Uniwersytet Mikołaja Kopernika
Wydział Nauk Biologicznych i Weterynaryjnych

Maria Duszyn

Rola cyklicznego GMP w odpowiedzi na stres biotyczny wywołany przez *Fusarium pseudograminearum* u kłosownicy dwukłoskowej (*Brachypodium distachyon*)

Rozprawa doktorska

Praca doktorska wykonana
w Katedrze Fizjologii Roślin i Biotechnologii,
pod kierunkiem
prof. dr hab. Adriany Szmidt-Jaworskiej
Promotor pomocniczy:
dr Brygida Świeżawska-Boniecka

Toruń 2022

Składam serdeczne podziękowania mojej Promotor, prof. dr hab. Adrianie Szmidt-Jaworskiej za obdarzenie mnie zaufaniem w czasie realizacji badań, poświęcony czas, cierpliwość i wsparcie okazane mi podczas kilkuletniej opieki naukowej. Dziękuję za ogrom przekazanej wiedzy i pomoc merytoryczną w trakcie przygotowywania tej pracy doktorskiej.

Dziękuję również dr Brygidzie Świeżawskiej-Bonieckiej za motywację do krytycznego spojrzenia nad podejmowaną problematyką badawczą, nieocenioną pomoc, rady, liczne owocne dyskusje oraz ogromne wsparcie udzielone podczas realizacji tej pracy doktorskiej.

Dziękuję dr hab. Krzysztofowi Jaworskiemu, prof. UMK, za cenne wskazówki, pomoc merytoryczną, poświęcony czas oraz wsparcie dobrym słowem w momentach wątpliwości.

Dziękuję wszystkim Pracownikom i Doktorantom Katedry Fizjologii Roślin i Biotechnologii UMK za nieocenioną pomoc, przekazaną wiedzę, cenne sugestie, okazaną życzliwość oraz wspaniałą i motywującą atmosferę w pracy.

Chciałabym podziękować również najbliższym, Rodzinie, przede wszystkim Rodzicom za ich wsparcie na całej drodze mojej edukacji, Rodzeństwu i Przyjaciołom, za lata cierpliwości, motywację i słowa otuchy w chwilach zwątpienia.

Badania będące podstawą niniejszej rozprawy doktorskiej zostały sfinansowane z grantu Narodowego Centrum Nauki:

„cGMP jako cząsteczka koordynująca procesy sygnalizacyjne uruchamiane w komórkach *Brachypodium distachyon* na skutek infekcji *Fusarium pseudograminearum*" kierownik grantu mgr Maria Duszyn (PRELUDIUM nr 2018/29/N/NZ9/00812).

Spis treści

Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Artykuł I	**Duszyn, M**.*; Świeżawska-Boniecka, B.; Wong, A.; Jaworski, K.; Szmidt-Jaworska, A. *In Vitro* Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach. Int. J. Mol. Sci. 2021, 22, 6243.	**IF (5-letni) - 6,132 punktacja MNiSW- 140**
Artykuł II	**Duszyn, M**.*; Świeżawska-Boniecka, B.; Skorupa, M.; Jaworski, K.; Szmidt-Jaworska, A. BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate. Int. J. Mol. Sci. 2022, 23, 2674.	**IF (5-letni) - 6,132 punktacja MNiSW- 140**

*autor korespondencyjny

Łączny IF – 12,234

Łącznie punkty MNiSW – 280

Wprowadzenie

Cykliczne nukleotydy (cNMP), do których zalicza się cykliczny 3',5'-adenozynomonofosforan (cAMP) oraz cykliczny 3',5' - guanozynomonofosforan (cGMP) to cząsteczki sygnalne, które pełnią istotną rolę zarówno w komórkach prokariotycznych, jak i eukariotycznych. Cykliczny AMP wyizolowany został po raz pierwszy w 1958 roku z wątroby psa [Rall, Sutherland 1958], a cGMP kilka lat później z moczu szczura [Ashman i in. 1963]. Od tamtej pory badania związane z cyklicznymi nukleotydami w komórkach zwierzęcych umożliwiły identyfikację enzymów syntetyzujących (cyklazy) i inaktywujących (fosfodiesterazy) cNMP, poznanie mechanizmów ich działania oraz scharakteryzowanie licznych procesów metabolicznych z ich udziałem. Dowiedziono, że u zwierząt cAMP zaangażowany jest w działanie hormonów i neurotransmiterów oraz regulację szeregu szlaków metabolicznych, np. w percepcję i rozpoznawanie zapachów, natomiast cGMP uczestniczy między innymi w procesie przetwarzania impulsu świetlnego na sygnał nerwowy, działa na NO-zależnym szlaku regulacji skurczu mięśni oraz bierze udział w regulacji transportu jonów Na^+ w poprzek błony [Newton, Smith 2004].

Poszukiwania cyklicznych nukleotydów w komórkach roślinnych rozpoczęły się na przełomie lat 60. i 70. XX wieku. Pierwsze odkrycia spotkały się z licznymi wątpliwościami oraz sceptycyzmem środowiska naukowego, które związane były głównie z faktem, iż poziom cyklicznych nukleotydów w komórkach roślinnych był wielokrotnie niższy niż w komórkach zwierzęcych, jak i wątpliwą istotnością biologiczną związaną z tak niskim stężeniem tych cząsteczek. Jednak, zastosowanie dokładniejszych technik chromatograficznych, radioimmunologicznych, bioluminescencji oraz spektrometrii mas ostatecznie dowiodło istnienia roślinnych cyklicznych nukleotydów i ich roli w licznych procesach (pato)fizjologicznych. Pierwsze prace wskazują na udział cGMP w reakcjach zachodzących w komórkach roślinnych i dotyczą między innymi zmian stężenia cGMP podczas podziałów i wzrostu komórek *Nicotiana tabacum* [Lundeen i in. 1973] oraz zaangażowania w rozwój chloroplastów [Bowler i in. 1994a; Bowler i in. 1994b; Wu i in. 1996]. Ponadto, cGMP został zidentyfikowany jako cząsteczka sygnalna biorąca udział w regulacji kiełkowania nasion u *Arabidosis thaliana* [Teng i in. 2010], pośrednicząca w formowaniu korzeni przybyszowych u *Cucumis sativus* [Pagnussat i in. 2003] oraz zaangażowana w indukcję kwitnienia kontrolowanego przez fitochrom u *Pharbits nil* [Szmidt-Jaworska i in. 2008]. Jednocześnie odnotowano

udział cGMP w reakcjach będących następstwem pojawienia się stresorów abiotycznych i biotycznych. Istnieją dowody, że u *Arabidopsis thaliana* i *Hippeastrum hybridum* cGMP jest zaangażowany w percepcję zewnątrzkomórkowych bodźców abiotycznych i biotycznych, a następnie wzmacnianie i transdukcję sygnałów [Donaldson i in. 2004; Świeżawska i in. 2015; Hussain i in. 2016].

Istotnym aspektem w poznaniu szlaków transdukcji sygnału z udziałem cyklicznych nukleotydów u roślin jest identyfikacja oraz charakterystyka zarówno enzymów biorących udział w ich syntezie (cyklazy), jak i inaktywacji (fosfodiesterazy) oraz efektorów cNMP, tj. kinaz zależnych od cyklicznych nukleotydów i kanałów bramkowanych cyklicznymi nukleotydami. Elementy te zostały szeroko omówione w pracach przeglądowych, których jestem współautorką [Świeżawska i in. 2018; Duszyn i in. 2019; Świeżawska-Boniecka i in. 2021b]. Cykliczny guanozyno-3':5'-monofosforan (cGMP) syntetyzowany jest w reakcji katalizowanej przez cyklazę guanylanową (GC), a degradowany przez fosfodiesterazy cyklicznych nukleotydów. Przeważająca ilość informacji na temat budowy, mechanizmów działania i roli cyklaz w procesach sygnalnych dotyczy komórek zwierzęcych. Jednakże, wiedza dotycząca roślinnych GC z każdym rokiem jest powiększana o nowe informacje, a zebrane dane wskazują na znaczne rozbieżności w budowie i funkcjonowaniu cyklaz u organizmów prokariotycznych i eukariotycznych oraz roślin i zwierząt. Przez wiele lat brakowało bezpośrednich dowodów biochemicznych i molekularnych na istnienie cyklaz guanylanowych w komórkach roślinnych. W związku z początkowymi trudnościami w poszukiwaniu białek o potencjalnej aktywności GC, przeprowadzono szereg analiz bioinformatycznych mających na celu ustalenie sekwencji ewolucyjnie zachowywanego motywu specyficznego dla tych enzymów. W oparciu o dotychczas poznane sekwencje GC z cyjanobakterii, niższych oraz wyższych organizmów eukariotycznych określono 14-aminokwasowy, wysoko zachowany ewolucyjnie, region teoretycznego centrum aktywnego domeny katalitycznej. W 2003 roku, dzięki analizie genomu *A. thaliana* zidentyfikowano siedem genów kodujących białka o przypuszczalnej aktywności GC. Jedno spośród nich charakteryzowało się obecnością motywu bogatego w glicynę na końcu aminowym i zostało wykorzystane do dalszych analiz, które zakończyły się zidentyfikowaniem oraz scharakteryzowaniem pierwszej roślinnej cyklazy guanylanowej (AtGC1). Enzym ten do swojej aktywności wymaga jonów Mg^{2+}, przejawia znacznie wyższe powinowactwo względem GTP aniżeli ATP i ma charakter cytozolowy. Ponadto,

AtGC1 znacząco różni się budową od zwierzęcych odpowiedników zarówno o charakterze błonowym (pGC, ang. *particulate Guanylyl Cyclase*), jak i cytoplazmatycznym (sGC, ang. *soluble Guanylyl Cyclase*). Jedną z cech różnicujących jest obecność domeny katalitycznej na końcu aminowym polipeptydu, a nie na karboksylowym jak ma to miejsce w przypadku cyklaz zwierzęcych. Ponadto, w roślinnej GC nie zidentyfikowano sekwencji wiążącej hem oraz tlenek azotu NO, a enzym jest aktywny w formie monomeru, a nie homo- czy heterodimeru jak sGC i pGC u zwierząt [Ludidi, Gehring 2003]. Kolejno, w 2008 roku scharakteryzowano homolog genu *AtGC1* u *Zea mays* (*ZmGC1*), a w 2009 roku u *Pharbitis nil* (*PnGC1*) [Yuan i in. 2008; Szmidt-Jaworska i in. 2009]. Także, te enzymy nie posiadają domeny dimeryzacyjnej, transmembranowej, kinazowej oraz wiążącej NO, a domena katalityczna ze wszystkimi charakterystycznymi aminokwasami znajduje się na końcu aminowym polipeptydu. Od tego czasu opisano szereg cyklaz guanylanowych, wśród których wyodrębniono dwie grupy. Cyklazy należące do pierwszej grupy występują jako samodzielne białka cytoplazmatyczne (np. HpGC1 [Świeżawska i in. 2015]). W drugiej grupie domena cyklazy jest częścią większego, wielodomenowego kompleksu, najczęściej zakotwiczonych w błonie białek pełniących funkcje receptorów wiążących ligandy. Ta druga grupa białek określana jest jako białka wielofunkcyjne (ang. *moonlight proteins*) [Turek, Irving 2021]. Wszystkie analizowane receptory z domeną cyklazową należą do klasy transmembranowych receptorów LRR-RLK (ang. *Leucine Rich Repeats – Receptor Like Kinases*), które posiadają zewnątrzkomórkową domenę bogatą w powtórzenia leucynowe odpowiedzialne za interakcje z peptydami na N-końcu oraz wewnątrzkomórkową domenę kinazową na C-końcu. W ostatnich latach to właśnie ta grupa jest głównym przedmiotem zainteresowania badaczy. Do tej pory zidentyfikowano cztery grupy receptorów o podwójnej aktywności, kinazy oraz cyklazy guanylanowej, u *A. thaliana*, *Oryza sativa*, *Hippeastrum hybridum*, *Solanum lycopersicum*. Przedstawione spektrum gatunków wskazuje na rozpowszechnienie tej grupy białek zarówno u roślin jedno- jak i dwuliściennych. Do grupy wspomnianych białek w komórkach roślinnych należą: receptory brassinosteroidów (BRI1, ang. *brassinosteroid insensitive* 1; AtBRI1 [Kwezi i in. 2007]), fitosulfokin (PSKR1, ang. *phytosulfokine receptor* 1; AtPSKR1 [Kwezi i in. 2011]), peptydów patogenowych (PepR, ang. *pathogen peptide receptor*; AtPepR1 [Qi i in. 2010], HpPepR1 [Świeżawska i in. 2017], SlGC17 i SlGC18 [Rahman i in. 2020]) oraz receptory WAKL (ang. *wall-associated kinase-like*; AtWAKL10 [Meier i in. 2010], OSWAKL21.2 [Malukani i in.

