. Our research is a rare example of the analysis of the DNA methylation in relation to the chromosome aberrations, namely micronuclei in plants. Previously similar studies were performed in humans [64]. The authors undoubtedly evidenced the contribution of epigenetic alterations to MN formation in human cells; micronuclei formation is induced epigenetically, mainly through the loss of DNA methylation.

The characteristic response of genomes, which are visible as changes in DNA methylation level for genotoxic agents, could be due to a specific mechanism of action of mutagens. MH is a well-described clastogenic mutagen that acts in the S phase, whereas MNU causes point mutations, although chromosome damage is also a characteristic for it. These specific effects regarding DNA methylation in response to two chemical mutagens need to be determined in studies with a larger number of chemical and physical compounds that are characterized by different mechanisms of action.

The number of studies on the epigenetic impact of mutagens on the plant genome and the contribution of epigenetic alterations in MN formation is limited and the molecular mechanisms of their influence remain unknown. However, some hypotheses considering DNA hypomethylation in MN formation are postulated. Hypomethylation of repeated DNA sequences, such as satellite DNA in the centromeric and pericentromeric regions of chromosomes, could be linked with chromosome instability. Specifically, hypomethylation of heterochromatin in the pericentromeric regions is associated with chromatin decondensation, which leads to improper chromosome segregation and exclusion into MN, whereas global hypomethylation is associated with more relaxed chromatin, increased gene expression, elevated DNA damage and chromosomal breaks that form MN with acentric chromosome fragments [64]. The obtained results indicated hypomethylation in nuclei that form MN and, additionally, in MN after treatment with MH. The effect of hypomethylation and MN formation was similar to other research in humans [65,66]. The current data indicate that DNA methylation is somehow involved in the genotoxicity effects in plants that are caused by MH and MNU. Thus, we proposed that this could be used as a genotoxicity endpoint together with micronuclei formation. Other epigenetic modifications,

such as H2AX phosphorylation, served as an early marker for DNA damage in cancer predictivity [55]. Bleomycin and MMS led to the exclusion of the H2AX phosphorylation foci from heterochromatin into the MNs within the majority of nuclei. H2AX foci have also been largely excluded from heterochromatin after irradiation [67]. DNA methylation should be integrated into current genetic toxicology in order to explain the mechanisms of action of genetic instability.

4. Materials and Methods

4.1. Plant Material, Mutagenic Treatment and Slide Preparation

The plant material used in the study was *Brachypodium distachyon* seeds (Brachypodium, 2*n* = 10, cv. B21). The seeds were pre-soaked in water for 6 h, then sown in Petri dishes on moist filter paper and germinated at 21 °C in the dark for 72 h. The seedlings were treated with maleic hydrazide (MH, 4 mM; Sigma, CAS 123-3301) or nitroso-N-methylurea (MNU, 3 mM, Sigma, CAS 684-93-5) for 3 h in the dark under aeration at 21 °C. The mutagenic treatment procedure was repeated twice. The treatment conditions with MH used in the study were used in previous experiments in which the cytogenetic effects were estimated in *B. distachyon* [17–19]. The control seedlings were incubated under the same conditions in distilled water. After the treatment, the seedlings were washed three times in distilled water and then germinated in Petri dishes. The material was fixed in ethanol–glacial acetic acid (3:1) at three postincubation times: 0 h, 10 h and 20 h after treatment. The roots of the seedlings were used as the source of the meristems for the investigations of the micronuclei. For the nuclei and micronuclei preparations, the material was washed with a 0.01 mM sodium citrate buffer (pH 4.8) for 30 min and digested with an enzyme mixture containing 1% pectinase (*v/v*, Sigma) and 2% cellulose (*w/v*, Sigma) for 1.5 h at 37 °C. After digestion, the material was washed again with a sodium citrate buffer for 30 min. The squash preparations were prepared in a drop of 45% acetic acid. After freezing and coverslip removal, the slides were dried.

4.2. Immunostaining

The immunostaining step in *B. distachyon* was carried out as was previously described for barley with minor modifications [68]. The immunodetection of **5**-methylcytosine (5 mC) within nuclei and MN was performed using the primary antibody-mouse anti-**5**-methylcytosine (1:100 dilution in 1% BSA in 1xPBS, Abcam, cat. no. ab73938), and then detected by the secondary antibody goat anti-mouse IgG antibodies (1:100 dilution in 1% BSA, Invitrogen, Molecular Probes, cat. no. A-11001), which had been conjugated with Alexa Fluor 488.

Prior to the immunodetection of 5 mC, a chemical denaturation was performed with a solution of 0.25 M sodium hydroxide (NaOH, Merck) and 1 M sodium chloride (NaCl, POCH) for 30 min at 4 °C. The preparations were washed three times in distilled water and then incubated in 1 M Tris-HCl (VWR) for 30 min. Then, the slides were dehydrated in an alcohol series: 70%, 90% and 100%. Before applying the anti-5 mC antibodies, the slides were blocked with 5% BSA (Sigma) at RT in a moist chamber for 1h. Immunostaining with the primary antibodies, anti-**5**-methylcytosine antibodies, was performed overnight in a moist chamber at 4 °C. The slides were washed three times in 1xPBS, after which the secondary antibodies goat anti-mouse IgG antibodies were applied and then incubated in a moist chamber for 1 h at 37 °C. After that, the slides were washed three times in 1xPBS. The preparations were mounted and counterstained in Vectashield (Vector Laboratories, Peterborough, UK) that contained 2.5 µg/mL DAPI (Serva).

4.3. Image Acquisition, Data Analysis and Statistics

The slides were analyzed using a Carl Zeiss Imager Z2 fluorescence microscope with fluorescent lighting HxP, 120W. The images were recorded with an AxioCam ICc5 digital camera and immersions lens with a ×100 magnification.

The frequencies of root cells with micronuclei were analyzed for the control (untreated) and the MH- and MNU-treated meristems. Based on the analyses of the presence or absence of methylated DNA in the micronuclei and their parental nuclei, the frequency of cells with the 5 mC signals was calculated. Additionally, the average fluorescence intensity of Alexa Fluor 488 was analyzed in order to estimate the global level of DNA methylation in the nuclei that were the parental nuclei for the micronuclei and nuclei that did not form micronuclei. The fluorescence intensity of Alexa 488 was measured as the mean values from the integrated density (IntDen) parameter per nuclei using ImageJ (National Institutes of Health; http://imagej/nig.gov (accessed on 18 April 2021)). The integrated density parameter is the sum of all of the pixels within the region of interest (ROI). The eight-bit images with Alexa 488 fluorescence were segmented with the threshold value parameter, and their fluorescence intensities were measured as the mean values from the integrated density parameter. The results of these measurements were estimated in relative units.

The mutagenic treatment experiments using MH and MNU were repeated twice. Three slides, each made from one meristem, were analyzed for each of the two mutagenic treatment experiments. The frequencies of the cells with micronuclei were estimated for 3000 cells for each treatment (control, MH, MNU). Two hundred nuclei per each slide were analyzed in order to estimate the average fluorescence intensity. About 100 micronuclei for each treatment group were analyzed for the presence of the 5 mC signal and average fluorescence intensity. An ANOVA test followed by Tukey HSD test, $p < 0.05$; mean $\pm$ SD, were used for statistics.

5. Conclusions

In this study, we presented the analyses of DNA methylation in *B. distachyon* nuclei and micronuclei after chemical mutagenic treatments. This is the first example of such a study in plants. To conclude, our results indicated that (1) the level of DNA methylation in mutagen-treated nuclei (both with and without micronuclei) is different if compared to a control and (2) the level of DNA methylation in mutagen-treated nuclei depends on the mutagen type. Our results indicate also that MH and MNU induce micronuclei with different 5 mC levels. The open question remains as to whether DNA methylation has an active role in micronuclei formation, or it is only a marker for chromatin exclusion into micronuclei. More data on the relationship between micronuclei and DNA methylation would be provided using a single-cell genome-wide bisulfite sequencing approach. However, this is technically unfeasible in single Brachypodium nuclei and micronuclei due to their extremely small size. The data on the response of plant genomes to other mutagens that are characterized by different mechanisms of action would also provide more confident conclusions.

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Review

Plant Cytogenetics in the Micronuclei Investigation—The Past, Current Status, and Perspectives

Jolanta Kwasniewska * and Adrianna Wiktoria Bara

Plant Cytogenetics and Molecular Biology Group, Faculty of Natural Sciences, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland; adriannabara@gmail.com
* Correspondence: jolanta.kwasniewska@us.edu.pl

Abstract: Cytogenetic approaches play an essential role as a quick evaluation of the first genetic effects after mutagenic treatment. Although labor-intensive and time-consuming, they are essential for the analyses of cytotoxic and genotoxic effects in mutagenesis and environmental monitoring. Over the years, conventional cytogenetic analyses were a part of routine laboratory testing in plant genotoxicity. Among the methods that are used to study genotoxicity in plants, the micronucleus test particularly represents a significant force. Currently, cytogenetic techniques go beyond the simple detection of chromosome aberrations. The intensive development of molecular biology and the significantly improved microscopic visualization and evaluation methods constituted significant support to traditional cytogenetics. Over the past years, distinct approaches have allowed an understanding the mechanisms of formation, structure, and genetic activity of the micronuclei. Although there are many studies on this topic in humans and animals, knowledge in plants is significantly limited. This article provides a comprehensive overview of the current knowledge on micronuclei characteristics in plants. We pay particular attention to how the recent contemporary achievements have influenced the understanding of micronuclei in plant cells. Together with the current progress, we present the latest applications of the micronucleus test in mutagenesis and assess the state of the environment.

Keywords: chromosome aberrations; cytogenetics; DNA damage; micronuclei; mutagenesis

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1. Introduction

Cytogenetics is the branch of genetics, cytology, and cell biology that analyses the nuclear genomes at the chromosome level. Cytogenetics makes the chromosome a substantial target in elementary plant cell biology and other fields such as mutagenesis and genotoxicity studies. Standard cytogenetic methods were, and are still, commonly used. Modern cytogenetic technologies involving advanced microscopy and imaging methods, that progress in the analyses on epigenetic DNA and histone modifications as well as DNA damage by using fluorescent antibodies benefit plant genome structure, dynamics, and evolution. They have also served the comprehensive evaluation of the effects of various mutagens on the plant genome that are observed as chromosome aberrations, including micronuclei (MN). Mutagens affect the structure of DNA and cause double-strand breaks (DSBs) leading to MN formation. The elimination of MN causes DNA loss. Micronuclei are induced by many mutagenic factors, both physical and chemical, as well as those of an environmental nature. The analysis of their frequency is the basis of the commonly used micronucleus test. We provide a comprehensive overview of the current knowledge on MN characteristics in plants. This paper focuses on critical scientific problems: Is the distribution of DNA damage that led to micronuclei formation random? What

is the origin of plant micronuclei? Are epigenetic processes involved in micronuclei formation? How could there be a role of the genetic activity of chromatin in the formation of micronuclei?

2. The Importance of the Micronucleus Assay in Plants

Micronuclei (MN) are structural chromosome aberrations that are detected in non-dividing cells during interphase. Among numerous genotoxicity assays, the micronucleus (MN) test is especially recommended to evaluate the genotoxic effects of chemical and physical agents, as well as mixtures of substances. Since 1959 when the MN assay was first applied in kidney beans, followed the treatment with gamma-ray [1], it served as a well-established, fast, and reliable routine system for measuring the genome damage that is caused by genotoxic agents in mitotic and meiotic plant cells [2,3]. Currently, the MN test is still successfully used in testing many agents, including pesticides, nitroaromatic compounds, polyaromatic hydrocarbons, nitrosamines, heavy metals, ionizing radiation, and industrial chemicals, as well as other environmental samples [4–6]. Nowadays, the interactions of nanoparticles with plants have become a new field in micronuclei assays [7–9]. Currently, the testing is mainly performed in *Allium*, *Nicotiana*, and *Vicia* [10–13] and other model plants [7,14–17].

Compared to the chromosomal aberrations (CA) assays that are applied to mitotically divided cells, the MN test is less time-consuming and easier to perform. Most mutagens decrease mitotic activity, thus making the chromosome aberrations analyses in dividing cells, especially in metaphases, challenging and often impossible.