2020]). W odróżnieniu od zwierzęcych receptorów posiadających aktywność cyklazy, w ich roślinnych odpowiednikach domena charakterystyczna dla GC znajduje się w obrębie wewnątrzkomórkowej domeny kinazowej badanego receptora. W przypadku zwierzęcego receptora domena homologiczna do kinazowej oraz katalityczna odpowiadająca za konwersję GTP do cGMP występują obok siebie lub są oddzielone krótką sekwencją, tworząc dwa przestrzenie odsunięta od siebie i funkcjonalnie zróżnicowane regiony.

Kolejne badania, skupione wokół analizy 14-aminokwasowego regionu centrum aktywnego GC ([RKS][YFW][CTGH][VIL][FV]G[DNA]X[VIL]X{4}[KR]), który przyczynił się do odkrycia AtGC1 w 2003 roku, doprowadziły do lepszego scharakteryzowania i wyselekcjonowania potencjalnych aminokwasów funkcyjnych dla konkretnej pozycji i funkcji w centrum katalitycznym. Przeprowadzono szereg analiz bioinformatycznych, symulacji dokowania oraz mutacji punktowych w domenie aktywnej celem sprawdzenia jak poszczególne aminokwasy wpływają na aktywność i specyficzność substratową GC [Kwezi i in. 2011; Muleya i in. 2014; Wong i in. 2015; Turek, Gehring 2016; Wheeler i in. 2017; Świeżawska-Boniecka i in. 2021a]. W rezultacie, aminokwasom w pozycjach 1, 3 i 14 przypisano funkcje ważne dla aktywności katalitycznej. Aminokwas w pozycji 1 wiąże się z guaniną GTP, aminokwas w pozycji 3 nadaje specyficzność substratową rozróżniając GTP od ATP, zaś aminokwas w pozycji 14 stabilizuje przemianę GTP do cGMP. Dodatkowo, potwierdzono, iż aminokwas odpowiedzialny za wiązanie kofaktora (Mg^{2+} lub Mn^{2+}) występuje dwie, trzy lub cztery pozycje aminokwasowe za centrum katalitycznym w kierunku C-końca [Wong i in. 2015, 2018].

Scharakteryzowane roślinne cyklazy guanylanowe odgrywają istotną rolę w licznych procesach fizjologicznych. Badania wykazały, że białka z potwierdzoną aktywnością GC są zaangażowane we wzrost roślin (AtBRI1 [Wheeler i in. 2017], AtPSKR1[Kwezi i in. 2011]), kierunkowy wzrost łagiewki pyłkowej (AtDGK4 [Wong i in. 2020]) i odpowiedź na infekcję (AtPepR1 [Qi i in. 2010], HpPepR1 [Świeżawska i in. 2017], OsWAKL21.2 [Malukani i in. 2020], SlGC17 i SlGC18 [Rahman i in. 2020], HpGC1 [Świeżawska i in. 2015]). Jednakże do chwili obecnej mechanizmy molekularne angażujące GC i cGMP nie zostały szczegółowo poznane. Wśród zidentyfikowanych u *A. thaliana* receptorów transbłonowych z rodziny LRR-RLK, charakteryzujących się obecnością wewnątrzkomórkowej domeny o aktywności cyklazy guanylanowej, znajdują się białka

AtPepR1 oraz AtWAKL-10. Wykazano, że domena zewnątrzkomórkowa tych białek ma zdolność do rozpoznawania molekularnych wzorców związanych z niebezpieczeństwem, DAMP (ang. *danger-associated molecular pattern*). U *A. thaliana* istnieje rodzina peptydów AtPep1-6, dla których receptorem jest prawdopodobnie AtPepR1. Rozpoznanie białka prowadzi do aktywacji części kinazowo-cyklazowej domeny receptora, syntezy cGMP i w efekcie miejscowego podwyższenia poziomu cGMP, co z kolei aktywuje zlokalizowany w błonie plazmatycznej kanał (CNGC) i prowadzi do kierunkowego transportu Ca^{2+}, a następnie uruchomienia kaskady zdarzeń zależnych od wapnia [Qi i in. 2010]. Wykazano, że podobnie jak AtPepR1, również receptor WAKL10 wiąże ligand za pomocą motywu EGF, co prowadzi do aktywacji domeny kinazowo-cyklazowej, która generuje szybką syntezę cGMP. W badaniach wykazano korelację pomiędzy ekspresją genu *WAKL10,* a ekspresją innych genów szybkiej odpowiedzi roślin na stres biotyczny, takich jak geny *PR1* (ang. *pathogenesis-related* 1), *PAL* (ang. *phenylalanine ammonia lyase*), geny biosyntezy kwasu salicylowego (SA) i kwasu jasmonowego (JA) [Meier i in. 2010]. Ponadto zaobserwowano, że w następstwie infekcji grzybiczej dochodzi do akumulacji transkryptu innego genu kodującego receptor transbłonowy z rodziny LRR-RLK, *HpPepR1*, podczas gdy mechaniczne uszkodzenia nie miały wpływu na profil ekspresji badanego genu [Świeżawska i in. 2017]. Łącznie wyniki te wskazują na udział szlaku zależnego od cGMP w alarmowych reakcjach roślin indukowanych infekcją patogenem.

Innymi cząsteczkami szeroko analizowanymi i zaangażowanymi w odpowiedź roślin na infekcję patogenem są fitohormony [Buhrow i in. 2021; Hemelíková i in. 2021; Ngou i in. 2021; Nunes da Silva i in. 2022]. Dowiedziono, że zarówno kwas salicylowy, jak i jasmoniany biorą udział w reakcjach stresowych i odporności na patogeny tworząc sieć interakcji, często działając zależnie od siebie. Wykazano, że gdy szlak SA jest aktywowany w miejscu infekcji, w innych częściach rośliny wyzwalana jest odpowiedź obronna, aby chronić nieuszkodzone tkanki. Ponadto, mutanty niewrażliwe na SA lub niezdolne do akumulacji SA wykazują zwiększoną podatność na patogeny, natomiast wzrost poziomu SA w tkankach narażonych na patogen prowadzi do indukcji genów związanych z patogenezą [van Loon i in. 2006]. U *A. thaliana* SA i JA oddziałują antagonistycznie, co może modulować ekspresję genów obronnych w odpowiedzi na zakażenie patogenem [Takahashi i in. 2004]. Jednak szczegółowe relacje między tymi

hormonami, a innymi cząsteczkami, w tym cyklicznymi nukleotydami, są wciąż nieznane.

Głównym celem cyklu prac przedstawionych w niniejszej rozprawie doktorskiej jest odpowiedź na pytanie o rolę cyklaz guanylanowych w reakcjach uruchamianych w komórkach roślin jednoliściennych w następstwie biotycznych czynników stresowych. Podjęłam się weryfikacji hipotezy w myśl której cykliczny GMP jest cząsteczką sterującą, odpowiedzialną za uruchomienie procesów obronnych w komórkach *Brachypodium distachyon* na skutek infekcji nekrotroficznym patogenem *Fusarium pseudograminearum.* Infekcja tym patogenem powoduje chorobę zwaną zgorzelą węzła krzewienia (ang. *Fusarium crown rot*, FCR), a typowymi jej objawami są brązowienie koleoptyla, międzywęźla podkorony, dolnych pochew liściowych i przyległych łodyg oraz tkanek węzłowych. To brązowienie może stać się widoczne w ciągu kilku tygodni po kiełkowaniu lub w trakcie rozwoju rośliny, a zainfekowane rośliny mogą rozwinąć białe kłosy bez lub z pomarszczonymi ziarniakami. Choroba ta jest jednym z problemów dotykającym uprawy zbóż i wpływającym na duże straty plonów i jakości. Zaplanowane i przeprowadzone doświadczenia pozwoliły odpowiedzieć na pytanie, jakie jest miejsce cyklaz guanylanowych (rozpuszczalnej BdGUCD1 i błonowej BdPepR2) oraz funkcja cyklicznych nukleotydów w procesach zachodzących w komórkach roślinnych pod wpływem infekcji grzybowej i określenie zależności przyczynowo - skutkowych pomiędzy cyklazami, cyklicznymi nukleotydami i wybranymi hormonami stresowymi. Jako model badawczy wykorzystałam kłosownicę dwukłoskową (*Brachypodium distachyon*), która tym się różni od wielu innych traw, że posiada bardzo mały genom jądrowy, o niewielkiej zawartości sekwencji powtarzalnych i składzie genów zbliżonym do tych, które posiadają niezwykle dla nas ważne gatunki zbóż i wiele traw o charakterze użytkowym. Najważniejszymi cechami tej rośliny są niewielkie rozmiary, krótki cykl życiowy oraz nieskomplikowane wymagania odżywcze. Dzięki temu kłosownica dwukłoskowa została określona „modelową trawą" i materiałem w wielu badaniach dotyczących mechanizmów reakcji obronnych u traw. Zmiany w transkryptomie *B. distachyon* podczas infekcji patogenem grzybowym wykazują podobny profil do tych odnotowanych u *Triticum aestivum*, co potwierdza, że B distachyon może być dobrym organizmem modelowym dla badania interakcji trawa-patogen [Powell i in. 2017]. Podsumowując, zaproponowałam, że po zakażeniu patogenem *Fusarium pseudograminearum* cGMP jest cząsteczką kontrolną odpowiedzialną za zmianę

podstawowego programu stabilizacji, na naprawczy i obronny, wpływając na hormony roślinne.

W pierwszej pracy (Artykuł I) analizowałam aktywność enzymatyczną potencjalnej cyklazy guanylanowej BdPepR2. Białko należy do klasy transbłonowych receptorów LRR-RLK (ang. *Leucine Rich Repeats-Receptor Like Kinases*), a sekwencja aminokwasowa wykazuje podobieństwo do scharakteryzowanych białek u *Arabidopsis thaliana* (AtPepR1, AtPepR2 [Qi i in. 2010]) oraz *Hippeastrum hybridum* (HpRepR1 [Świeżawska i in. 2017]). 14-aminokwasowe centrum aktywne cyklaz guanylanowych zostało scharakteryzowane, a kluczowe dla aktywności enzymatycznej aminokwasy opisane [Wong i in. 2015, 2018]. Co istotne, analizy informatyczne wykazały, że w sekwencji aminokwasowej BdPepR2 występuje różnica w kluczowym aminokwasie centrum katalitycznego, w stosunku do centrum opisanego w literaturze, gdzie po raz pierwszy w tym miejscu obserwowano metioninę. Przedstawiłam więc hipotezę, że metionina w centrum aktywnym BdPepR2 będzie miała wpływ na funkcjonowanie centrum katalitycznego. Porównywałam aktywność enzymatyczną *in vitro* białek uzyskanych metodą mutagenezy kierunkowej z BdPepR2 w celu identyfikacji optymalnego aminokwasu w tym kluczowym miejscu centrum aktywnego cyklazy guanylanowej oraz zweryfikowania, czy funkcjonalny motyw rdzenia można rozszerzyć przez dodanie metioniny. Testowałam również jak zmiany te wpływają na aktywność kinazową białka.

W drugiej pracy (Artykuł II) testowałam hipotezę mówiącą, że cyklazy guanylanowe BdGUCD1 i BdPepR2 oraz cGMP uczestniczą w odpowiedzi na infekcję *Fusarium pseudograminearum* u *Brachypodium distachyon,* oraz, że to cGMP jest cząsteczką odpowiedzialną za uruchomienie kaskady obronnej i aktywację szlaku związanego z hormonami roślinnymi, tzn. analizowałam czy istnieją zależności przyczynowo skutkowe pomiędzy cGMP, a jasmonianami, kwasem salicylowym i kwasem abscysynowym, w następstwie infekcji.

Posumowanie i wnioski

Badania molekularne dotyczące 14-aminokwasowego centrum aktywnego cyklaz guanylanowych doprowadziły do scharakteryzowania aminokwasów wchodzących w skład centrum i przypisania im potencjalnej roli. Porównując centrum katalityczne GC BdPepR2 [1052-SYGVVELLCRKMPVD-1070] do danych literaturowych można wskazać, że wszystkie aminokwasy z wyjątkiem metioniny w pozycji 14 są rozpoznane jako kluczowe dla aktywności GC [Wong i in. 2015, 2018]. W przypadku BdPepR2 przewiduje się, że seryna zlokalizowana w pozycji 1 tworzy wiązanie wodorowe z guaniną, glicyna w pozycji 3 nadaje specyficzność substratową dla GTP, a aminokwas w pozycji 14 wiąże się do fosforanowej grupy acylowej i stabilizuje konwersję od GTP do cGMP. W BdPepR2 w pozycji 14 znajduje się metionina i zgodnie z dostępnymi danymi literaturowymi, taki aminokwas nigdy nie był badany jako potencjalny aminokwas w centrum GC. Dlatego moim celem było zweryfikowanie, czy funkcjonalny motyw rdzenia można rozszerzyć przez uwzględnienie metioniny. Zidentyfikowana sekwencja białka BdPepR2 zawiera również kwas asparaginowy, czyli resztę aminokwasową odpowiedzialną za oddziaływanie z jonami Mg^{2+}/Mn^{2+} zlokalizowaną dwie pozycje aminokwasowe za 14-aminokwasowym motywem w kierunku C-końca [Wong i in. 2015]. W opisywanym motywie zlokalizowane w pozycji 14 lizyna lub arginina wydają się być najkorzystniejszym funkcjonalnie rozwiązaniem i optymalnym aminokwasem dla aktywności GC białka [Wong i in. 2018]. Jednak modelowanie struktury połączone z dokowaniem substratu sugeruje, że w przypadku BdPepR2 metionina może przejąć rolę lizyny lub argininy z dodatnim powinowactwem dokowania GTP w centrum GC (Artykuł I). Dodatkowo, aby zbadać, czy metionina w pozycji 14 w centrum GC BdPepR2 jest preferowana, zaprojektowałam dwa białka z różnymi mutacjami w centrum GC: BdPepR2M1066A (obniżona aktywność GC) i BdPepR2M1066R (według literatury najbardziej korzystny aminokwas w tej pozycji dla aktywności enzymatycznej GC) i zbadałam ich aktywność enzymatyczną *in vitro*. Zaproponowane mutacje punktowe bazują na wcześniejszych odkryciach, które wskazywały, że konwersja ze zdefiniowanej lizyny lub argininy do alaniny lub treoniny powoduje spadek aktywności GC [Wheeler i in. 2017]. W rezultacie badanie aktywności GC, wyrażanej jako poziom cGMP, metodą LC-MS/MS, ujawniło, że białko BdPepR2M1066A wykazuje istotne zmniejszenie produkcji cGMP w porównaniu z rekombinowanym białkiem BdPepR2 typu dzikiego. Z kolei rekombinowane białko

BdPepR2M1066R wykazywało porównywalną aktywność cyklazy guanylanowej jak BdPepR2 typu dzikiego, co jest zgodne z danymi literaturowymi, wskazującymi że dodatnio naładowana reszta lizyny lub argininy jest wymagana do interakcji z grupą fosforanową GTP w centrach katalitycznych, jak zaobserwowano w innych roślinnych GC. Jednak uzyskane wyniki wykazały, że w przypadku BdPepR2 obojętna niepolarna reszta metioniny może przejąć rolę lizyny lub argininy w tej pozycji, a nawet wpływać na większą aktywność enzymatyczną, co może być unikalną cechą cyklaz guanylanowych roślin jednoliściennych (Artykuł I). Ponadto, potwierdziłam, że enzym BdPepR2 wymaga do swojej aktywności kofaktora w postaci jonów manganu oraz katalizuje konwersję zarówno ATP, jak i GTP do cyklicznych form tych nukleotydów. Jednakże w przypadku GTP reakcja była znacznie bardziej wydajna, a poziom oznaczanego cGMP jako produktu był trzykrotnie wyższy niż poziom cAMP w analogicznej reakcji z użyciem ATP jako substratu (Artykuł I). Wszystkie te dane dowodzą, że BdPepR2 jest kolejną, trzecią cyklazą guanylanową scharakteryzowaną u roślin jednoliściennych, a unikalna reszta aminokwasowa jaką jest metionina w centrum aktywnym pozytywnie wpływa na aktywność GC. Porównanie sekwencji aminokwasowej BdPepR2 z innymi przewidywanymi białkami PEPR1/2 z rodziny *Poaceae* wykazało zgodność wszystkich 14 aminokwasów w domenie katalitycznej GC, co może sugerować na różnice w budowie aminokwasowej domeny w różnych rodzinach roślin. BdPepR2 należy do klasy transmembranowych receptorów LRR-RLK z wewnątrzkomórkową domeną kinazową, która zawiera 14-aminokwasowe centrum GC. Potwierdziłam, że BdPepR2 oprócz aktywności GC ma również aktywność kinazy serynowo/treoninowej. Ponadto wykazałam, że wszystkie trzy białka, BdPepR2, BdPepR2M1066A i BdPepR2M1066R, mają podobną aktywność kinazową. Obie mutacje nie miały wpływu na aktywność kinazy, co oznacza, że aminokwas w pozycji 14 w centrum katalitycznym GC BdPepR2 nie wpływa na aktywność kinazową białka, co sugeruje, że główną funkcją tego regionu jest synteza cGMP. Ponadto, wykazałam, że cGMP w istotny sposób obniża aktywność kinazy BdPepR2 (Artykuł I). Podobny efekt hamowania aktywności kinazy przez cGMP zaobserwowano w aktywności kinazy AtBRI1 [Kwezi i in. 2007] i AtPSKR1 [Kwezi i in. 2011]. Podsumowując, wyniki te potwierdzają, że oprócz funkcjonalnej domeny GC, rekombinowany BdPepR2 zawiera również funkcjonalną domenę kinazy, co czyni go cząsteczką dwufunkcyjną, przy czym cGMP jest odpowiedzialny za hamowanie aktywności kinazy na zasadzie ujemnego sprzężenia zwrotnego (Artykuł I).

Scharakteryzowany BdPepR2 należy do cyklaz, w których region katalityczny GC jest częścią większego, wielodomenowego kompleksu. Jak wspomniano wcześniej wyodrębniono również grupę cyklaz o charakterze cytozolowy, które funkcjonują jako białka bez aktywności kinazowej. Do grupy tej należy BdGUCD1, u którego mimo braku charakterystycznej 14-aminokwasowej domeny potwierdziłam aktywność cyklazy guanylanowej oraz niższą aktywność cyklazy adenylanowej (AC). Analiza kinetyki enzymu pozwoliła na wykazanie różnic w wymaganiach względem kofaktora. Dla aktywności GC niezbędne są zarówno magnez, jak i mangan, natomiast dla aktywności AC konieczny jest tylko mangan (Artykuł II). Różne wymagania mogą być związane z regulacją aktywności BdGUCD1 w komórkach *B. distachyon.* Co istotne, aktywność enzymatyczna *in vitro* obu białek, BdPepR2 i BdGUCD1, badana była tą samą metodą, w podobnym czasie, co pozwala potwierdzić, że oba białka niezaprzeczalnie są cyklazami o podobnej aktywności, mimo, że tylko BdPepR2 zawiera domenę charakterystyczną dla roślinnych cyklaz. Sugeruje to, że istnieje grupa cyklaz o innej budowie strukturalnej oraz składzie aminokwasowym domeny katalitycznej. Warto w tym miejscu wspomnieć, że białka o potwierdzonej aktywności GC często mają dodatkową aktywność fosfodiesterazy, cyklazy adenylanowej lub kinazy modulowanej przez cykliczne nukleotydy lub wapń, co przypuszczalnie może wpływać na aktywność GC i może mieć znaczenie dla funkcjonowania tych enzymów w komórkach roślinnych w określonych procesach. Często trudno porównywać wszystkie scharakteryzowane cyklazy pod kątem ich aktywności w związku z odnotowanymi różnicami w kinetyce reakcji. Główne przyczyny tych różnic upatruje się w czułości metod pomiarowych stosowanych do określenia stężenia cGMP. W ostatnich latach stosowano różne metody, w tym test radioimmunologiczny [3H] cGMP, system testowy cGMP [125I], system immunoenzymatyczny (EIA) cGMP oraz metodę z wykorzystaniem LC-MS/MS. Jednak niezależnie od zastosowanej metody, obserwowana aktywność GC, a co za tym idzie, poziom cGMP w roślinach jest zawsze niższy niż rejestrowany dla komórek zwierzęcych [Rogers i in. 2014]. Przypuszcza się, że głównym powodem tej różnicy jest fakt, że roślinne GC do uzyskania wyższej aktywności mogą wymagać kofaktorów (np. wapnia, białek opiekuńczych i koprotein) lub niepoznanych jeszcze modyfikacji potranslacyjnych.