Although the knowledge on different aspects of the origin, structure, genetic activity, and micronuclei in plants has been explored in recent years, there is still much less that is known than in humans and animals. There are many reviews of MN in humans and animals, also from the last few years [18–27]. Micronuclei have become a potential linkage biomarker to cancer and aging-related diseases [28]. The MN test is now quite widely described in plants [29], although there are still no reviews that summarize all the data on MN, with particular reference to the latest methodological developments in the field of molecular cytogenetics.

3. Micronuclei—The Formation and Fate

Micronuclei (MN) are small, extranuclear bodies that are located next to the parental nucleus in the cytoplasm. Micronuclei are detected in the meristematic interphase cells of the shoots or roots, in the next cell cycle, followed by treatment with mutagen.

MN can originate in two ways. They can arise from acentric fragments resulting from double-strand breaks (DSBs) which are not repaired or repaired improperly. The micronuclei could also occur from the entire chromosome(s) that does not attach to the mitotic spindle at metaphase. Likewise, micronuclei that have arisen from entire chromosomes could result from kinetochore damage, failure of the cell cycle control system, or centromeric DNA hypomethylation. Thus, the knowledge on the origin of micronuclei allows for assessing the mutagen's mechanism of action as clastogenic or aneugenic. Changes in the structure of the chromosomes, such as chromosome fragments and delayed chromosomes, can also be detected during mitosis; however, their detection is not as straightforward as during interphase. Some micronuclei might also be derived from the breakage of anaphase bridges that are formed from dicentric chromosomes, concatenated ring chromosomes, the union of sister chromatids, unresolved sister chromatid connections, or chromosomes that have merged by telomere fusion.

The number of micronuclei in a single cell is most often one, but sometimes cells with a higher MN number are observed, depending on the number of chromosome fragments or delayed chromosomes. It still needs to be emphasized that, taking into account the mechanism of micronucleus formation, the frequency of dividing cells after the mutagenic

treatment influences the frequency of micronuclei. Cells need to divide so that chromosome fragments can be removed outside the newly formed daughter nuclei and create an MN.

From the data that are available for animals and humans, micronuclei can be lost from the cells and incorporated into the nucleus [30]. There are no specific data on the fate of micronuclei in plants cells.

4. Conventional Cytogenetics

The conventional cytogenetic is recognized as the approach for the detection and basic description of the MN after mutagenic treatment. Changes in chromosome morphology are usually detected using the basic chromosome-staining techniques, such as the Feulgen method, acetoorcein, and Giemsa stainings. Among these staining methods, the Feulgen technique (Figure 1) is characterized by the best contrast of chromatin staining; however, this procedure needs a longer time and a more complicated process. Together with slide scoring, these techniques allow the analysis of the frequency of MN. Possibly too small MN are not detected with these methods, and only those that arise from whole chromosomes or large chromosome fragments are visible.

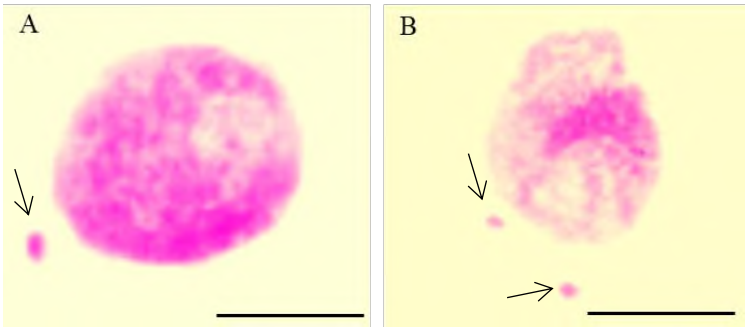

Figure 1. Nuclei with micronuclei (MN): one MN (**A**) and two MN in one cell (**B**) after maleic acid hydrazide (MH)-treatment in *Crepis capillaris* root meristematic cells; Feulgen technique. Arrows show the micronuclei. The bars represent 5 μm.

Nowadays, fluorescent methods, e.g., DAPI (4′,6-diamidino-2-phenylindole staining) (Figure 2) or acridine orange stainings rather than traditional methods, are recommended for micronuclei detection and scoring instead of conventional methods.

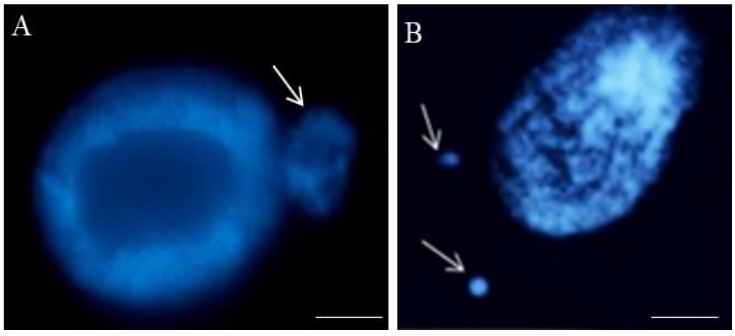

Figure 2. Nuclei with micronuclei: one (**A**) and two in one cell (**B**) after maleic acid hydrazide (MH)-treatment in the root cells of *Crepis capillaris* seedlings. The micronuclei differ in size; DAPI staining. Arrows show the micronuclei. The bars represent 20 μm.

The fluorescence methods are quick and precise, and even small micronuclei can be detected [31]. Still, the analysis of micronuclei using these simple methods does not provide any information on the localization of the DNA breaks and the mechanisms that lead to their formation.

5. Molecular Cytogenetics

The early and current achievements of molecular cytogenetics have led to progress in the detection and detailed characterization of micronuclei (MN) [32,33]. Modern cytogenetics techniques have revolutionized knowledge on the composition and genetic activity of the chromatin that is involved in micronuclei. The knowledge on the specific genetic content of the micronuclei is essential as they could be related to the ability of chromatin in the micronuclei to exert proper DNA expression and DNA repair. Among techniques, fluorescence in situ hybridization (FISH) and all its modifications have been successfully used in the modern generation era of DNA damage characterization. Molecular cytogenetics that is based on the multi-fluorescence detection of the specific chromosomes landmarks or painting whole chromosomes represents a milestone in DNA damage analyses in relation to genome organization. Additionally, it enables the studies of even minute details of the chromosome, providing the analyses of DNA damage more accurately and precisely. In the time of sequencing of plant genomes, FISH becomes even more important as many new chromosome-specific probes become available. Moreover, the cytogenetic analyses of DNA and histone epigenetic modifications on plant chromosomes and nuclei provide new possibilities to learn the role of plant chromatin dynamics in response to mutagens [34]. Currently, the involvement of the histone modifications was proven to be closely related to plant environmental stress [35].

5.1. Fluorescence in situ Hybridization Serves to Understand the Origin of Micronuclei

A breakthrough in the analyses of the localization of DNA damage at the chromosomal level in plants came with applying the fluorescence in situ hybridization (FISH). It provides information on the possible 'hot spots' in plant genomes for DNA damage after the action of mutagens. Also, it gives information on the mechanisms of the biological effect of the individual agents that induce DNA damage. This knowledge is particularly crucial in plant mutagenesis, as the use of the chemical and physical mutagens is the most common way to obtain mutants. This technique could detect even extremely small aberrations in dividing and non-dividing cells.

There is only one morphological type of micronuclei that may differ in size (Figure 2). The size of the micronucleus does not provide any information on whether it originated from chromosome fragments or entire chromosome(s), as the size may be related to the different degrees of the chromatin condensation. A more detailed analysis of the involvement of a specific chromosome or chromosome fragments in micronuclei formation is possible using fluorescence in situ hybridization (FISH). So far, FISH has not found such a wide application in the study of chromosome aberrations, including MN, in plants, as it has in humans [36–38]. Different types of DNA probes for FISH are applied in plants, e.g., repetitive DNA sequences, single-locus chromosome-specific BAC clones, partial (e.g., arm), and whole chromosome paints. The limitations of the chromosome-specific DNA sequences in plants make the comprehensive identification of chromosome fragments in micronuclei using FISH still limited to a few species. Among the repetitive DNA sequences, centromere, *Arabidopsis thaliana* (Arabidopsis)-type (TTTAGGG)n telomeric sequences, and ribosomal DNA (rDNA), which give strong and easily observed FISH signals, have found application in the detailed characterization of MN. These DNA sequences' advantages are evolutionary conservation and location at a specific chromosome region. Repetitive dispersed DNA sequences are not a good source for probe pool for fluorescence in situ hybridization to study the origin of micronuclei.

FISH using 45S rDNA as the probe was first applied to localize the chromatin aberrations, such as translocations [39] and anaphase bridges [40], in *Arabidopsis thaliana.* Applying the rDNA as probes showed rules regarding gamma-ray–induced MN formation in barley (*Hordeum vulgare*) (Figure 3).

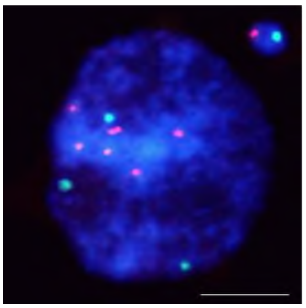

Figure 3. *Hordeum vulgare* interphase nuclei with the micronucleus induced by X-radiation. The nucleus was subjected to mcFISH with 5S rDNA (red) and 25S rDNA (green) probes. The micronucleus has one 5S rDNA and one 25S rDNA. Chromatin is stained with DAPI (blue). The bar represents = 10μm.

5S rDNA-bearing chromosomes are shown to be more often involved in MN formation than NOR chromosomes in barley [41,42]. Similar rules regarding radiation-induced MN formation have been found in *Brachypodium distachyon* [43]. The hot spots for chromosome breakage in *Lolium multiflorum* were not correlated with rDNA sites [44].

The use of the centromere and telomere-specific DNA sequences for FISH also provided some rules regarding the origin of MN. It confirmed that the gamma ray-induced MN may originate from acentric fragments or whole lagging chromosomes. Thus, this approach allows the distinguishing of the micronuclei being a clastogenic and aneugenic effect of mutagens. However, most MN had only telomeric DNA signals, indicating that terminal deletion is the primary type of chromosome aberration leading to their formation (Figure 4).

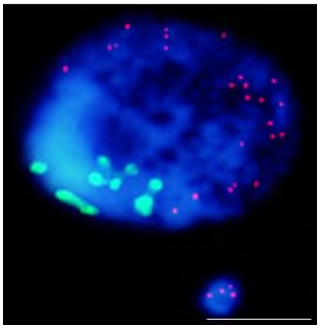

Figure 4. Results of mcFISH with telomeric (red) and centromeric (green) probes. *Brachypodium distachyon* interphase nuclei with micronucleus that were induced by X-radiation; micronucleus shows only telomeric DNA signals. The bar represents 5 μm. Micrograph by A. Kus.

Comparing the contribution of particular chromosome fragments in MN that are induced by different chemical clastogens, the maleic acid hydrazide (MH) and nitroso-N-methyl-urea (MNU) have shown the difference in the size of the chromosome fragments that are involved in the MN. Most MH-induced MN originated from large acentric fragments, whereas MNU-induced MN is from small terminal chromosome fragments [41,42].

FISH provides much more information about MN formation with DNA probes that are dedicated to different chromosomes or particular chromosomes. Standard A- and B-chromosome-specific probes were successfully used in the rye gamma-irradiated cells (*Secale cereale* L.) [45] for the detection of the translocations between the A- and B-chromosomes.

One of the FISH approaches that is used to detect and characterize micronuclei in plants is multicolor FISH (mcFISH). It is based on the two consecutive FISH experiments that use a pair or pairs of probe sets that are removed after each experiment and include the reprobing step. Combining more than two differently labeled DNA probes on the same nuclei slide makes this technique more informative [46]. For the first time, mcFISH

has been applied in human carcinogenicity studies [47], then it has found application in mammalian cells [48]. mcFISH is a common technique that is widely used in plants; however, it has narrow application in plant mutagenesis and genotoxicity. For the first time, this approach was applied in the analysis of the involvement of four different DNA sequences: 5S rDNA, 25S rDNA, the Arabidopsis-type (TTTAGGG)n telomeric sequence, and the Brachypodium-originated centromeric BAC clone CB33J12 in the micronuclei formation in *Brachypodium distachyon* root-tip cells that were subjected to a chemical mutagen [43].

The most advanced FISH-based approach in plants is chromosome painting (CP), which permits the selective visualization of entire chromosomes or their specific segments during mitosis as well the interphase [49–54]. The wide use of this technique for humans and mammals to determine the involvement of specific chromosomes in the formation of micronuclei showed that they preferentially comprise particular chromosomes that are related to the chromatin organization [55]. The large amounts of repetitive DNA on all chromosomes are obstacles to CP on plants. CP is limited to a few plant species: Arabidopsis [56], Brachypodium [57], and few other species that are characterized by a small genome. mcFISH and CP with low repeat (small and large pools of bacterial artificial chromosomes (BAC)) clones that are specific for selected chromosomes, were applied to improve the 'standard' MN test in *Brachypodium distachyon* (Figure 5).

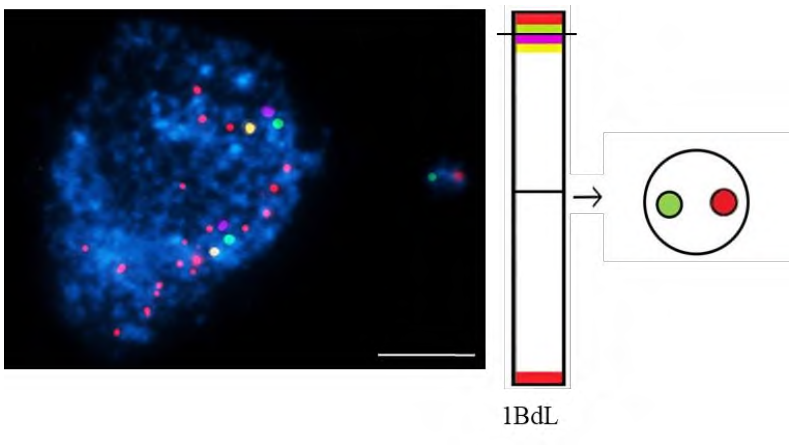

Figure 5. *Brachypodium distachyon* interphase nuclei with micronuclei that were induced by MH-treatment that were subjected to mcFISH with the following probes: telomeric sequence (red), I BAC pool (green), II BAC pool (violet) , and III BAC pool (yellow). Chromatin is stained with DAPI (blue). The diagram next to the photomicrographs shows the putative origins of the micronuclei. Transverse dashed lines indicate chromosome breakpoint. The scale bar = 5μm**.** Micrographs by A. Kus.

BAC-FISH-based chromosome painting provides new information on the composition, origin, and mechanisms of micronuclei formation that is induced by MH-treatment and X-radiation in Brachypodium by showing the 'fragile spots' of DNA breaks [58]. Site-specific DNA breaks in chromosomes Bd4 and Bd5 were shown [59].

To summarize, FISH provides new insights into the localization of DNA breaks on plant chromosomes, proving the non-random distributions of chromosome aberrations. The reasons for this non-random distribution may be the spatial organization of the nucleus at the interphase, the diverse transcriptional activity of specific chromosome regions, and chromosome size. Single BAC-FISH-based chromosome barcoding and 'chromosome painting' approaches have proven to be effective in analyzing the mechanism of micronuclei formation in plants after mutagenic treatment. The advantages of the FISH technique in terms of accuracy and quality of quantitative analyses make the technique one that is likely to become more widespread in DNA damage studies in plants.

5.2. Genetic Activity and DNA Replication

The nucleolus, whose primary function is ribosomal RNA (rRNA) synthesis and ribosome biogenesis, plays a crucial role in the response to biotic and abiotic stress. This aspect has not been extensively studied in plants [60]. Various stresses can lead to alterations in the protein content and organization of plant nucleoli due to alterations in nucleolar transcriptional activity [61]. The nucleolus, including rRNA genes that are arranged in tandem DNA arrays, is observed during interphase. Then nucleoli are reconstituted on NOR sites during mitosis. Its activity differs depending on the environmental conditions [62].

The p53 transcription factor plays a significant role in the DNA damage response (DDR) in mammalian cells to maintain genome stability [63]. Plants developed their unique system for stress response that involved nucleolar proteins; many plant proteins are involved in DDR [64,65].

In plants, cytogenetic studies of the activity of rRNA genes in MN seem to be particularly important as the frequent involvement of the rRNA genes in their formation was shown for a few species: barley (Figure 6), Brachypodium, and *Crepis capillaris* [42,43].

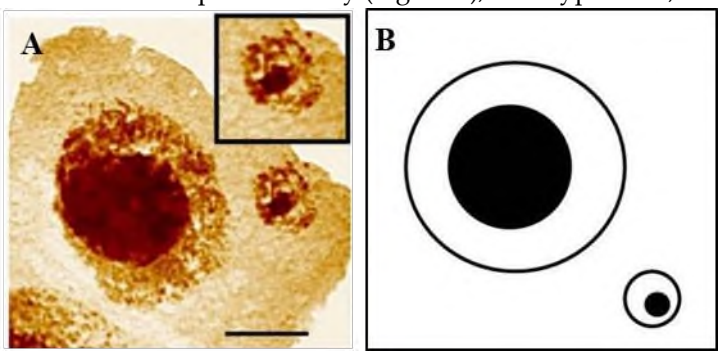

Figure 6. *Hordeum vulgare* interphase cell with micronucleus after treatment with MH. Staining with the silver-staining method (**A**) and scheme (**B**). The bar represents 10 μm. Micrographs by J. Jaskowiak.

The transcriptional activity of 35S rRNA genes that are present in MN that were analyzed using silver-staining is always maintained in barley [66]. MN in *Vicia faba*, with a nucleolar organizer, could synthesize protein and replicate DNA [67]. Studies on the transcriptional activity in plants in the main nuclei after being subjected to different stresses are more common than in the micronuclei. The changes in the number and size of nucleoli, their disintegration, and leakage into cytoplasm were detected in plant cells in response to various stress factors [68,69]. The molecular aspects of nucleolar stress responses in plants were reviewed by Ohbayashi et al. [70].

Many studies on the transcriptional activity in micronuclei were performed in humans [71]. It depends on the micronuclear content; the micronuclei that originate from the whole chromosome show transcriptional activity, whereas MN containing acentric fragments do not. The role of nuclear pore complexes is being considered in cancer cells [72].

Precise genome replication is crucial in maintaining the stability of genomes and any replication errors are critical for living cells. The studies on the genetic activity of chromatin in MN also includes the ability to replicate DNA. The early studies on the micronuclear chromatin replication indicate the heterogenous behavior of MN in animal cells [73]. DNA synthesis was studied using pulse labeling of cells with bromodeoxyuridine (BrdUrd, BrdU) followed by the immunofluorescence detection with anti-BrdUrd antibodies. If the micronuclear DNA can replicate, it also usually occurs in the main nucleus. DNA synthesis in micronuclei corresponds with nuclei during the S-phase in approximately 98% of the micronuclei.

Nowadays, there has been progress in detecting S-phase nuclei and DNA replication in the MN. BrdU, with many disadvantages, such as a denaturation step and low specify

that is correlated with the size of antibody signals, has been replaced by modern labeling higher resolutions techniques - click" reaction with 5-ethynyl-2'-deoxyuridine (EdU) [74,75] (Figure 7).

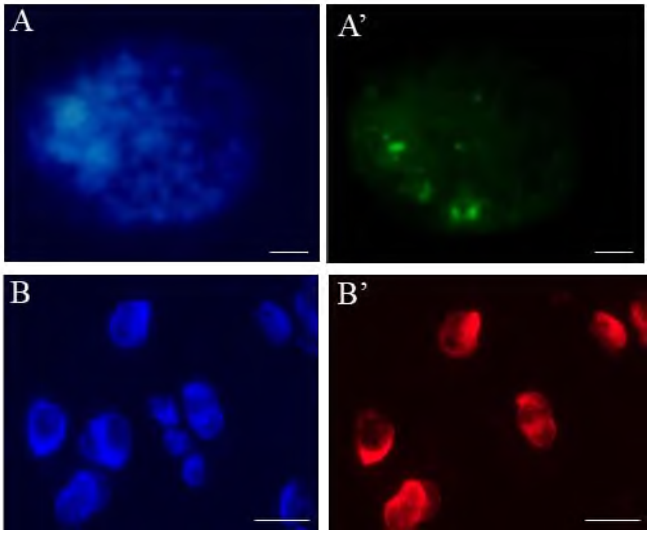

Figure 7. Localization of the replication sites in *Hordeum vulgare* root-tip nuclei using (**A'**) EdU and (**B'**) BrdU labelling. Nuclei are counterstained with DAPI (**A**,**B**). The bars represent 5 μm.

Distinct rules have been observed in plant cells when the replication ability of micronuclei was analyzed using the pulse EdU labeling method [76]. The presence of S-phase labeling characterized only 1% of the micronuclei. The ability of micronuclear chromatin to be replicated is greatly influenced by the specific genetic content of the micronucleus.

5.3. DNA Damage and Repair

The DNA damage response (DDR) plays a role in maintaining the genome integrity in response to abiotic and biotic stresses [77–81]. The final effect of a mutagenic treatment is the primary DNA damage and the process of DNA repair. Strand breaks, which can lead to changes in the chromosome structure, including MN, are the most important types of damage that have been observed at the DNA level. Of the 5000 single DNA breaks that were generated during one cell cycle, only 1% are converted into double DNA breaks (dsDNA); micronuclei constitute a significant result of dsDNA.

Many methods have been developed to detect and localize DNA damage in a genome, quantify the repair processes, and thus provide better insight into the mutagenesis process in various organisms [82]. DNA breakage after mutagenic treatment can be quickly evaluated using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) test to analyze the frequencies of cells that have fragmented DNA [76]. It detects single and double DNA strand breaks in interphase nuclei. The 3`-OH termini are enzymatically-labeled with a modified nucleotide such as fluorescein dUTP. The reaction is catalyzed by the terminal deoxynucleotidyl transferase (TdT), and the signals are detected using fluorescence microscopy. All the nuclei are simultaneously stained with another fluorochrome, e.g., DAPI (4',6-Diamidino-2-phenylindole), and, therefore, the percentage of damaged nuclei using positive labeling is possible. The advantages of the TUNEL test are its ability to detect DNA breaks in a single nucleus with the possible analysis of specific localization within it as well as the short time that is required for an assay and the easy screening of the labeled nuclei. This test has been recommended for the preliminary evaluation of the genotoxicity of any newly tested agent, both in the main nuclei and the micronucleus.

5.4. Chromatin Structure and Its Role in Response to Mutagens

Epigenetic modifications of chromatin, which are defined as being mitotically- and meiotically-heritable changes in the gene expression patterns that arise independent of the changes in DNA sequence, are essential for many biological processes, including growth and reproduction. Post-translational modifications of histones and DNA methylation are the main epigenetic modifications that have a causal role in establishing different chromatin states. Chromatin is a dynamic complex of DNA and proteins. The two main chromatin states can be distinguished: compacted and repressed, the so-called heterochromatin, or the less condensed and gene-rich euchromatin. Earlier studies indicated that the heterochromatic regions represent 'hot spots' for the aberrations that are induced by S-phase-dependent mutagens.

DNA methylation is one of the epigenetic modifications that has been studied in plants most intensively. At the chemical level, this process involves the covalent addition of a methyl group to the 5th position of cytosine in a pyrimidine ring. It is catalyzed by the methyltransferase enzymes using S-adenosyl methionine as the methyl group donor. In plants, the heterochromatin domains are determined by the methylation of cytosines (5mC), and there is a close link between DNA and histone methylation. DNA methylation is highly concentrated in the heterochromatin domains, mainly in the centromeric regions and repetitive sequences. Cytologically, heterochromatin, which has a high level of methylated DNA, can be defined as intensively DAPI-stained chromocenters during the interphase.