Dane literaturowe wskazują na udział cGMP i enzymów z potwierdzoną *in vitro* aktywnością GC w odpowiedzi na stresowe czynniki biotyczne między innymi

u *H. hybridum* [Świeżawska i in. 2015, 2017], *A. thaliana* [Qi i in. 2010], *S. lycopersicum* [Rahman i in. 2020]. Także w swoich badaniach skupiłam się na określeniu roli wybranych elementów szlaków cNMP, tj. cyklaz (BdGUCD1, BdPepR2) i cyklicznych nukleotydów w regulacji reakcji w odpowiedzi na stres biotyczny. Endogenny poziom cGMP i cAMP oraz poziom ekspresji genów *BdGUCD1* i *BdPepR2* określiłam w czterech punktach czasowych po inokulacji *F. pseudograminearum* (1, 3, 5 i 7 dni po traktowaniu patogenem). Uzyskane wyniki wykazały na brak zmian w poziomie cAMP po inokulacji w porównaniu z wariantami kontrolnymi. Natomiast stężenie cGMP po zakażeniu *F. pseudograminearum* uległo istotnym zmianom gdyż w 1 i 3 dobie poziom wzrósł dwukrotnie w stosunku do roślin niezainfekowanych. Dane te potwierdziły moje przewidywania, że cGMP jest cząsteczką zaangażowaną w reakcje obronne *B. distachyon.* Aby potwierdzić fakt, że za odnotowane zmiany odpowiadają cyklazy nukleotydów purynowych testowałam zmiany w poziomie dwóch enzymów syntetyzujących cGMP, *BdPepR2* oraz *BdGUCD1*. Analizy bioinformatyczne w ostatnich latach, dostarczyły danych wskazujących na istnienie w wielu białkach domen odpowiedzialnych za reakcję cyklizacji nukleotydów, a tylko u nielicznych z nich udało się potwierdzić aktywność enzymatyczną *in vitro.* Stąd też, do dalszych badań wybrałam reprezentantów z dwóch grup roślinnych GC. BdPepR2 jest receptorem błonowym peptydowych cząsteczek sygnałowych o potwierdzonej podwójnej aktywności kinazowej i cyklazy guanylanowej (Artykuł I). W poprzednich latach opisano zmiany w poziomie transkryptów genów kodujących inne białka tej rodziny, *HpPepR1* [Świeżawska i in. 2017], *AtWAKL10* [Meier i in. 2009], a wyciszenie homologów *AtPepR1* u *S. lycopersicum*, *SlGC17* i *SlGC18*, znacząco osłabiło odporność na wybrane patogeny. Drugim analizowanym genem był, *BdGUCD1*, kodujący aktywną cyklazę guanylanową, bez domen transmembranowych oraz 14-aminokwasowej domeny charakterystycznej dla roślinnych GC. Uzyskane dane wykazały, że podczas 7-dniowego cyklu badawczego po infekcji grzybiczej ekspresja obu genów ulegała zmianom. W obu przypadkach odnotowano szybki, trzykrotny wzrost poziomu transkryptów tych genów w pierwszej dobie. Kolejny znaczący wzrost obserwowałam w piątej dobie po inokulacji w przypadku *BdPepR2* i siódmej dla *BdGUCG1*. Ta znacząca stymulacja ekspresji genu *BdPepR2* indukowana zakażeniem *F. pseudograminearum* może sugerować rolę białka BdPepR2 w aktywacji odpowiedzi obronnej roślin. Co ważne, profil ekspresji genu *BdPepR2* pokrywa się ze szczytem endogennego poziomu cGMP dzień po zainfekowaniu *F. pseudograminearum*. Jednocześnie obserwowane zmiany w poziomach mRNA

są zgodne z wcześniejszą hipotezą, ponieważ gen *BdPepR2* koduje białko prawdopodobnie działające jako receptor odpowiedzialny za rozpoznawanie białka patogenu.

Przedstawione powyżej dane wskazują na udział cGMP i cyklaz guanylanowych w odpowiedź na biotyczny czynnik stresowy u *B. distachyon*. Kolejnym interesującym zagadnieniem jest ustalenie jak te elementy współdziałają z innymi cząsteczkami zaangażowanymi w procesy obronne, takimi jak hormony roślinne. Wykazano, że zarówno kwas salicylowy (SA), jak i kwas jasmonowy (JA), biorą udział w reakcjach stresowych oraz podstawowej i/lub indukowanej odporności na patogeny roślinne. Co ważne, nie działają one niezależnie od siebie, a raczej tworzą sieć interakcji w odpowiedzi na różne patogeny. Gdy szlak SA jest aktywowany w miejscu infekcji, w dystalnych częściach rośliny wyzwalana jest odpowiedź obronna, mająca na celu ochronę nieuszkodzonych tkanek [Takahashi i in. 2004; Zhang i in. 2020]. Mutanty niewrażliwe na SA lub niezdolne do akumulacji SA wykazują zwiększoną podatność na patogeny, a wzrost poziomu SA w tkankach eksponowanych na patogen prowadzi do indukcji genów związanych z patogenezą [van Loon i in. 2006]. Dokonano analizy wzorców ekspresji genów u *Triticum aestivum* po traktowaniu jasmonianem metylu i wykazano, że indukowało to geny obronne, które zmieniały wzorzec ekspresji również po infekcji *F. pseudograminearum* [Desmond i in. 2005]. Biorąc pod uwagę doniesienia literaturowe, zaplanowane doświadczenie miało na celu określenie zależności przyczynowo - skutkowych pomiędzy cyklicznymi nukleotydami i wybranymi hormonami stresowymi. Pierwszym etapem analiz było zweryfikowanie czy infekcja patogenem *F. pseudograminearum* zmienia poziomy fitohormonów (JA, SA, ABA) u *B. distachyon*. Wyniki pokazały, że do wzrostu stężenia JA doszło w pierwszej dobie po inokulacji, ale nie w późniejszych punktach czasowych. Ponadto, stężenie SA nieznacznie wzrósło 1 dobę po traktowaniu patogenem. Natomiast poziom ABA wzrósł znacznie tylko trzeciego dnia (Artykuł II). Wyniki te są zgodne z poprzednim doniesieniem, sugerującym, że JA działa jako pierwotna reagująca cząsteczka sygnałowa podczas infekcji *F. pseudograminearum* u roślin jednoliściennych [Desmond i in. 2005, 2008]. Kolejnym krokiem było zbadanie, czy podczas reakcji na stres występują jakiekolwiek interakcje między cGMP, a badanymi hormonami roślinnymi. Jednym z narzędzi w tego typu badaniach jest zastosowanie inhibitorów aktywności enzymatycznej. W tym wypadku wykorzystałam inhibitor cyklazy guanylanowej

(NS 2028) podany dzień przed infekcją, a następnie zmierzyłam poziom wszystkich badanych cząsteczek w tym samym czasie, co poprzednio opisywane warianty. Obserwowałam, że endogenne poziomy cGMP w wariantach traktowanych inhibitorem były znacznie niższe niż w roślinach kontrolnych, co potwierdza, że inhibitor NS 2028 blokował aktywność roślinnych GC, nie doprowadzając do syntezy cGMP. W roślinach poddanych infekcji cyklaza guanylanowa była nadal hamowana, a poziomy cGMP pozostały niskie. Zastosowanie inhibitora spowodowało wzrost stężenia JA w porównaniu z kontrolą w pierwszym analizowanym punkcie czasowym (pierwsza doba po inokulacji), natomiast w kolejnych wartości były podobne lub niższe. Stężenia pozostałych analizowanych hormonów, SA i ABA, utrzymały się na podobnym poziomie, wzorzec zmian stężenia w czasie był podobny jak w roślinach kontrolnych. Opisane wyniki uzupełniają dotychczasowa wiedzę na temat odpowiedzi roślin na biotyczny czynnik stresowy. Ponadto wykazałam, że istnieje zależność pomiędzy cGMP, a szlakiem jasmonowym gdyż komórkowa redukcja poziomu cGMP modulowała stężenie JA w roślinach. Na podstawie dostępnych danych można stwierdzić, że jest to efekt pośredni. Hamowanie poziomu cGMP może wzmocnić wytwarzanie elementów obronnych za pośrednictwem transdukcji sygnały JA.

Najważniejsze wyniki uzyskane w czasie badań

Wyniki badań otrzymane podczas realizacji prezentowanej rozprawy doktorskiej umożliwiły sformułowanie następujących wniosków końcowych:

- BdPepR2 jest aktywną cyklazą guanylanową zdolną do konwersji GTP do cGMP (Artykuł I)
- BdPepR2 posiada aktywność kinazową, która jest regulowana przez cGMP (Artykuł I)
- W centrum katalitycznym BdPepR2, w pozycji odpowiedzialnej za stabilizację przemiany GTP do cGMP, znajduje się metionina powodująca wyższą aktywność cyklazy guanylanowej *in vitro* w porównaniu z wzorcowym centrum katalitycznym (Artykuł I)
- BdGUCD1 jest białkiem o podwójnej, AC/GC, aktywności *in vitro*, wyższej aktywności cyklazy guanylanowej i niższej aktywności cyklazy adenylanowej (Artykuł II)
- U *Brachypodium distachyon*, infekcja *Fusarium pseudograminearum* prowadzi do oscylacyjnych zmian profilu ekspresji obu badanych genów kodujących cyklazy guanylanowe (*BdGUCD1* i *BdPepR2*) (Artykuł II)
- W wyniku infekcji *Fusarium pseudograminearum* u *Brachypodium distachyon* dochodzi do zmian w poziomie tylko jednego z cyklicznych nukleotydów tj. cGMP (Artykuł II)
- Obniżenie poziomu cGMP, na skutek zablokowania aktywności cyklaz guanylanowych, wpłynęło na poziom kwasu jasmonowego u *Brachypodium distachyon* po infekcji *Fusarium pseudograminearum* (Artykuł II)

Bibliografia

Ashman D. F., Lipton R., Melicow M. M., Price T. D. 1963. Isolation of adenosine 3', 5'-monophosphate and guanosine 3', 5'-monophosphate from rat urine. Biochemical and Biophysical Research Communications, 11, 330–334. DOI: 10.1016/0006-291x(63)90566-7

Bowler C., Yamagata H., Neuhaus G., Chua N. H. 1994a. Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. Genes & Development, 8 (18), 2188–2202. DOI: 10.1101/gad.8.18.2188

Bowler C., Neuhaus G., Yamagata H., Chua N.-H. 1994b. Cyclic GMP and calcium mediate phytochrome phototransduction. Cell, 77 (1), 73–81. DOI: 10.1016/0092-8674(94)90236-4

Buhrow L. M., Liu Z., Cram D., Sharma T., Foroud N. A., Pan Y., Loewen M. C. 2021. Wheat transcriptome profiling reveals abscisic and gibberellic acid treatments regulate early-stage phytohormone defense signaling, cell wall fortification, and metabolic switches following Fusarium graminearum-challenge. BMC Genomics, 22. DOI: 10.1186/s12864-021-08069-0

Desmond O. J., Edgar C. I., Manners J. M., Maclean D. J., Schenk P. M., Kazan K. 2005. Methyl jasmonate induced gene expression in wheat delays symptom development by the crown rot pathogen Fusarium pseudograminearum. Physiological and Molecular Plant Pathology, 67 (3), 171–179. DOI: 10.1016/j.pmpp.2005.12.007

Desmond O. J., Manners J. M., Schenk P. M., Maclean D. J., Kazan K. 2008. Gene expression analysis of the wheat response to infection by Fusarium pseudograminearum. Physiological and Molecular Plant Pathology, 73 (1), 40–47. DOI: 10.1016/j.pmpp.2008.12.001

Donaldson L., Ludidi N., Knight M. R., Gehring C., Denby K. 2004. Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. FEBS Letters, 569 (1–3), 317–320. DOI: 10.1016/j.febslet.2004.06.016