Additionally, the same specific patterns of 5mC can be found along the metaphase chromosomes [83]. Also, DNA demethylation occurs in plants. This phenomenon can be achieved through passive DNA demethylation, e.g., during the replication process or active DNA demethylation via the action of specific demethylating enzymes. DNA methylation is involved in the plant's response to environmental stresses. Recent studies have shown the differential regulation of genes encoding epigenetic regulators and chromatin and DNA methylation changes in response to various abiotic stresses, including cold, salinity, drought, and osmolality [84]. There are some studies on epigenetic modifications' involvement in the MN formation in mammals, and only single study in plants to date. Based on the studies on humans, it was shown that the MN formation was induced epigenetically mainly through the loss of DNA methylation. Specifically, the hypomethylation of heterochromatin in the pericentromeric regions was associated with chromatin decondensation, which leads to incorrect chromosome segregation and exclusion into the MN [19]. Our previous study on the impact of two mutagenic agents: chemical – maleic acid hydrazide (MH) and physical – gamma rays on the global epigenetic modifications of chromatin H3K9me2, H4K5ac, and 5mC in barley revealed that MN in barley could have a low or high level of specific epigenetic modifications (Figure 8).

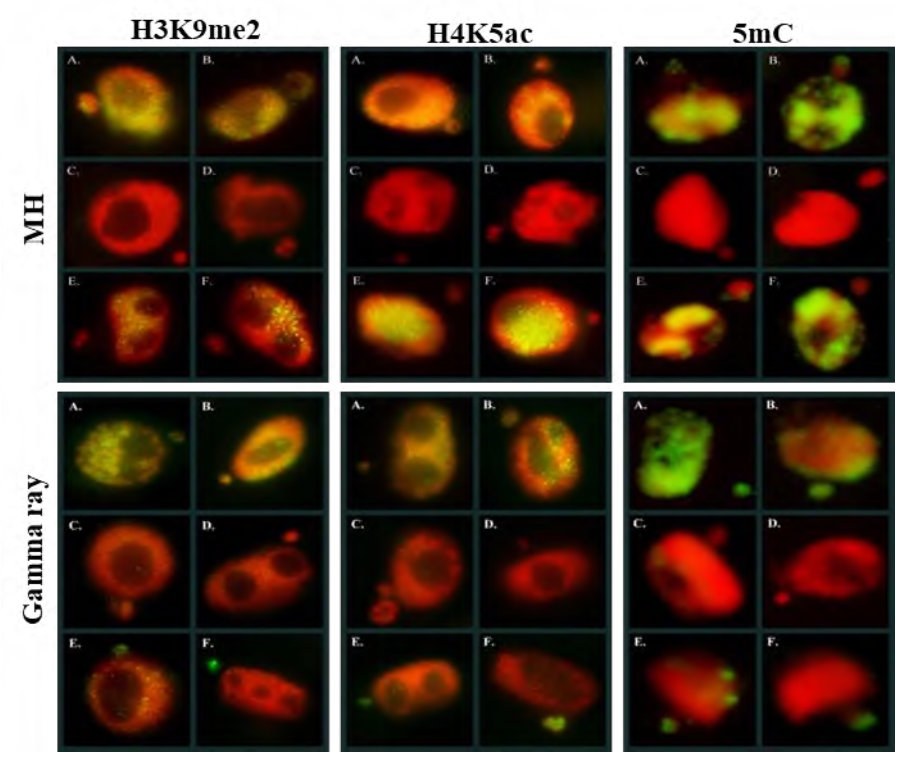

Figure 8. Histone and DNA epigenetic modifications in *Hordeum vulgare* nuclei and micronuclei after MH and gamma rays treatments. DAPI – red, histone-modifications (H3K9me2, H4K5ac) and 5mC – green. Micrographs by A. Braszewska.

However, similar levels of histone H3 methylation, histone H4 acetylation, and 5mC in the MN and its parental nucleus were observed more often. Rarely, the differences in the level of epigenetic modification between the MN and its parental nuclei were observed [85]. The evaluation of DNA methylation in a single nucleus and micronucleus *in B. distachyon* genome was studied. DNA methylation might respond to mutagenic treatments [86]. It demonstrates that analyses of the epigenetic modifications should be integrated into current plant genetic toxicology and mutagenesis.

5.5. Imaging Approaches

For the MN test, the microscope is obligatorily used for the visual detection of micronuclei and their manual counting based on observing a significant number of cells. Despite the many advantages of MN, their analysis by picking out and manually counting with microscopy is time-consuming, requires the proper skills, and is prone to subjectivity. On the other hand, the visual confirmation of MN and visualization of cytoplasm to associate MN to a particular cell is an advantage of this method. The use of high definition fluorescence microscopy that is equipped with a high-sensitivity camera allows the precise detection and quantification of micronuclei and automatically captures images. The development of microscopic and bioimaging techniques enables the rapid and versatile assessment of MN. These approaches improved the statistical power of this method and the robustness of the MN assay. Previously, laser scanning cytometry (LSC) and conventional flow cytometry methods were successfully applied to identify and enumerate MN [87,88]. This method was fraught because MN is not correctly distinguished from other DNA bodies, debris, and nuclei [89]. A flow cytometry-based approach testing micronucleus induction (FCMN assay) was also tested for humans to detect nanomaterials-induced MN [90]. Due to many scored cells and the compatibility of the results with other tests, the FCMN approach can serve as a speed assay to evaluate the potential genotoxicity as MN formation. A fully automated Image Stream Imaging Flow Cytometer has been

developed to perform the in vitro micronucleus assay [91]. It combines the speed of the high-throughput nature of conventional flow cytometry with the visual information of high-resolution microscopy. Another method, single cell quantitative imaging microscopy (scQuantIM) accurate for quantifying the frequency of micronuclei formation for biomedical research; so far was optimized and tested for cancer cells, treated by genotoxic agents, etoposide, or bleomycin [92]. All the above technical innovations have been developed and used for human research; however, they hopefully can be applied to plant cells in the future.

6. Concluding Remarks and Future Perspectives

In conclusion, we have highlighted the possibilities of the detection and detailed analysis of MN in plants, emphasizing the research directions using modern molecular cytogenetic approaches. These collected approaches provide future directions to study MN in plants.

The most important advancement from the development of the molecular cytogenetic techniques for the MN analysis is based on fluorescence in situ hybridization (FISH) and its variants. With the application of FISH, many obstacles that are connected to the small size and uniformity of chromosomes were overcome. Thus micronuclei, which are very small, could be analyzed even in species that are characterized by a small genome. The other cytogenetic advances, e.g., in chromosome preparation, such as extended fiber-FISH, are in no way needed for the study of MN, although they are very helpful in the analysis of plant genome structure. The imaging and signal amplification technologies have improved the ability to detect small gene-sized probes in micronuclei. Recently, the main driver of plant cytogenetics are next generation sequencing (NGS) platforms, as well as bioinformatics that enables analyses of DNA sequences. Up to date, based on the whole genome sequencing achievements for many species, the linking DNA sequence to the physical chromosomes enable the development of new areas of plant genomics, epigenetics, and evolution. The integration of the big data and next-/third-generation sequencing, with the cytogenetics offers possibilities for new insight into micronuclei structure in the future.

Although several advances have recently been made in the studies of MN in plants (Figure 9), a number of important questions still need to be addressed, namely whether micronuclei can be re-engulfed by the cell nucleus and whether the micronuclear content can be degraded independently of further cell divisions.

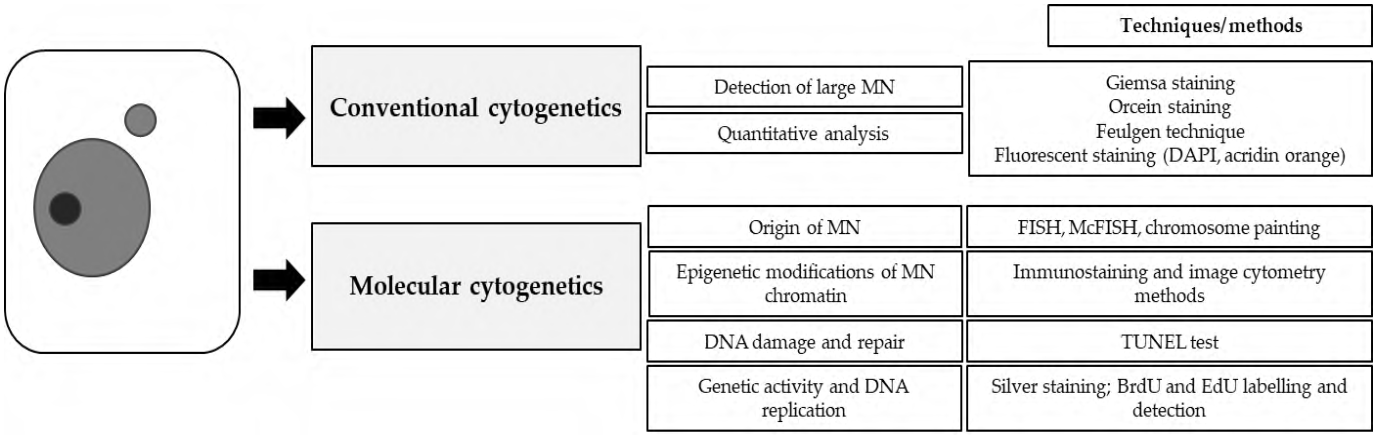

Figure 9. Possibilities of micronuclei assessment in plants.

There is no knowledge on the possible cell lethal events and their risk to the organism. These new approaches may help to clarify whether micronuclei and genomic instability are related to other cellular mechanisms that have not been described so far.

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II.3.

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Unraveling the DNA Methylation in the rDNA Foci in Mutagen-Induced *Brachypodium distachyon* Micronuclei.

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Unraveling the DNA Methylation in the rDNA Foci in Mutagen-Induced *Brachypodium distachyon* Micronuclei

Adrianna W. Bara-Halama, Dominika Idziak-Helmcke and Jolanta Kwasniewska *

Plant Cytogenetics and Molecular Biology Group, Faculty of Natural Sciences, University of Silesia in Katowice, Jagiellonska 28, 40-032 Katowice, Poland; adriannabara@gmail.com (A.W.B.-H.); dominika.helmcke@us.edu.pl (D.I.-H.)
* Correspondence: jolanta.kwasniewska@us.edu.pl

Abstract: Many years have passed since micronuclei were first observed then accepted as an indicator of the effect of mutagens. However, the possible mechanisms of their formation and elimination from the cell are still not fully understood. Various stresses, including mutagens, can alter gene expression through changes in DNA methylation in plants. In this study we demonstrate for the first time DNA methylation in the foci of 5S and 35S rDNA sequences in individual *Brachypodium distachyon* micronuclei that are induced by mutagenic treatment with maleic acid hydrazide (MH). The impact of MH on global epigenetic modifications in nuclei and micronuclei has been studied in plants before; however, no in situ analyses of DNA methylation in specific DNA sequence sites are known. To address this problem, we used sequential immunodetection of 5-methylcytosine and fluorescence in situ hybridization (FISH) with 5S and 25S rDNA probes on the non-dividing cells of *B. distachyon*. Such investigations into the presence or absence of DNA methylation within specific DNA sequences are extremely important in plant mutagenesis in the light of altering gene expression.

Keywords: *Brachypodium distachyon*; DNA methylation; FISH; maleic acid hydrazide; micronuclei; rDNA

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1. Introduction

In eukaryotic cells, chromatin exists in two forms: as loosely structured euchromatin and condensed heterochromatin. The access of mutagens to chromatin depends on the state of chromatin condensation. Chromatin structure is an essential element in the control of gene expression. The organization of chromatin, i.e., the degree of its condensation, strongly depends on the post-translational modification of histones and DNA methylation [1]. Previous studies have shown that epigenetic modifications, such as DNA methylation and histone modifications, change gene expression without modifying the DNA sequence and participate in the organism's response to various environmental factors, including those having a mutagenic effect [2–4]. In the case of abiotic stresses, such as salinity, drought, heavy metals, heat, and cold, DNA hypo- or hypermethylation depends on the type of stress, its intensity or dose, and the plant species subjected to the stress [2,5].