Duszyn M., Świeżawska B., Szmidt-Jaworska A., Jaworski K. 2019. Cyclic nucleotide gated channels (CNGCs) in plant signalling—Current knowledge and perspectives. Journal of Plant Physiology, 241, 153035. DOI: 10.1016/j.jplph.2019.153035

Hemelíková N., Žukauskaitė A., Pospíšil T., Strnad M., Doležal K., Mik V. 2021. Caged Phytohormones: From Chemical Inactivation to Controlled Physiological Response. Journal of Agricultural and Food Chemistry, 69 (41), 12111–12125. DOI: 10.1021/acs.jafc.1c02018

Hussain J., Chen J., Locato V., Sabetta W., Behera S., Cimini S., Griggio F., Martínez-Jaime S., Graf A., Bouneb M., Pachaiappan R., Fincato P., Blanco E., Costa A., De Gara L., Bellin D., de Pinto M. C., Vandelle E. 2016. Constitutive cyclic GMP accumulation in Arabidopsis thaliana compromises systemic acquired resistance induced by an avirulent pathogen by modulating local signals. Scientific Reports, 6 (1), 36423. DOI: 10.1038/srep36423

Kazan K., Gardiner D. M. 2018. Fusarium crown rot caused by Fusarium pseudograminearum in cereal crops: recent progress and future prospects. Molecular Plant Pathology, 19 (7), 1547–1562. DOI: 10.1111/mpp.12639

Kwezi L., Meier S., Mungur L., Ruzvidzo O., Irving H., Gehring C. 2007. The Arabidopsis thaliana Brassinosteroid Receptor (AtBRI1) Contains a Domain that

Functions as a Guanylyl Cyclase In Vitro. PLoS ONE, 2 (5), e449. DOI: 10.1371/journal.pone.0000449

Kwezi L., Ruzvidzo O., Wheeler J. I., Govender K., Iacuone S., Thompson P. E., Gehring C., Irving H. R. 2011. The Phytosulfokine (PSK) Receptor Is Capable of Guanylate Cyclase Activity and Enabling Cyclic GMP-dependent Signaling in Plants. Journal of Biological Chemistry, 286 (25), 22580–22588. DOI: 10.1074/jbc.M110.168823

Ludidi N., Gehring C. 2003. Identification of a Novel Protein with Guanylyl Cyclase Activity in Arabidopsis thaliana. Journal of Biological Chemistry, 278 (8), 6490–6494. DOI: 10.1074/jbc.M210983200

Lundeen C. V., Wood H. N., Braun A. C. 1973. Intracellular Levels of Cyclic Nucleotides during Cell Enlargement and Cell Division in Excised Tobacco Pith Tissues. Differentiation, 1 (4), 255–260. DOI: 10.1111/j.1432-0436.1973.tb00120.x

Malukani K. K., Ranjan A., Hota S. J., Patel H. K., Sonti R. V. 2020. Dual Activities of Receptor-Like Kinase OsWAKL21.2 Induce Immune Responses. Plant Physiology, 183 (3), 1345–1363. DOI: 10.1104/pp.19.01579

Meier S., Madeo L., Ederli L., Donaldson L., Pasqualini S., Gehring C. 2009. Deciphering cGMP signatures and cGMP-dependent pathways in plant defence. Plant Signaling & Behavior, 4 (4), 307–309. DOI: 10.4161/psb.4.4.8066

Meier S., Ruzvidzo O., Morse M., Donaldson L., Kwezi L., Gehring C. 2010. The Arabidopsis Wall Associated Kinase-Like 10 Gene Encodes a Functional Guanylyl Cyclase and Is Co-Expressed with Pathogen Defense Related Genes. PLoS ONE, 5 (1), e8904. DOI: 10.1371/journal.pone.0008904

Muleya V., Wheeler J. I., Ruzvidzo O., Freihat L., Manallack D. T., Gehring C., Irving H. R. 2014. Calcium is the switch in the moonlighting dual function of the ligand-activated receptor kinase phytosulfokine receptor 1., 5.

Newton R. P., Smith C. J. 2004. Cyclic nucleotides. Phytochemistry, 65 (17), 2423–2437. DOI: 10.1016/j.phytochem.2004.07.026

Ngou B. P. M., Jones J. D. G., Ding P. 2021. Plant immune networks. Trends in Plant Science, S1360-1385(21)00243–0. DOI: 10.1016/j.tplants.2021.08.012

Nunes da Silva M., Carvalho S. M. P., Rodrigues A. M., Gómez-Cadenas Aurelio, António C., Vasconcelos M. W. 2022. Defence-related pathways, phytohormones and primary metabolism are key players in kiwifruit plant tolerance to Pseudomonas syringae pv. actinidiae. Plant, Cell & Environment, 45 (2), 528–541. DOI: 10.1111/pce.14224

Pagnussat G. C., Lanteri M. L., Lamattina L. 2003. Nitric oxide and cyclic GMP are messengers in the indole acetic acid-induced adventitious rooting process. Plant Physiology, 132 (3), 1241–1248. DOI: 10.1104/pp.103.022228

Powell J. J., Carere J., Sablok G., Fitzgerald T. L., Stiller J., Colgrave M. L., Gardiner D. M., Manners J. M., Vogel J. P., Henry R. J., Kazan K. 2017. Transcriptome analysis of Brachypodium during fungal pathogen infection reveals both shared and distinct defense responses with wheat. Scientific Reports, 7 (1), 17212. DOI: 10.1038/s41598-017-17454-3

Qi Z., Verma R., Gehring C., Yamaguchi Y., Zhao Y., Ryan C. A., Berkowitz G. A. 2010. Ca2+ signaling by plant Arabidopsis thaliana Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca2+ channels. Proceedings of the National Academy of Sciences, 107 (49), 21193–21198. DOI: 10.1073/pnas.1000191107

Rahman H., Wang X.-Y., Xu Y.-P., He Y.-H., Cai X.-Z. 2020. Characterization of tomato protein kinases embedding guanylate cyclase catalytic center motif. Scientific Reports, 10 (1), 4078. DOI: 10.1038/s41598-020-61000-7

Rall T. W., Sutherland E. W. 1958. Formation of a cyclic adenine ribonucleotide by tissue particles. The Journal of Biological Chemistry, 232 (2), 1065–1076.

Rogers N. M., Seeger F., Garcin E. D., Roberts D. D., Isenberg J. S. 2014. Regulation of soluble guanylate cyclase by matricellular thrombospondins: implications for blood flow. Frontiers in Physiology, 5, 134. DOI: 10.3389/fphys.2014.00134

Szmidt-Jaworska A., Jaworski K., Kopcewicz J. 2008. Involvement of cyclic GMP in phytochrome-controlled flowering of Pharbitis nil. Journal of Plant Physiology, 165 (8), 858–867. DOI: 10.1016/j.jplph.2007.02.010

Szmidt-Jaworska A., Jaworski K., Pawełek A., Kopcewicz J. 2009. Molecular Cloning and Characterization of a Guanylyl Cyclase, PnGC-1, Involved in Light Signaling in Pharbitis nil. Journal of Plant Growth Regulation, 28 (4), 367–380. DOI: 10.1007/s00344-009-9105-8

Świeżawska B., Duszyn M., Jaworski K., Szmidt-Jaworska A. 2018. Downstream Targets of Cyclic Nucleotides in Plants. Frontiers in Plant Science, 9, 1428. DOI: 10.3389/fpls.2018.01428

Świeżawska B., Jaworski K., Duszyn M., Pawełek A., Szmidt-Jaworska A. 2017. The Hippeastrum hybridum PepR1 gene (HpPepR1) encodes a functional guanylyl cyclase and is involved in early response to fungal infection. Journal of Plant Physiology, 216, 100–107. DOI: 10.1016/j.jplph.2017.05.024

Świeżawska B., Jaworski K., Szewczuk P., Pawełek A., Szmidt-Jaworska A. 2015. Identification of a Hippeastrum hybridum guanylyl cyclase responsive to wounding and pathogen infection. Journal of Plant Physiology, 189, 77–86. DOI: 10.1016/j.jplph.2015.09.014

Świeżawska-Boniecka B., Duszyn M., Hammer K., Wong A., Szmidt-Jaworska A., Jaworski K. 2021. Brachypodium distachyon ERECTA-like1 protein kinase is a functional guanylyl cyclase. Frontiers in Bioscience-Elite, 13 (2), 249. DOI: 10.52586/E882

Świeżawska-Boniecka B., Duszyn M., Kwiatkowski M., Szmidt-Jaworska A., Jaworski K. 2021. Cross Talk Between Cyclic Nucleotides and Calcium Signaling Pathways in Plants–Achievements and Prospects. Frontiers in Plant Science, 12, 643560. DOI: 10.3389/fpls.2021.643560

Takahashi H., Kanayama Y., Zheng M. S., Kusano T., Hase S., Ikegami M., Shah J. 2004. Antagonistic interactions between the SA and JA signaling pathways in Arabidopsis modulate expression of defense genes and gene-for-gene resistance to cucumber mosaic virus. Plant & Cell Physiology, 45 (6), 803–809. DOI: 10.1093/pcp/pch085

Teng Y., Xu W., Ma M. 2010. cGMP is required for seed germination in Arabidopsis thaliana. Journal of Plant Physiology, 167 (11), 885–889. DOI: 10.1016/j.jplph.2010.01.015

Turek I., Gehring C. 2016. The plant natriuretic peptide receptor is a guanylyl cyclase and enables cGMP-dependent signaling. Plant Molecular Biology, 91 (3), 275–286. DOI: 10.1007/s11103-016-0465-8

Turek I., Irving H. 2021. Moonlighting Proteins Shine New Light on Molecular Signaling Niches. International Journal of Molecular Sciences, 22 (3), 1367. DOI: 10.3390/ijms22031367

van Loon L. C., Rep M., Pieterse C. M. J. 2006. Significance of Inducible Defense-related Proteins in Infected Plants. Annual Review of Phytopathology, 44 (1), 135–162. DOI: 10.1146/annurev.phyto.44.070505.143425

Wheeler J. I., Wong A., Marondedze C., Groen A. J., Kwezi L., Freihat L., Vyas J., Raji M. A., Irving H. R., Gehring C. 2017. The brassinosteroid receptor BRI 1 can generate cGMP enabling cGMP -dependent downstream signaling. The Plant Journal, 91 (4), 590–600. DOI: 10.1111/tpj.13589

Wong A., Donaldson L., Portes M. T., Eppinger J., Feijó J. A., Gehring C. 2020. Arabidopsis DIACYLGLYCEROL KINASE4 is involved in nitric oxide-dependent pollen tube guidance and fertilization. Development, 147 (8), dev183715. DOI: 10.1242/dev.183715

Wong A., Gehring C., Irving H. R. 2015. Conserved Functional Motifs and Homology Modeling to Predict Hidden Moonlighting Functional Sites. Frontiers in Bioengineering and Biotechnology, 3. DOI: 10.3389/fbioe.2015.00082

Wong A., Tian X., Gehring C., Marondedze C. 2018. Discovery of Novel Functional Centers With Rationally Designed Amino Acid Motifs. Computational and Structural Biotechnology Journal, 16, 70–76. DOI: 10.1016/j.csbj.2018.02.007

Wu Y., Hiratsuka K., Neuhaus G., Chua N.-H. 1996. Calcium and cGMP target distinct phytochrome-responsive elements. The Plant Journal, 10 (6), 1149–1154. DOI: 10.1046/j.1365-313X.1996.10061149.x

Yuan J., Ali M. L., Taylor J., Liu J., Sun G., Liu W., Masilimany P., Gulati-Sakhuja A., Pauls K. P. 2008. A guanylyl cyclase-like gene is associated with Gibberella ear rot resistance in maize (Zea mays L.). Theoretical and Applied Genetics, 116 (4), 465–479. DOI: 10.1007/s00122-007-0683-1

Zhang N., Zhou S., Yang D., Fan Z. 2020. Revealing Shared and Distinct Genes Responding to JA and SA Signaling in Arabidopsis by Meta-Analysis. Frontiers in Plant Science, 11, 908. DOI: 10.3389/fpls.2020.00908

Streszczenie

Cykliczny 3',5' – guanozynomonofosforan (cGMP) to cząsteczka sygnalna, która pełni istotną rolę zarówno w komórkach prokariotycznych, jak i eukariotycznych. Enzymami odpowiedzialnymi za syntezę cGMP są cyklazy guanylanowe (GC). Roślinne GC charakteryzują się znacząco różną budową od lepiej poznanych i scharakteryzowanych cyklaz zwierzęcych. Do tej pory opisano enzymy te u kilku gatunków roślin oraz zbadano rolę GC i cGMP w procesach wzrostu, rozwoju i w odpowiedzi na zewnętrzne czynniki stresowe.