One of the stress factors for plant organisms is mutagens. The genotoxic effects of mutagens in all eukaryotes could be observed at the gene/DNA, chromosome, and organism level [6]. Cytogenetic tests are commonly used to detect the clastogenic effects of mutagens in plant chromosomes. Among these tests, the micronucleus test is widely used for the detection and quantitative analysis of the effects of mutagens. Micronuclei (MN) are small outside-nuclear bodies located next to the interphase nucleus. They can arise from acentric chromosome fragments due to single- and double-strand breaks in DNA or from entire chromosomes due to the action of a mutagenic agent on the microtubules [7].

Maleic acid hydrazide (MH) is a commonly used chemical mutagen in plant mutagenesis [7]. MH is a uracil isomer that integrates into DNA and inhibits DNA synthesis, although the exact mechanism of its action is still not well understood. Its potential to

cause chromosomal aberrations, including the formation of MN, is well documented in plant cells. Its genotoxic activity was previously confirmed in many plant species including *Hordeum vulgare*, *Allium cepa*, *Vicia faba*, and *Brachypodium distachyon*, in which the formation of MN has been observed [8–11]. MH has also been used in studies of epigenetic modifications in the response of plant cells to stress factors [3,12]. MH and gamma radiation change the level of various global epigenetic modifications, including DNA methylation, in barley cells. Interestingly, gamma radiation strongly influenced changes in the level of DNA methylation, while changes in histone methylation and acetylation were mainly noticed after treatment with MH [3]. Studies on DNA methylation and other epigenetic modifications after mutagenic treatments were carried out on the single *B. distachyon* nuclei and micronuclei [12]. The level of DNA methylation in the nuclei after mutagenic treatment differed from the level of DNA methylation present in control nuclei and strongly depended on the type of applied mutagen, MH or N-nitroso-N-methylurea (MNU). Differences in the 5-methylcytosine (5mC) levels in MH- and MNU-induced micronuclei were also observed. Our previous research suggested that DNA methylation could potentially have an active role in MN formation or be a marker for chromatin exclusion into MN [12].

Previous studies have demonstrated that the distribution of chromosome aberrations is not random. Micronuclei preferentially comprise particular chromosomes or their parts [13–16], which may be related to chromosome size, gene density [17], and other aspects of chromatin organization [18]. The hot spots of MH-induced DNA damage within the nuclei could be detected using multicolor fluorescence in situ hybridization (FISH). Among repetitive DNA sequences, ribosomal DNA (rDNA) sequences are widely used as probes for FISH in plant cells, mainly due to their availability and evolutionarily conserved nature. In *B. distachyon*, the application of rDNA sequences as probes for FISH showed that 5S-rDNA-bearing chromosomes are involved in MN formation more frequently than 35S-rDNA-bearing chromosomes [19]. The application of rDNA sequences as probes for FISH has also been useful in determining the origin of micronuclei in other plant species, e.g., barley [6,10,20]. It has been shown that the micronuclei of some plant species [21] and humans [22] are more often formed with the participation of nucleolar organizing regions. These cytogenetic approaches improve the sensitivity of the MN test.

Until now, the DNA methylation of particular DNA sequences or chromosome regions, both in the cell nucleus and micronucleus, has not been analyzed in single plant cells within the context of mutagenesis. Such an approach would provide further useful information about the possible mechanisms that govern MN formation and elimination followed by mutagenic treatment in plants. This strategy has not yet been successfully applied to studies of micronuclei in plants.

Here, for the first time, we demonstrate the DNA methylation in the foci of 5S and 35S rDNA sequences in the individual *B. distachyon* micronuclei that are induced by mutagenic treatment with MH. Although the impact of MH on the level of global epigenetic modifications has been studied in plants, to date no in situ analyses regarding the rDNA sites are known. For this purpose, immunodetection of 5-methylcytosine and FISH with 5S and 25S rDNA probes were applied sequentially to the nuclei and micronuclei of *B. distachyon* cells treated with MH.

2. Results

2.1. The Presence of rDNA Signals in MH-Induced B. distachyon Micronuclei

Fluorescence in situ hybridization (FISH) with 5S and 25S rDNA as probes was applied to the interphase nuclei of *B. distachyon* plants treated with 4 mM MH and control (not-treated) plants. The examples of control nuclei and nuclei with micronuclei from MH-treated cells are shown in Figure 1.

In the control nuclei two signals from 5S rDNA and two signals from 35S rDNA were observed (Figure 1A), which corresponds to the number of rDNA loci that are present in *B. distachyon* genome. In the case of MH-treated nuclei with MN, four situations were discerned: (1) no rDNA signal in MN; (2) one 5S rDNA signal in MN; (3) one 35S rDNA

signal in MN; and (4) both 5S and 35S rDNA signals in MN (Figure 1B–E, respectively). We analyzed the frequencies of MN with signals of 5S and/or 35S rDNA in *B. distachyon* cells subjected to different post-treatment times: 0 h, 10 h, 20 h, following MH treatment (Figure 2).

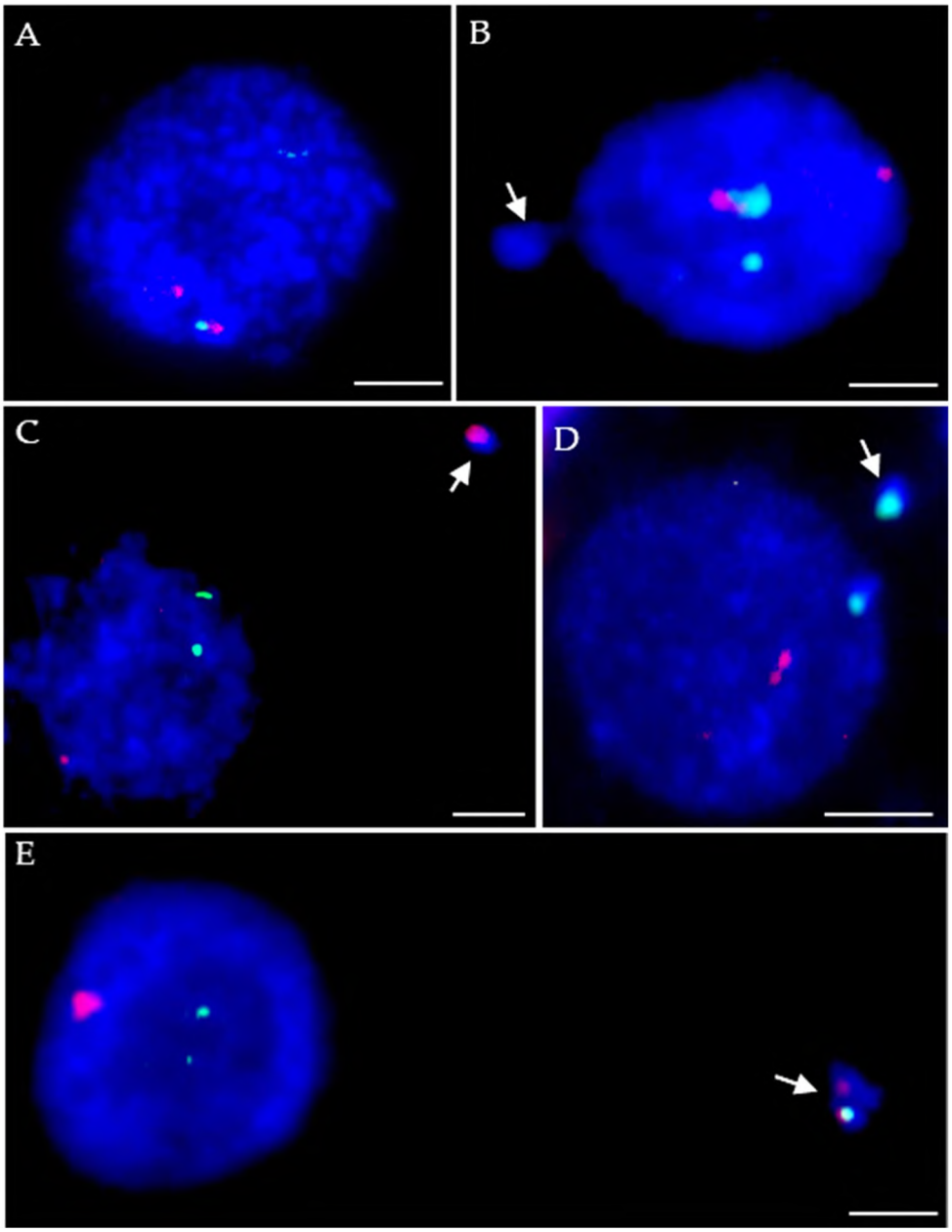

Figure 1. *B. distachyon* interphase nuclei: control (**A**) and with micronuclei (MN) induced by MH treatment (**B**–**E**), FISHed with 5S rDNA (red) and 25S rDNA probes (green). The chromatin was stained with DAPI (blue). Arrows indicate the micronuclei. Types of micronuclei are described in the main text. Scale bars = 5 µm.

No differences in the frequencies of MN with rDNA signals were observed with regard to the post-treatment time, so the results have been combined into one dataset. MN with 5S rDNA signals were observed with the highest frequency of 39%, whereas MN with both 5S and 35S rDNA foci had a slightly lower frequency of 35%. MN with 25S rDNA signals only were the least frequent (26%).

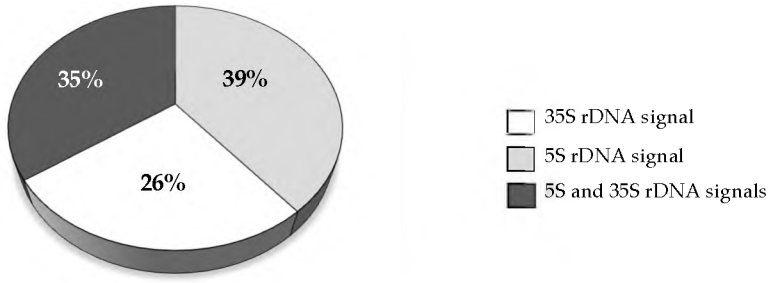

Figure 2. The frequencies of MN induced by MH treatment displaying signals of 5S and/or 35S rDNA. The results obtained after different post-treatment times, 0 h, 10 h, 20 h, are combined into one dataset.

2.2. The Presence of 5S and/or 35S rDNA and 5mC Signals in B. distachyon Micronuclei

The FISH with 5S and 25S rDNA as probes and immunocytochemical detection of 5-methylcytosine (5mC) applied to the same nuclei have enabled the analysis of the occurrence of DNA methylation precisely in the rDNA sites (Figure 3). Based on the diversity of signals we distinguished two main categories of MN with rDNA: with 5mC signals (Figure 3B,B`,D,D`,F,F`) and without 5mC signals (Figure 3A,A`,C,C`,E,E`). The MN without rDNA foci are shown in Figure 3G,H. The last group could also be divided into two subsets: with and without 5mC signals (Figure 3G`,H`, respectively).

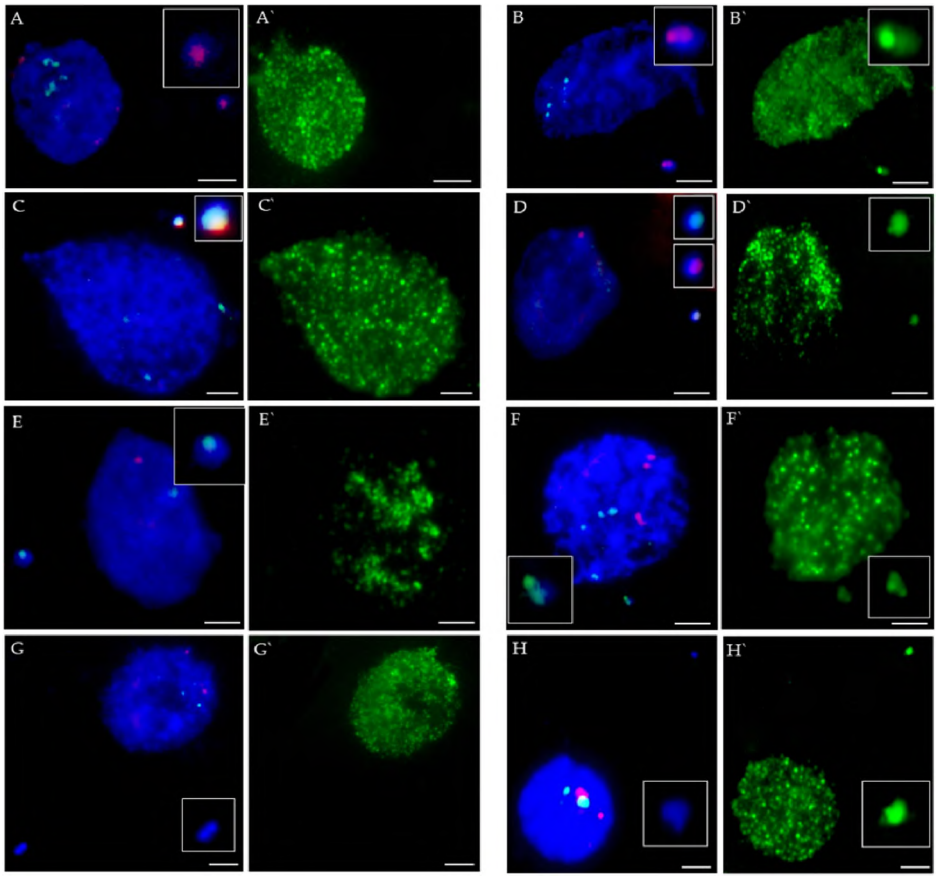

Figure 3. *B. distachyon* interphase nuclei with MN, FISHed with 5S rDNA (red) and 25S rDNA probes (green), stained with DAPI (blue) (**A**–**H**), and subjected to the immunocytochemical detection of 5mC (green) (**A`**–**H`**). Types of micronuclei are described in the main text. Small images of magnified micronuclei are shown in each picture. Scale bars = 5 µm.

The frequencies of MN with different signal compositions were analyzed. Figure 4 presents the frequencies of MN with different signals after different post-treatment times combined into one dataset. Among all analyzed MN, only 15% had the signals of rDNA. Within this group, 41% of MN had 5mC signals within these foci. The MN lacking both the presence of rDNA and 5mC foci constituted 61% of the total MN number, while the frequency of MN displaying 5mC foci but no rDNA signals was 24% (Figure 4).

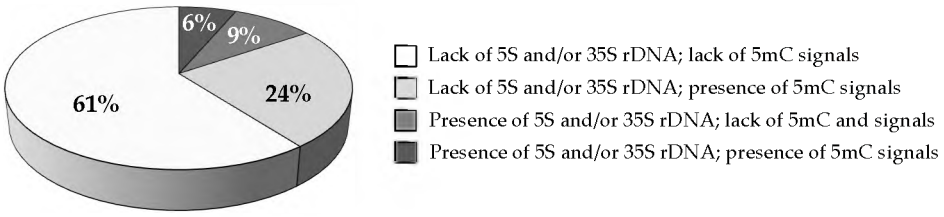

Figure 4. The frequencies of MN induced by MH treatment classified into different groups related to the presence or lack of both rDNA and 5mC signals. The results obtained after different post-treatment times, 0 h, 10 h, 20 h, are combined into one dataset.

We observed mostly minor differences in the frequency of MN with specific signal composition between nuclei subjected to different post-treatment lengths (Figure 5). The MN lacking the rDNA signals dominated in all cases, with rates of 86%, 83%, and 85% for 0 h, 10 h, and 20 h of post-treatment, respectively. Interestingly, we noted a significant difference in the frequencies of MN with and without 5mC signals in the rDNA regions (3% and 12%, respectively) only in the MN subjected to 20 h of post-treatment. In contrast, only slight differences in the rates of rDNA-bearing MN with and without DNA methylation foci were observed after 0 h and 10 h of post-treatment.

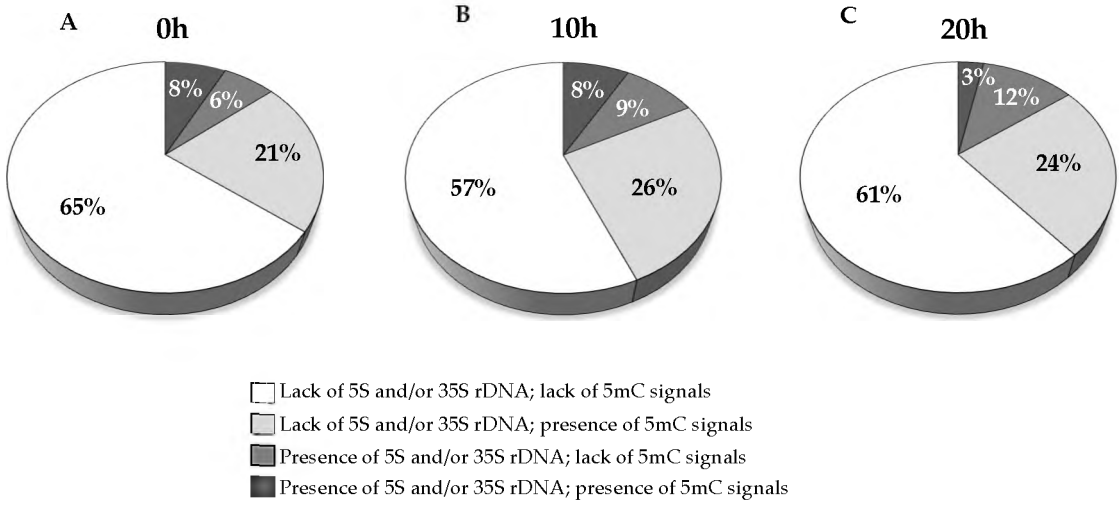

Figure 5. The frequencies of MN induced by MH treatment classified into different groups related to the presence or lack of rDNA and 5mC signals. The results for (**A**) 0 h, (**B**) 10 h, (**C**) 20 h of post-treatment time are presented.

2.3. Differences in DNA Methylation of 5S and 35S rDNA Foci in B. distachyon Micronuclei

The MN displaying rDNA signals were subjected to a more detailed analysis in order to investigate the differences in DNA methylation between 5S and 35S rDNA foci in MN. We compared the frequencies of MN bearing either one or both rDNA signals, which were additionally immunostained with anti-5mC antibodies conjugated with Alexa 488 (Figure 6).

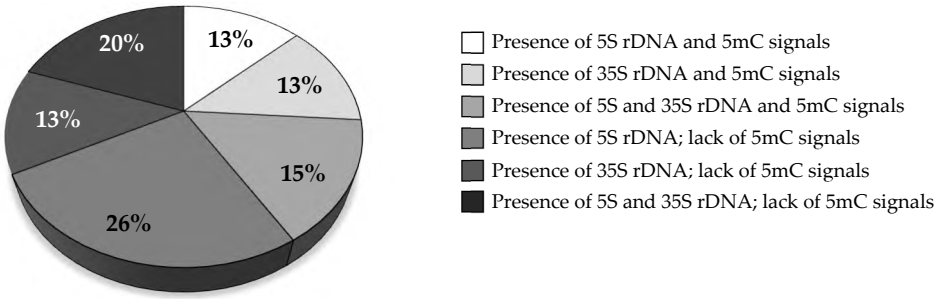

Figure 6. The frequencies of MN induced by MH treatment, with rDNA foci classified into different groups related to the presence or lack of 5mC signals. The graph shows the results for the different types of rDNA separately. The results obtained after different post-treatment times, 0 h, 10 h, 20 h, are combined into one dataset.

We observed that the frequency of MNs carrying only 5S rDNA without 5mC signals in this region was two times higher than of those with 5mC signals (26% vs. 13%, respectively). In contrast, no differences in terms of the presence or absence of 5mC signals were observed in the MN bearing only 35S rDNA foci. In both cases the frequencies equaled 13%. The rates of the presence or absence of 5mC signals in MN with both 5S and 25S rDNA signals were relatively similar and equaled 15% and 20%, respectively.

3. Discussion

Micronuclei (MN) are considered to be hallmarks of genome instability. Despite the long tradition of using the MN test [23], the mechanism of the formation and elimination of micronuclei is still unknown. The occurrence of MN is commonly attributed to a failure in the repair of DNA double-strand breaks (DSB). A number of studies on human MN indicate that the contribution of particular chromosomes or their parts to MN is not entirely random [24,25], suggesting the existence of 'hot spots' more prone to DSB. The spatial organization of the nucleus at interphase, the diverse transcriptional activity of specific chromosome regions, chromosome size, or the presence of large heterochromatin blocks are among the factors that could be responsible for the non-random distribution of DSB that lead to MN formation [26].

The identification of the origin of MN in plants is possible by using FISH with repetitive DNA sequences, such as centromeric, telomeric, and rDNA, as probes. In our experiment only 15% of all MN displayed rDNA signals. This result confirms that 5S rDNA and 35S rDNA loci are not "hot spots" for DNA breaks in the *B. distachyon* genome after the application of MH, as was previously stated by Kus et al. [27].

The 5S rDNA site is localized proximally in the long arm of chromosome Bd4, while a nucleolar organizing region with transcriptionally active 35S rDNA loci is found distally in the short arm of chromosome Bd5 [28]. The interstitial localization of the 5S rDNA site means that two breaks in the double-stranded DNA flanking the site would be required in order to incorporate the 5S rDNA sequence into MN. In comparison, in the case of the terminally located 35S rDNA site, only one DSB is necessary. Considering only chromosomal position, one should expect that 35S rDNA will be more sensitive to mutagens than 5S rDNA. Remarkably, our study demonstrated that MN with 5S rDNA signals were observed at a higher frequency than MN with 35S rDNA. Kus et al. [19] also showed that 5S-rDNA-bearing chromosomes were involved in *B. distachyon* MN formation more frequently than 35S-rDNA-bearing chromosomes. Similar differences in the sensitivities of 5S and 35S rDNA to MH treatment were previously reported in *Hordeum vulgare* [29] and *Crepis capillaris* [30,31]. In the latter species, the application of comet–FISH demonstrated that 5S rDNA was present in the comet tail more frequently than 35S rDNA. These phenomena can be explained by the association of the active 35S rDNA with the nucleolus [30,32]. The authors postulated that 35S rDNA, which represents housekeeping genes, associated with the nucleolus, is less prone to fragmentation and migration into

the comet tail. The combination of the MN test with analysis of the transcriptional activity of 35S rRNA genes in MH-treated *H. vulgare* showed that the expression of 35S rDNA is always maintained in MN, even though they are eliminated during the next cell cycle [29].

A number of studies have shown that epigenetic DNA modifications play a key role in gene expression under different stresses in plants. Although epigenetic regulation is not associated with changes in the nucleotide sequence, it can lead to heritable changes in gene expression [33]. DNA methylation can protect the plant genome from a variety of mutations [34], for example by deactivation of transposable elements [35]. Very recently epigenetic modifications in response to abiotic stresses such as high or low temperature, high salt exposure, and deficient or flood water conditions over generations has been reported [36–39]. Natural variation in DNA methylation associated with environmental changes was observed for example in tomato and soybean [40,41]. Epigenetics has attained great success for its applications in plant breeding, where it has been used to assess the propagation of epigenetic marks across generations to improve desirable crop traits [42].

However, the correlation between epigenetic modifications and changes that are induced by mutagens is still not well understood. Most recent research on DNA methylation relies on molecular and biochemical techniques. For the determination of methylation in the DNA molecule, cloning and sequencing are widely used methods that may even provide single-nucleotide resolution of methylation. Newly developed high-throughput sequencing tools for identifying RNA alterations have greatly advanced the practical study of RNA epitranscriptomics [43,44]. The methodical approach applied in this paper, based on the detection of methylated sites in situ on cell nucleus preparations, is not common, but in some studies, e.g., those aimed at explaining the mechanism of micronucleus formation, it is the only method.

However, in some respects, complementary cytological studies are necessary to analyze the 5mC distribution in situ in plant chromosomes and nuclei [45,46]. Examining the distribution of highly methylated chromosomal regions by using antibodies directed against 5mC enables an insight into the correlation between the instability of the genome and its structure at the cytological level. Such analyses appear to be particularly important in studies on the effects of mutagens that manifest themselves as micronuclei.