Celem niniejszej pracy była charakterystyka dwóch cyklaz guanylanowych u *Brachypodium distachyon,* transmembranowej BdPepR2 i cytozolowej (rozpuszczalnej) BdGUCD1, oraz odpowiedź na pytanie o rolę cyklaz guanylanowych w reakcjach uruchamianych w komórkach roślin jednoliściennych w następstwie biotycznych czynników stresowych wywołanych infekcją *Fusarium pseudograminearum,* określenie funkcji cyklicznych nukleotydów w procesach zachodzących w komórkach roślinnych pod wpływem infekcji i ustalenie zależności przyczynowo - skutkowych pomiędzy cyklazami, cyklicznymi nukleotydami i wybranymi hormonami stresowymi.

W toku prac dowiodłam, iż rekombinowane białka BdPepR2 i BdGUCD1 charakteryzują się aktywnością enzymatyczną cyklazy guanylanowej *in vitro*. Ponadto, potwierdziłam aktywność kinazy serynowo/treoninowej u BdPepR2, która jest regulowana przez cGMP. Powyższe doświadczenia biochemiczne uzupełniłam analizami *in silico*, które wykazały, że BdPepR2 zawiera 14-aminokwasowe centrum katalityczne charakterystyczne dla roślinnych GC. Jednak w przypadku BdPepR2 w jednej z funkcjonalnie istotnych pozycji występuje metionina, nigdy wcześniej nie analizowana jako potencjalny aminokwas wchodzący w skład centrum aktywnego. Modelowanie struktury połączone z dokowaniem substratu sugeruje, że w przypadku BdPepR2 metionina może wykazywać dodatnie powinowactwo względem GTP w centrum GC, dane te wymagały jednak weryfikacji poprzez wykonanie serii doświadczeń. Wykorzystując mutagenezę kierunkową zaprojektowałam białka z różnymi mutacjami w centrum GC, a następnie potwierdziłam, że metionina jest aminokwasem najbardziej preferowanym spośród analizowanych. Jej obecność wiązała się z najwyższą aktywnością GC *in vitro*, co może być unikalną cechą cyklaz guanylanowych roślin jednoliściennych. Kolejno, testowałam

czy cyklazy guanylanowe, BdGUCD1 i BdPepR2, oraz cGMP uczestniczą w odpowiedzi *Brachypodium distachyon* na infekcję *Fusarium pseudograminearum* oraz analizowałam czy istnieją zależności przyczynowo skutkowe pomiędzy cGMP, a jasmonianami, kwasem salicylowym i kwasem abscysynowym, w następstwie infekcji. W wyniku przeprowadzonych analiz wykazałam, że wzrost poziomu cGMP inicjowany jest infekcją *F. pseudograminearum*, a analizy poziomu transkryptu ukazały dynamiczne zmiany ekspresji genów *BdGUCD1* i *BdPepR2*, wykazując, że podlegają one regulacji przez infekcję grzybową. Ponadto, odnotowałam, że w pierwszej dobie po inokulacji *F. pseudograminearum* w komórkach *B. distachyon* dochodzi do znaczącego wzrostu poziomu kwasu jasmonowego , natomiast stężenie kwasu abscysynowego wzrasta tylko trzeciego dnia. Ponadto, dzięki zastosowaniu inhibitora cyklaz guanylanowych przed inokulacją *F. pseudograminearum*, wykazałam, że istnieje zależność pomiędzy cGMP, a szlakiem jasmonowym gdyż zahamowanie syntezy cGMP modulowało poziom tylko jednego z analizowanych hormonów, kwasu jasmonowego. Opisane wyniki pozwoliły na wkomponowanie dokonanych odkryć do istniejącego stanu wiedzy o metabolizmie cyklicznych nukleotydów u roślin oraz uzupełniły dotychczasowa wiedzę na temat odpowiedzi *B. distachyon* na biotyczny czynnik stresowy.

Streszczenie w języku angielskim (Summary)

Guanosine 3',5'-cyclic monophosphate is a signaling molecule that plays an important role in both prokaryotic and eukaryotic cells. Guanylate cyclase (GCs) are enzymes that catalyze the synthesis of cGMP from GTP, and plant GCs are significantly different in structure from better known and characterized animal cyclases. So far, cyclases have been described in several plant species, and role of GC and cGMP in plant growth, development and response to external stress factors has been investigated.

The aim of this study was to characterize two guanylate cyclases in *Brachypodium distachyon*, transmembrane BdPepR2 and cytosolic (soluble) BdGUCD1. In addition, to answer the question about the role of guanylate cyclases in the reactions triggered in monocotyledonous plant cells as a result of biotic stress factors caused by *Fusarium pseudograminearum* infection. Moreover, the aim was to determine the function of cyclic nucleotides in processes in plant cells after infection and to determine the cause and effect relationships between cyclases, cyclic nucleotides and selected stress phytohormones.

In the course of the work, I proved that the recombinant BdPepR2 and BdGUCD1 proteins exhibited *in vitro* guanylate cyclase activity. Furthermore, I confirmed the serine/threonine kinase activity in BdPepR2 and demonstrated that this activity is regulated by cGMP. The above biochemical analyzes were supplemented with *in silico* analyzes, which showed that the amino acid sequence of BdPepR2 contains a 14-amino acid catalytic center characteristic of plant GCs. However, in the case of BdPepR2, there is a methionine at one of the functionally relevant positions, never previously analyzed as a potential amino acid at this active site. Structure modeling combined with substrate docking suggests that in the case of BdPepR2, methionine may have positive GTP docking affinity at the GC center, although this data needed to be verified by a series of experiments. Using site-direct mutagenesis, I created proteins with different mutations in the GC center. Then I confirmed that methionine is the most preferred amino acid among the analyzed ones, its presence was associated with the highest GC *in vitro* activity. This may be a unique feature of monocotyledonous guanylate cyclases. Subsequently, I tested whether guanylate cyclases, BdGUCD1 and BdPepR2, and cGMP were involved in the response to *Fusarium pseudograminearum* infection in *Brachypodium distachyon*. As well as, I analyzed whether there are cause-and-effect relationships between cGMP and jasmonates, salicylic acid and abscisic acid following infection. As a result of the

analyzes, I showed that the increase in the level of cGMP is initiated by *F. pseudograminearum* infection, and analyzes of the transcript level showed dynamic changes in the expression of *BdGUCD1* and *BdPepR2* genes, showing that they are regulated by fungal infection. I noticed that as a result of the *F. pseudograminearum* inoculation the level of jasmonic acid increased only at 1 dpi (days post-infection), the level of salicylic acid slightly increased at 1 dpi, while the level of abscisic acid increased significantly only at 3 dpi. In addition, I used a guanylate cyclase inhibitor prior to the *F. pseudograminearum* inoculation, which inhibited the activity of plant GCs and did not lead to cGMP synthesis. Then, by analyzing the level of hormones, I showed that there is a relationship between cGMP and the jasmonate pathway because the cellular reduction of cGMP modulated the level of only one of the analyzed hormones, jasmonic acid. The results incorporated into the existing state of knowledge about the metabolism of cyclical nucleotides in plants and supplemented the existing knowledge on the response of *B. distachyon* to the biotic stress factor.

Publikacje wchodzące w skład rozprawy doktorskiej

Artykuł I

Duszyn, M.; Świeżawska-Boniecka, B.; Wong, A.; Jaworski, K.; Szmidt-Jaworska, A. *In Vitro* Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach. Int. J. Mol. Sci. 2021, 22, 6243.

International Journal of
Molecular Sciences

MDPI

Article

In Vitro Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach

Maria Duszyn [1,*], Brygida Świeżawska-Boniecka [1], Aloysius Wong [2,3], Krzysztof Jaworski [1] and Adriana Szmidt-Jaworska [1]

1 Chair of Plant Physiology and Biotechnology, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń, Lwowska St. 1, PL 87-100 Torun, Poland; swiezawska@umk.pl (B.Ś.-B.); jaworski@umk.pl (K.J.); asjawors@umk.pl (A.S.-J.)
2 Department of Biology, College of Science and Technology, Wenzhou-Kean University, 88 Daxue Road, Ouhai, Wenzhou 325060, China; alwong@kean.edu
3 Zhejiang Bioinformatics International Science and Technology Cooperation Center, Wenzhou-Kean University, Ouhai, Wenzhou 325060, China
* Correspondence: duszyn@doktorant.umk.pl

Abstract: In recent years, cyclic guanosine 3′,5′-cyclic monophosphate (cGMP) and guanylyl cyclases (GCs), which catalyze the formation of cGMP, were implicated in a growing number of plant processes, including plant growth and development and the responses to various stresses. To identify novel GCs in plants, an amino acid sequence of a catalytic motif with a conserved core was designed through bioinformatic analysis. In this report, we describe the performed analyses and consider the changes caused by the introduced modification within the GC catalytic motif, which eventually led to the description of a plasma membrane receptor of peptide signaling molecules—BdPepR2 in *Brachypodium distachyon*. Both in vitro GC activity studies and structural and docking analyses demonstrated that the protein could act as a GC and contains a highly conserved 14-aa GC catalytic center. However, we observed that in the case of BdPepR2, this catalytic center is altered where a methionine instead of the conserved lysine or arginine residues at position 14 of the motif, conferring higher catalytic activity than arginine and alanine, as confirmed through mutagenesis studies. This leads us to propose the expansion of the GC motif to cater for the identification of GCs in monocots. Additionally, we show that BdPepR2 also has in vitro kinase activity, which is modulated by cGMP.