There is evidence suggesting that increased chromatin condensation may play a role in DNA elimination from the nucleus. In the crosses between wheat and pearl millet, uniparental genome elimination of pearl millet occurs during the early stages of embryo development. The pearl millet chromosomes form micronuclei, which are generally more condensed than the nuclei, and contain either heterochromatin and euchromatin or exclusively heterochromatin [47]. In sciarid flies, the paternal chromosome set undergoing elimination shows low levels of histone H4/H3 acetylation, which is usually an indicator of heterochromatinization [48]. Another hallmark of heterochromatin, histone H3K9 methylation, is required for the chromatin elimination that accompanies the development of the somatic macronucleus in *Tetrahymena* [49]. In our case, the presence of 5mC signals was noted in only 30% of the micronuclei, suggesting that heterochromatinization might not play such an important part in the DNA exclusion resulting from the mutagenic treatment. However, cytosine methylation is only one of heterochromatin's signature features, and other markers, such as the level of histone H3K9 and H3K27 methylation, should be involved in future studies on micronuclei formation.

We show that among all MN that were bearing rDNA, 41% had 5mC signals within these foci. Our results can be interpreted in relation to the DNA methylation patterns in the *B. distachyon* submetacentric chromosomes Bd4 and Bd5, which carry 5S and 35S rDNA loci, respectively. Individual *B. distachyon* chromosomes exhibit a characteristic DNA methylation pattern, with numerous differences in the distribution of the methylated sites between homologous chromosomes as well as between the arms of a given chromosome [28]. However, some chromosome sites, such as pericentromeric regions, are invariably highly methylated in all chromosomes. Chromosomes Bd4 and Bd5 have characteristic 5mC foci distribution. In the case of chromosome Bd4, two peaks with a high

density of 5mC foci are observed with a slight decrease in the proximal region of the long arm carrying 5S rDNA locus. A survey of methylation patterns in Arabidopsis indicates that highly repetitive sequences, such as rDNA sequence arrays, are usually densely methylated [50,51]. The high DNA methylation level is often correlated with transcriptional inhibition. 5S rDNA is an exception to that rule since it remains an active genetic region despite being highly methylated [51]. We found that unmethylated 5S rDNA sequences participated in MN formation two times more often than methylated 5S rDNA. Since a decrease in DNA methylation is linked to its decondensation, such a result might indicate that the unmethylated, less-condensed 5S rDNA sites are more likely to suffer from mutagen-induced DSB. This hypothesis can be corroborated by the results of studies on human lymphocytes, which demonstrated that the decondensation of heterochromatic regions on chromosomes 1 and 9 caused by idoxyuridine treatment strongly correlated with MN formation by these chromosomes [52].

The 5S rDNA sequence has been reported to have a higher methylation and condensation level than 35S rDNA in Arabidopsis [53] and tobacco [54]. 5S rDNA also appeared to be more resistant to hypomethylation in Brassica [55]. However, despite the presumably less-condensed structure of 35S rDNA sites, we demonstrated that their occurrence in MN was not more frequent than 5S rDNA, suggesting that in this case factors other than DNA methylation and condensation level play a role in the inclusion of specific chromosomes in MN.

The 35S-rDNA-bearing Bd5 chromosome is characterized by having the highest levels of DNA methylation in pericentromeric regions, which decreases towards both chromosome termini. This chromosome displays two different methylation patterns depending on chromosome condensation, with less-condensed Bd5 showing a considerably lower methylation level in 35S rDNA sites [28]. In contrast to the 5S-rDNA-containing MN, we did not observe differences in the rates of MN carrying 35S rDNA sites with or without 5mC signals. Our results suggest that the chromosomes with both types of the methylation pattern contribute equally to the formation of MN.

Future studies should focus on studying the factors determining the presence of methylated or unmethylated sequences in the MN. It would be also interesting to find a connection between the DNA methylation level of MN and their future fate, i.e., whether or not they are eliminated during subsequent cell cycles.

4. Materials and Methods

4.1. Plant Material, Mutagenic Treatment, and Slide Preparation

The experiments were performed with the *Brachypodium distachyon* ($2n = 10$) reference genotype Bd21. The seed material was obtained from the collection held by the United States Department of Agriculture's National Plant Germplasm System. *B. distachyon* seeds were presoaked in tap water for 6 h. Then the seeds were germinated on moist filter paper in Petri dishes for 72 h in the dark at 21 °C. The seedlings were treated with 4 mM maleic acid hydrazide (MH, Sigma, St Louis, MO, USA) for 3 h in the dark at 21 °C under aerated conditions. At the same time, control seedlings were incubated for 3 h in distilled water under the same aerated conditions. After the treatment, the seedlings were washed three times in distilled water and then grown in Petri dishes. The experiment with MH treatment was repeated two times. Whole seedlings were fixed in 3:1 (v/v) methanol:glacial acetic acid at three post-treatment times: 0 h, 10 h, and 20 h after treatment and stored at −20 °C until use. Cytogenetic preparations were made using a previously described procedure [56]. To make cytogenetic preparations, the root meristems were washed in 0.01 mM citrate buffer for 3 × 5 min and then digested in a mixture of maceration enzymes: 1% pectinase (v/v, Sigma) and 2% cellulose (w/v, Sigma) for 1.5 h at 37 °C. The roots were washed again in the citrate buffer and then nuclei preparations were made using 45% acetic acid. Slides were incubated on dry ice to remove the coverslips and then stored at 4 °C.

4.2. Immunodetection of 5-Methylcytosine

The immunodetection of 5-methylcytosine (5mC) was carried out as previously described for *B. distachyon* [12]. DNA denaturation was performed prior to the immunodetection of 5mC within nuclei and micronuclei. For the chemical denaturation of DNA, the preparations were soaked in 0.25 M sodium hydroxide (NaOH, Merck) and 1 M sodium chloride (NaCl, POCH) for 30 min at 4 °C. Then, the slides were washed 3 × 5 min in cold distilled water, and then in 1 M Tris-HCl (VWR) for 30 min at 4 °C. The preparations were washed in an alcohol series (70%, 90%, and 100%) and allowed to dry. Blocking serum (5% BSA, Sigma) in 1 × PBS was applied to the slides and incubated at room temperature (RT) in a humid chamber for 1 h. Then, the primary mouse anti-5mC antibody (Abcam, Cambridge, UK) (1:100 in 5% BSA) was applied to the slides and left in a humid chamber overnight at 4 °C. The preparations were washed 3 × 5 min in 1 × PBS. Then a secondary goat anti-mouse IgG antibody, conjugated with Alexa Fluor 488 (Invitrogen, Molecular Probes, Eugene, OR, USA), was applied to the slides and left in a humid chamber for 1 h at 37 °C. The slides were washed again for 3 × 5 min in 1 × PBS. The preparations were mounted and counterstained in Vectashield (Vector Laboratories, Peterborough, UK) that contained 2.5 μg/mL DAPI (Serva).

4.3. Probes Labeling and FISH Procedure

The labeling of the probes and FISH were performed according to the methodology described by Jenkins and Hasterok [56]. Two probes were used: 5S rDNA and 25S rDNA (Roche Diagnostics, Basel, Switzerland), which were labeled with digoxigenin-11-dUTP (Roche Diagnostics) and tetramethylrhodamine-5-dUTP (Roche Diagnostics) by the nick-translation method using nick-translation mix (Roche Diagnostics).

Initially, RNAse was applied to the slides and incubated at 37 °C for 1 h in a humid chamber. After this time, the slides were washed 3 × 5 min in 2 × SSC and then transferred to 1% formaldehyde (v/v) in 1 × PBS for 10 min at RT, and washed again 3 × 5 min at 2 × SSC at RT. For DNA denaturation and hybridization, a mix consisting of 50% formamide, 10% dextran sulphate (w/v), 2 × SSC, 0.5% (w/v) SDS, 75–200 ng of the DNA probes/slide, and water was applied to the slides. The slides and the mix were incubated at 70 °C for 4.5 min. The slides were cooled to 37 °C and stored overnight at 37 °C. Post-hybridization washing was performed in 20% v/v formamide in 0.1 × SSC at 42 °C for 2 × 5 min, then in 2 × SSC at 42 °C for 3 × 5 min, and finally in 2 × SSC at RT for 3 × 5 min. Then the slides were washed in Tween20/4 × SSC at RT. A blocking agent (5% milk) was applied to the slides and incubated for 30 min at RT in a humid chamber. Digoxigenated probe immunodetection was performed with a FITC-conjugated anti-digoxigenin antibody (Roche Diagnostics) and milk solution (1:11) applied to the slides and incubated for 1 h at 37 °C in a humid chamber. Then the slides were washed 3 × 8 min in Tween20/4 × SSC at 42 °C. The preparations were dehydrated in 70%, 90%, and 100% ethanol for 1 min each and allowed to dry. The air-dried preparations were mounted and counterstained in Vectashield (Vector Laboratories) containing 2.5 μg/mL DAPI (Serva, Heidelberg, Germany).

4.4. Image Acquisition

After 5mC immunodetection, slides were analyzed using a Carl Zeiss Imager Z2 fluorescence microscope with fluorescent lighting HSP, 120 W. The images were recorded with an AxioCam ICc5 digital camera and immersion lens with a ×100 magnification. The specific location of the cell nuclei with the micronuclei was also recorded. After subsequent FISH, the same slides were analyzed using the same equipment. The specific locations for each previously recorded cell nucleus and micronucleus were found after FISH and hybridization signals were captured. The frequencies of MN with specific signals and without signals were calculated. For the group treated with MH, a total of 350 nuclei with MN were evaluated. In order to analyze the presence of DNA methylation signals within the rDNA foci, 50 MN with rDNA signals were evaluated.

5. Conclusions

In this study, we presented the analyses of DNA methylation in the rDNA sites in *B. distachyon* micronuclei (MN) induced by treatment with maleic acid hydrazide (MH). This is the first cytological study of DNA methylation within specific DNA sequences in plant mutagenesis. To conclude, our results indicated that: (1) 5S and 35S rDNA in the MN can be either methylated or not; (2) the frequency of MN carrying only 5S rDNA (no 35S rDNA) without 5mC signals in this region was two times higher than the frequency of those with a 5mC signal; (3) no differences in the frequency of MN bearing methylated or unmethylated 35S rDNA were observed. Analyses of DNA methylation within other specific DNA sequences would provide more data to establish the involvement of DNA methylation in the incorporation of various sequences into micronuclei and their subsequent elimination from the genome.

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III. PODSUMOWANIE I WNIOSKI

Na podstawie wyników badań przedstawionych w rozprawie doktorskiej zaproponowano następujące wnioski:

1. Zastosowanie immunocytochemicznej metody wykrywania 5mC umożliwiło analizę obecności i poziomu metylacji DNA w jądrach i mikrojądrach komórek merystematycznych korzeni *B. distachyon,* kontrolnych oraz po traktowaniu chemicznymi czynnikami mutagennymi: hydrazydem kwasu maleinowego (MH) oraz N-nitrozo-N- metylomocznikiem (MNU).
2. Metylacja DNA zmienia się w odpowiedzi genomu jądrowego *Brachypodium distachyon* na działanie wybranych mutagenów chemicznych, a tym samym może odgrywać rolę w powstawaniu i naprawie uszkodzeń DNA.
3. Zmiany w metylacji DNA w jądrach komórkowych i mikrojądrach zależą od rodzaju zastosowanego mutagenu i prawdopodobnie mechanizmu jego działania.
4. Sekwencyjne zastosowanie metody immunocytochemicznego wykrywania 5mC i FISH, z sondami 5S i 25S rDNA, umożliwiło analizę metylacji DNA w miejscach loci rDNA, w mikrojądrach *B. distachyon,* po traktowaniu MH.
5. Loci 5S i 35S rDNA w poszczególnych mikrojądrach, indukowanych działaniem mutagenów DNA różnią się obecnością metylacji DNA, co może wskazywać, że ich udział w tworzeniu mikrojąder zależy od obecności metylacji w ich obrębie.
6. Metylowany DNA w obrębie loci 5S rDNA w MN występował dwukrotnie rzadziej niż niemetylowany DNA. Niemetylowane, mniej skondensowane loci 5S rDNA są bardziej narażone na powstawanie pęknięć podwójnej nici DNA, a tym samym udział w tworzeniu mikrojąder. Nie stwierdzono podobnej zależności dla loci 35S rDNA.
7. Metylacja/demetylacja DNA w genomie jądrowym *B. distachyon* może brać udział w utrzymaniu integralności i stabilności genomu w odpowiedzi na działanie mutagenów.