Keywords: guanylate cyclase; guanylyl cyclase; moonlighting proteins; 3′,5′-cyclic guanosine monophosphate; cGMP; PepR2; *Brachypodium distachyon*

Citation: Duszyn, M.; Świeżawska-Boniecka, B.; Wong, A.; Jaworski, K.; Szmidt-Jaworska, A. *In Vitro* Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach. *Int. J. Mol. Sci.* **2021**, *22*, 6243. https://doi.org/10.3390/ijms22126243

Academic Editor: Karen Skriver

Received: 12 May 2021
Accepted: 7 June 2021
Published: 10 June 2021

1. Introduction

Guanosine 3′,5′-cyclic monophosphate (cGMP) is a key signaling molecule that is involved in signal transduction and controls different physiological responses and processes in numerous prokaryotic and eukaryotic cells. The concentration of cyclic GMP is regulated by guanylyl cyclases (GCs) that catalyze the synthesis of cGMP from GTP and phosphodiesterases (PDEs) that hydrolyze cGMP to GMP. The role and importance of cGMP and GC in mammals and prokaryotes was known for a long time, while their presence in plants was questionable, as they show less activity in vitro than in animal cells [1,2]. However, in recent years, an increasing number of reports on proteins with GC activity have appeared, which indicates that they play important roles in signal transduction and in several physiological processes in plants [3,4]. The cGMP-dependent signaling pathway is involved in root gravitropism of *Glycine max* [5], phytochrome-controlled induction of flowering of *Pharbitis nil* [6], and regulation of *Arabidopsis thaliana* seed germination [7]. Studies showed that proteins with confirmed in vitro GC activity are involved in plant

growth (AtBRI1 [8], AtPSKR1 [9,10]), pollen tube guidance (AtDGK4 [11]), and response to pathogens (AtPepR1 [12], HpPepR1 [13], OsWAKL21.2 [14], SlGC17, and SlGC18 [15]).

Significant progress was made in identifying and characterizing new GCs after deducing the pattern search motif of the catalytic center of GCs. The motif includes a highly conserved 14-amino-acid catalytic motif, and it was the first alignment of designated catalytic regions from prokaryotes and eukaryotes [16]. In subsequent research, this motif was the basis for the search for new plant GCs. Moreover, with increasing knowledge, the motif was steadily improved, and new amino acids were added, which enables us to distinguish between strict and relaxed GC motifs [17,18].

In this study, we investigated PepR2 from *Brachypodium distachyon* (BdPepR2), which belongs to the leucine-rich repeat receptor-like protein kinase family and is the orthologs of the PepR1 from *A. thaliana* and *Hippeastrum hybridum,* which were experimentally confirmed to be functional GCs [12,13]. Based on in vitro analysis, the recombinant BdPepR2 protein was determined to have GC activity. The previously described GC catalytic center contains conserved residues lysine or arginine [KR] at position 14, which are involved in stabilizing the transition to cGMP. In the case of BdPepR2, methionine [M] is at this position in the center; [M] was never considered or tested as one of the key amino acids for GC activity. However, our results indicated that mutating this amino acid to arginine [R] or alanine [A] lowers GC activity. This result implies first that [M] at position 14 of BdPepR2 is optimal for GC activity, and second that it is worth expanding the GC motif to identify new cyclases, especially from monocotyledonous plants. To the best of our knowledge, BdPepR2 is only the third active GC that contain a highly conserved 14-aa search center in the GC catalytic site reported from monocotyledonous plants, after HpPepR1 [13] and OsWAKL21.2 [14]. Additionally, we show that BdPepR2 also has in vitro kinase activity, which is modulated by cGMP, indicating that it is a twin-domain molecule.

2. Results and Discussion

2.1. Sequence Analysis of BdPepR2

The amino acid sequence of BdPepR2 is referred to in the NCBI database as a putative leucine-rich repeat receptor-like protein kinase PEPR2 (NCBI: XP_003571653.2). Analysis of the 3526-bp ORF sequence corresponding to the 1146-aa polypeptide using the Blast program demonstrated similarity with other receptor-like protein kinases. The amino acid sequence of BdPepR2 exhibited only 38.17% amino acid identity with AtPepR2, and 36.26% amino acid identity with AtPepR1, a protein with confirmed GC activity from *A. thaliana* [12] (as illustrated in Figure 1C). *B. distachyon* is a monocotyledonous plant, whereas *A. thaliana* is a dicotyledonous plant; thus, divergences in the amino acid sequences of these proteins may result from their separate evolutionary development. The *BdPepR2* gene is predicted to encode a protein with 1146 amino acid residues with domain organization typical of LRR receptor-like kinases (RLKs) and highly conserved LRR domains [19] (as illustrated in Figure 1A). The N-terminus contains a hydrophobic secretion signal followed by an extracellular domain with 24 tandem copies of a 24-residue LRR (residues 121 to 745). A single transmembrane domain (residues 810 to 832) is predicted to separate the extracellular domain from an intracellular Ser-Thr kinase domain (residues 870 to 1146) with an embedded GC motif [16]. The mentioned motif [RKS] [YFW] [GCTH] [VIL] [FV] x(3) [VIL] x(4) [KR] is present in the catalytic center of guanylyl cyclases of several *A. thaliana* proteins, including brassinosteroid receptor AtBRI1 [8], wall-associated kinase like 10 AtWAKL10 [20], Pep1 receptor AtPepR1 [12], phytosulfokine receptor AtPSKR1 [9], nitric oxide-binding GC AtNOGC1 [21], plant natriuretic peptide receptor AtPNP-R1 [22], *H. hybridum* HpPepR1 [13], and 99 GC candidates in *Solanum lycopersicum* [15].

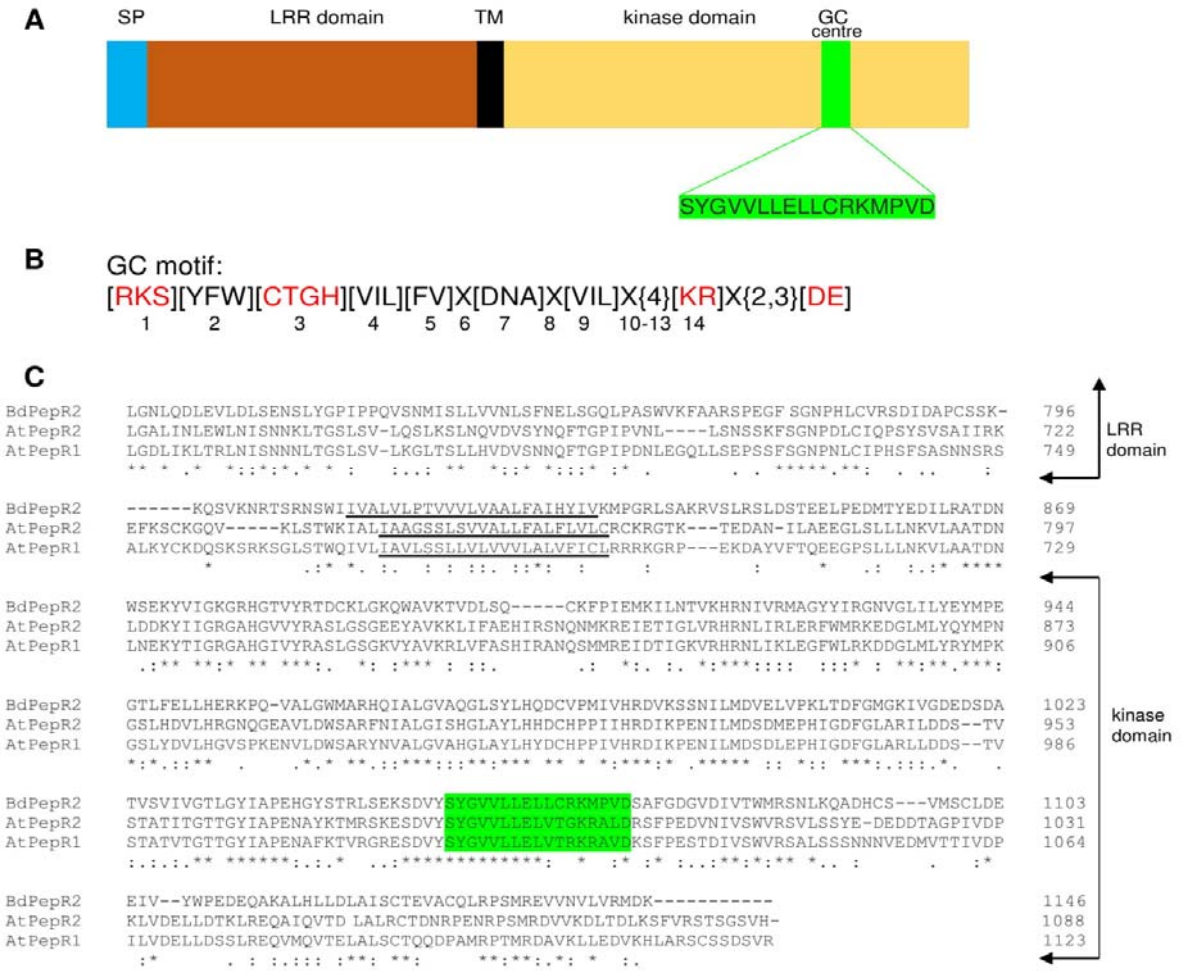


Figure 1. Structural features of GC catalytic domain of BdPepR2. (**A**) Representation of domain organization of BdPepR2 containing a signal peptide (SP), leucine-rich repeats (LLRs), transmembrane domain (TM), and GC center embedded in kinase domain. (**B**) Fourteen amino acid long original search motif; red amino acids are functionally assigned residues of the catalytic center. Residue in position 1 can form hydrogen bond with purine; residue in position 3 confers substrate specificity; residue in position 14 stabilizes transition state from GTP to cGMP; amino acid [D or E] at 2–3 residue downstream from position 14 participates in Mg^{2+}/Mn^{2+}-binding. (**C**) Amino acid sequence alignment of BdPepR2 (XP_003571653.1), AtPepR1 (OAP14914.1), and AtPepR2 (OAP12577.1) using the Clustal Omega. Predicted protein domains are indicated on the right side, TM is underlined, GC motif is highlighted in green. An * (asterisk) indicates positions that have a single, fully conserved residue. A: (colon) indicates conservation between groups of strongly similar properties. A. (period) indicates conservation between groups of weakly similar properties.

As presented in Figure 1B, the red amino acids at positions 1, 3 and 14 within the search motif are functionally assigned residues [16]. In the case of BdPepR2, the serine [S] localized at the 1 position is predicted to form a hydrogen bond with guanine, the glycine [G] at position 3 confers substrate specificity for GTP, and the amino acid at position 14 binds to the phosphate acyl group and stabilizes the transition state from GTP to cGMP. In BdPepR2, there is a methionine [M] in this position, and to the best of our knowledge, such an amino acid was never studied as a potential amino acid in the GC domain. Therefore, our objective was to verify whether the functional core motif could be extended by the addition of methionine. The identified BdPepR2 protein sequence also contains aspartic acid [D], a residue responsible for interaction with Mg^{2+}/Mn^{2+} ions located two amino acids behind the conserved motif [17].

2.2. Purification of BdPepR2 Recombinant Protein and In Vitro GC Activity

The truncated 942-bp fragment of the BdPepR2 cDNA, including full kinase domain with the GC motif, was cloned into the pGEX-6P-2 vector in frame with a glutathione S-transferase (GST) tag and expressed in *E. coli* BL21 cells as a GST-BdPepR2 recombinant protein. The molecular mass of the 313-aa-long BdPepR2 polypeptide was predicted in silico to be 35.35 kDa, and the isoelectric point was predicted to be 5.76 (http://web.expasy.org/compute_pi; accessed on 9 January 2021). The recombinant BdPepR2 protein was purified and used for GC enzymatic activity determination. Affinity chromatography enabled the purification of the GST-BdPepR2 fusion protein as a clear main 60 kDa band. Removing the GST tag from the fusion protein by digestion with PreScission protease resulted in the appearance of one distinct protein measuring approximately 36 kDa, which corresponded well with the predicted molecular mass of 35.35 kDa for the truncated BdPepR2 protein (as illustrated in Figure 5B).