IV. STRESZCZENIE

Ważną rolę w powstawaniu i naprawie uszkodzeń DNA odgrywa stopień kondensacji chromatyny, który zależy między innymi od metylacji DNA. Tym samym metylacja DNA może decydować o wrażliwości genomu jądrowego na działanie mutagenów - hypometylowane regiony są bardziej wrażliwe. Ponadto w odpowiedzi na stres dochodzi do zmiany ekspresji genów, co skorelowane jest z obniżeniem lub podniesieniem poziomu metylacji DNA w loci tych genów.

Celem pracy doktorskiej było próba zrozumienia roli metylacji DNA w odpowiedzi komórek *Brachypodium distachyon* na traktowanie mutagenami. Odpowiedź komórek na działanie wybranych mutagenów chemicznych, charakteryzujących się różnymi mechanizmami działania: hydrazydu kwasu maleinowego (MH) i N-nitrozo-N-metylomocznika (MNU) obserwowano na poziomie cytologicznym jako powstawanie mikrojąder. Analizowano obecność fluorescencji przeciwciała Alexa Fluor 488 skoniugowanego pośrednio z 5-metylocytozyną (5mC) oraz jej intensywność w jądrach i mikrojądrach komórek *B. distachyon* kontrolnych oraz po traktowaniu MH lub MNU. Badania obejmowały porównawczą analizę zmian metylacji DNA w jądrach komórkowych po traktowaniu wybranymi mutagenami oraz udziału metylowanego DNA w powstałych mikrojądrach. Podjęto również próbę poznania udziału i porównania metylacji DNA w loci 5S i 35S rDNA w mikrojądrach *B. distachyon*, po traktowaniu MH, z sekwencyjnym wykorzystaniem immunocytochemicznego wykrywania 5mC oraz techniki FISH, z sondami 5S i 25S rDNA.

Badania wykazały różnice w obecności i poziomie metylacji DNA w komórkach kontrolnych oraz po traktowaniu mutagenami. Intensywność fluorescencji 5mC w jądrach komórkowych zmieniała się po traktowaniu mutagenami i zależała od rodzaju mutagenu oraz zastosowanego czasu postinkubacji. Intensywność fluorescencji 5mC w jądrach matecznych dla mikrojąder była niższa niż w jądrach komórek bez mikrojąder. Ponadto wykazano, że mikrojądra różnią się obecnością metylacji DNA w loci 5S i 35S rDNA. Częstość mikrojąder z sygnałem 5S rDNA, z nieobecną metylacją DNA w tym rejonie, była dwukrotnie wyższa niż częstość mikrojąder z metylacją DNA obecną w tym loci.

Poziom metylacji DNA w genomie jądrowym *B. distachyon* zmienia się po działaniu wybranych mutagenów chemicznych, a tym samym może odgrywać rolę w powstawaniu i naprawie uszkodzeń DNA, decydując o utrzymaniu integralności i stabilności genomu.

V. SUMMARY

An important role in the DNA damage and repair is played by the degree of chromatin condensation, which depends on DNA methylation. Thus, DNA methylation may determine the sensitivity of the nuclear genome to mutagens - hypomethylated regions are more sensitive. Moreover, in response to stress, gene expression changes, which is correlated with a decrease or an increase in the level of DNA methylation at the loci of these genes.

The aim of the dissertation was to try to understand the role of DNA methylation in the response of *Brachypodium distachyon* cells to treatment with mutagens. The response of cells to the action of selected chemical mutagens characterized by different mechanisms of action: maleic acid hydrazide (MH) and N-nitroso-N-methylurea (MNU) was observed at the cytological level as micronucleus formation. The presence of the Alexa Fluor 488 antibody indirectly conjugated with 5-methylcytosine (5mC) and its intensity in the nuclei and micronuclei of control *B. distachyon* cells and after treatment with MH or MNU were analyzed. The research included a comparative analysis of changes in DNA methylation in cell nuclei after treatment with selected mutagens and the participation of methylated DNA in the micronuclei. An attempt was also made to understand the contribution and comparison of DNA methylation at the 5S and 35S rDNA loci in *B. distachyon* micronuclei after MH treatment, with sequential use of 5mC immunocytochemical detection and FISH technique with 5S and 25S rDNA probes.

The studies showed differences in the presence and level of DNA methylation in control cells and after treatment with mutagens. The 5mC fluorescence intensity in the cell nuclei changed after the treatment with mutagens and depended on the type of mutagen and the post-incubation time used. The fluorescence intensity of 5mC in the mother nuclei for micronuclei was lower than in the nucleus of cells without micronuclei. In addition, it was shown that micronuclei differ in the presence of DNA methylation at the 5S and 35S rDNA loci. The frequency of micronuclei with 5S rDNA signal, absent DNA methylation in this region, was twice as high as the frequency of micronuclei with DNA methylation present at this loci.

The level of DNA methylation in the *B. distachyon* nuclear genome changes after the action of selected chemical mutagens, and thus may play a role in DNA damage and the repair, determining the integrity and stability of the genome.

VI. OŚWIADCZENIA AUTORÓW

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

Katowice, 20.10.2022 r.
miejscowość, data

mgr Adrianna Halama

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009389

email: adrianna.bara@us.edu.pl

DNA Methylation—An Epigenetic Mark in Mutagen-Treated *Brachypodium distachyon*
Cells. Plants. 2021, 10, 1408

Adrianna Wiktoria Bara, Agnieszka Braszewska, Jolanta Kwasniewska

Niniejszym oświadczam, że w ww. publikacji mój udział polegał na:

przygotowaniu materiału roślinnego; traktowaniu mutagenami; wykonaniu analiz laboratoryjnych: immunocytochemicznym wykrywaniu 5mC z wykorzystaniem przeciwciał I i II-rzędowych, barwieniu DAPI, wykonaniu dokumentacji fotograficznej preparatów cytogenetycznych; analizie wyników: analizie obecności sygnałów 5mC w jądrach komórkowych i mikrojądrach, analizie średniej intensywności fluorescencji Alexa Fluor 488 w jądrach komórkowych i mikrojądrach; uczestniczeniu w przygotowaniu oryginalnego manuskryptu, w tym w przygotowaniu rycin, uczestniczeniu w korekcie manuskryptu po recenzjach

Adrianna Halama
podpis

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

Katowice, 20.10.2022 r.
miejscowość, data

mgr Adrianna Halama

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009389

email: adrianna.bara@us.edu.pl

Plant Cytogenetics in the Micronuclei Investigation—The Past, Current Status, and Perspectives. International Journal of Molecular Sciences. 2022, 23, 1306

Jolanta Kwasniewska, **Adrianna Wiktoria Bara**

Niniejszym oświadczam, że w ww. publikacji mój udział polegał na:

przygotowaniu pozycji literaturowych, uczestniczeniu w pisaniu oryginalnego manuskryptu oraz w korekcie manuskryptu po recenzjach

Adrianna Halama
podpis

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

Katowice, 20.10.2022 r.
miejscowość, data

mgr Adrianna Halama

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009389

email: adrianna.bara@us.edu.pl

Unraveling the DNA Methylation in the rDNA Foci in Mutagen-Induced *Brachypodium distachyon* Micronuclei. International Journal of Molecular Sciences. 2022, 23, 6797

Adrianna W. Bara-Halama, Dominika Idziak-Helmcke, Jolanta Kwasniewska

Niniejszym oświadczam, że w ww. publikacji mój udział polegał na:

przygotowaniu materiału roślinnego; traktowaniu mutagenami; wykonaniu analiz laboratoryjnych: immunocytochemicznym wykrywaniu 5mC z wykorzystaniem przeciwciał I i II-rzędowych, barwieniu DAPI, przygotowaniu sond do fluorescencyjnej hybrydyzacji *in situ* (FISH), przeprowadzeniu fluorescencyjnej hybrydyzacji *in situ*, wykonaniu dokumentacji fotograficznej preparatów cytogenetycznych; analizie wyników: analizie obecności sekwencji 5S i 35S rDNA oraz sygnałów 5mC w jądrach komórkowych i mikrojądrach; uczestniczeniu w przygotowaniu oryginalnego manuskryptu, w tym w przygotowaniu rycin, uczestniczeniu w korekcie manuskryptu po recenzjach

Adrianna Halama
podpis

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

Katowice 20.10.2022
miejscowość, data

dr hab. Jolanta Kwaśniewska, prof. UŚ

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009468

email: jolanta.kwasniewska@us.edu.pl

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Cells. Plants. 2021, 10, 1408

Adrianna Wiktoria Bara, Agnieszka Braszewska, **Jolanta Kwasniewska**

Niniejszym oświadczam, że w ww. publikacji mój udział polegał na:

opracowaniu koncepcji i metodyki badań, analizie i opracowaniu wyników, przygotowaniu rycin, pisaniu oraz korekcie oryginalnej i końcowej wersji manuskryptu, odpowiedzi na recenzje

podpis

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

Katowice, 20.10.2022
.....
miejscowość, data

dr hab. Jolanta Kwaśniewska, prof. UŚ

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009468

email: jolanta.kwasniewska@us.edu.pl

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Jolanta Kwasniewska, Adrianna Wiktoria Bara,

Niniejszym oświadczam, że w ww. publikacji mój udział polegał na:

opracowaniu koncepcji manuskryptu, analizie i opracowaniu wyników, pisaniu oraz korekcie oryginalnej i końcowej wersji manuskryptu, odpowiedzi na recenzje, przygotowaniu rycin

.....
podpis

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

Katowice 20.10.2022
.....
miejscowość, data

dr hab. Jolanta Kwaśniewska, prof. UŚ

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009468

email: jolanta.kwasniewska@us.edu.pl

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Adrianna W. Bara-Halama, Dominika Idziak-Helmcke, **Jolanta Kwasniewska**

Niniejszym oświadczam, że w ww. publikacji mój udział polegał na:

opracowaniu koncepcji badań, pozyskaniu środków na badania, analizie i opracowaniu wyników, przygotowaniu rycin, pisaniu i korekcie manuskryptu, odpowiedzi na recenzje

.....
podpis

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

20.10.2022 r. Katowice
miejscowość, data

dr Agnieszka Brąszewska

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009553

email: agnieszka.braszewska@us.edu.pl

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Cells. Plants. 2021, 10, 1408

Adrianna Wiktoria Bara, **Agnieszka Braszewska**, Jolanta Kwasniewska

Niniejszym oświadczam, że w ww. publikacji mój udział polegał na:

analizie wyników, pisaniu oraz korekcie oryginalnej i końcowej wersji manuskryptu

Agnieszka Braszewska
podpis

OŚWIADCZENIE WSPÓŁAUTORA
O WKŁADZIE PRACY

Katowice, 20.10.2022
miejscowość, data

dr Dominika Idziak-Helmcke

Wydział Nauk Przyrodniczych
Instytut Biologii, Biotechnologii i Ochrony Środowiska
ul. Jagiellońska 28
40-032 Katowice
tel. 32 2009553

email: dominika.idziak-helmcke@us.edu.pl

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Adrianna W. Bara-Halama, **Dominika Idziak-Helmcke**, Jolanta Kwasniewska

Niniejszym oświadczam, że w ww. pracy mój udział polegał na:

udziale w wykonaniu analiz laboratoryjnych z wykorzystaniem fluorescencyjnej hybrydyzacji *in situ*, uczestniczeniu w przygotowaniu oryginalnej manuskryptu oraz w korekcie manuskryptu po recenzjach

Dominika Idziak-Helmcke
podpis