Subsequently, the purified intracellular domain of the BdPepR2 protein was tested for its ability to convert GTP substrate to cGMP in the presence of magnesium and/or manganese ions as cofactors. The maximal BdPepR2 activity was reached at 1.5 mM GTP 15 min after starting the reaction, and the generated level of cGMP was 72.1 pmol mg protein^{-1} min^{-1} ($\pm$9.48) (as illustrated in Figures 2A and 3). The kinetic parameters determined for BdPepR2 were a Vmax of 112.1 fmole min^{-1} ug^{-1} protein and a Km value of 1.189 mM. The BdPepR2 GC activity was higher compared to that of the results previously determined for other plant guanylyl cyclases, e.g., HpPepR1 (17 pmol cGMP mg protein^{-1} min^{-1}) [13], AtPepR1 ($\sim$2.5 pmol mg^{-1} min^{-1}) [12], and AtBRI1 ($\sim$4.3 pmol mg^{-1} min^{-1}) [8]. However, compared to that of the activity described for PnGC1 (78.1 pmol mg^{-1} min^{-1}) [23] and for HpGC1 (600 pmol mg^{-1} min^{-1}) [24], the observed maximum GC activity of BdPepR2 was similar or considerably lower. At present, there are two groups of GCs: the first with the canonical GC domains, that often appears as a stand-alone molecule, and in the second group, the cyclase domain is part of a larger, multidomain protein complex. Of the proteins mentioned above, only PnGC1 and HpGC1 do not have a complete motif and are canonical GCs. The remaining proteins are so-called moonlight proteins. In addition to the GC domain, these proteins also have kinase domains, many of which have confirmed in vitro activity that presumably may affect GC activity. Moonlighting proteins have multiple tasks and have significant roles that contribute to the whole plant [4]. Therefore, it is possible that the GC activity of these proteins is lower than that of PnGC1 and HpGC1, which are presumed cytoplasmic proteins with unknown activity other than GC. Furthermore, such large differences in GC activity levels may be associated with the sensitivity of the measurement methods utilized to determine cGMP concentration. Over the past years, various methods were used, including [3H] cGMP radioimmunoassay, [125I] the cGMP assay system, cGMP enzyme immunoassay (EIA) system, and the high-resolution detection method LC-MS/MS. Due to the use of different methods and their different means of optimization, it is difficult to compare the activities of the guanylyl cyclases present in plants. However, regardless of the method used, the level of GC activity, and thus, the level of cGMP in plants, is always lower than that recorded for animal cells [25]. The main reason for this difference is that higher GC activities might require cofactors (e.g., calcium ions, chaperones, and coproteins) or unknown posttranslational modifications.

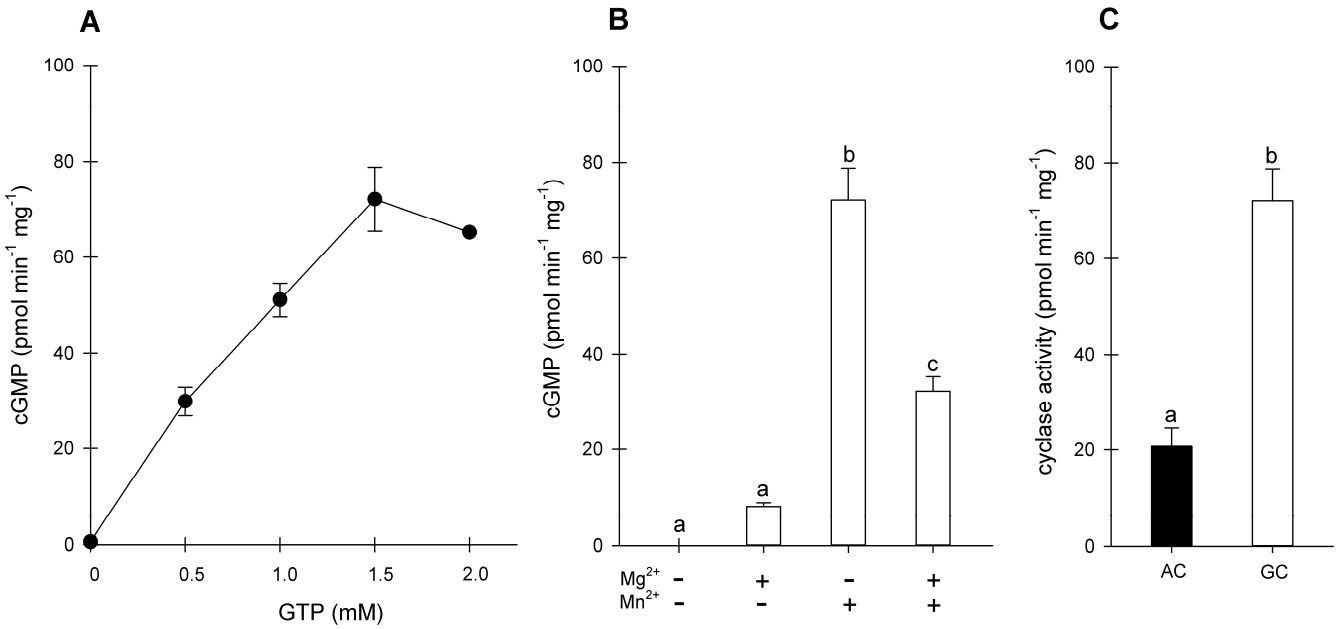


Figure 2. Enzymatic activity of recombinant BdPepR2. (**A**) Recombinant BdPepR2 activity in response to various concentrations of GTP. Reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$, GTP (0.5–2 mM), and 5 µg of purified protein (without GST-tag) in a final volume of 100 µL. (**B**) Determination of BdPepR2 cofactor specificity. Reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$ and/or 5 mM $MgCl_2$, 1.5 mM GTP and 5 µg of purified protein (without GST-tag) in a final volume of 100 µL. (**C**) Determination of BdPepR2 substrate specificity. Reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$, 1.5 mM GTP or 1.5 mM ATP, and 5 µg of the purified protein (without GST-tag) in a final volume of 100 µL. Data are mean values (n = 3), and error bars show standard error of mean. Statistical analysis was performed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test. Different letters indicate significantly different data.

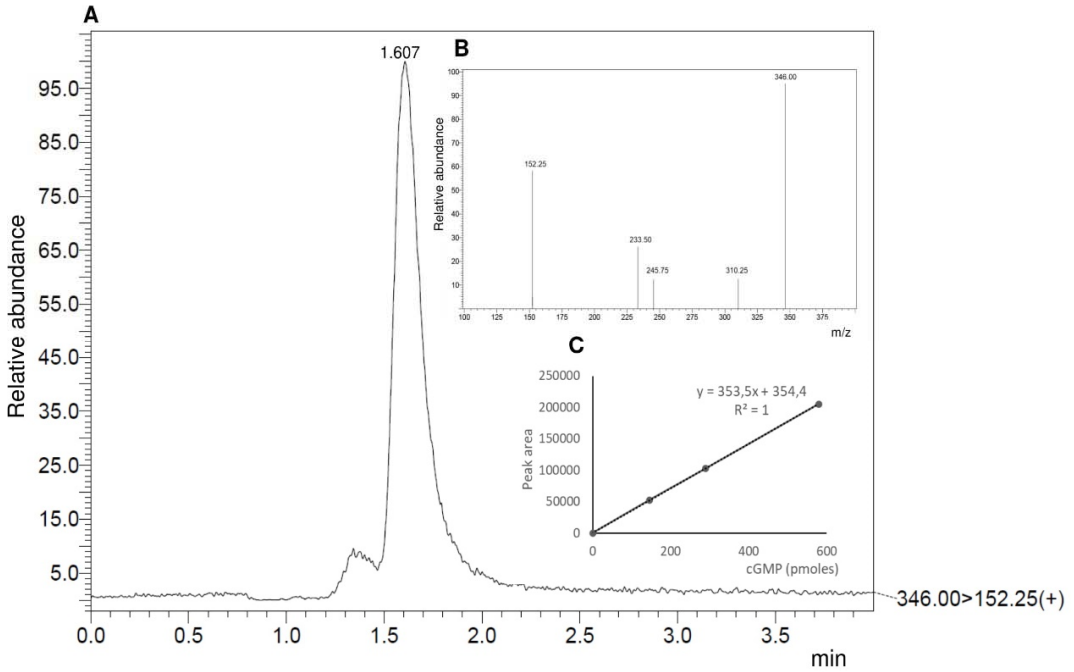


Figure 3. Detection of cGMP generated by BdPepR2 by LC-MS/MS. (**A**) Determination of GC activity of the BdPepR2 protein by LC-MS/MS. Ion chromatogram of cGMP was generated from a reaction mixture containing 5 µg of purified protein and GTP as a substrate in the presence of 5 mM Mn^{2+}. (**B**) Inset showing parent cGMP ion at *m*/*z* 346.00 [M + H]+ and corresponding fragmented daughter ion at *m*/*z* 125.25 [M + H]+. Fragmented product ion was used for quantitation. (**C**) Inset showing the cGMP calibration curve performed with 0–0.58 pmoles of pure cGMP on the column.

In the BdPepR2 sequence, aspartic acid [D] is located two amino acids behind the conserved motif. Based on the findings reported in the available literature, [DE] residues at this position are responsible for interactions with Mg^{2+}/Mn^{2+} ions [17]. Thus, we predicted that divalent cations are the cofactors necessary for the CG activity of BdPepR2, and we tested whether it is Mn^{2+}-dependent or Mg^{2+}-dependent. The results showed that the studied GC domain of BdPepR2 has a higher affinity for Mn^{2+} than Mg^{2+} ions (as illustrated in Figure 2B). The same situation was observed in the case of previously examined AtPepR1-GC [12], PnGC1 [23], and AtNOGC1 [21]. Other recombinant GCs, such as AtWAKL-10 [20], AtBRI1 [8], AtGC1 [16], AtPNP-R1 [22], and HpPepR1 [13], exhibited a preference for Mg^{2+} over Mn^{2+}.

The catalytic centers of plant GCs and adenylyl cyclases (ACs) share a high level of amino acid similarity and differ only in the residue at position 3, which is responsible for substrate recognition [16]. We tested BdPepR2 for AC activity in the presence of 1.5 mM ATP as the substrate. Mass spectrometry analysis showed more than fourfold lower BdPepR2 AC activity (~16.26 pmol mg protein^{-1} min^{-1}), clearly indicating that the analyzed protein has GTP preference (as illustrated in Figure 2C).

2.3. Mutational Analysis

In the GC catalytic center of BdPepR2 [1052-SYGVVELLCRKMPVD-1070], all amino acids except [M] were confirmed to be essential for cyclase activity [17,18]. In the conserved model at this position, [KR] is preferable over [M] [18]. However, structural analysis suggests that in the case of BdPepR2, [M] can assume the role of [KR] with positive docking affinity of GTP at the GC center. Molecular docking of GTP to the catalytic center of the BdPepR2 [Met834→Lys1146] model elucidated the substrate pose, orientation, and interactions with the key residues serine, arginine, and methionine of the GC center (as illustrated in Figure 4). Homology model predictions also showed that neighboring R1064 (position 12) and K1065 (position 13) were unlikely to bind GTP because they were facing away from the GC center (as illustrated in Figure 4).

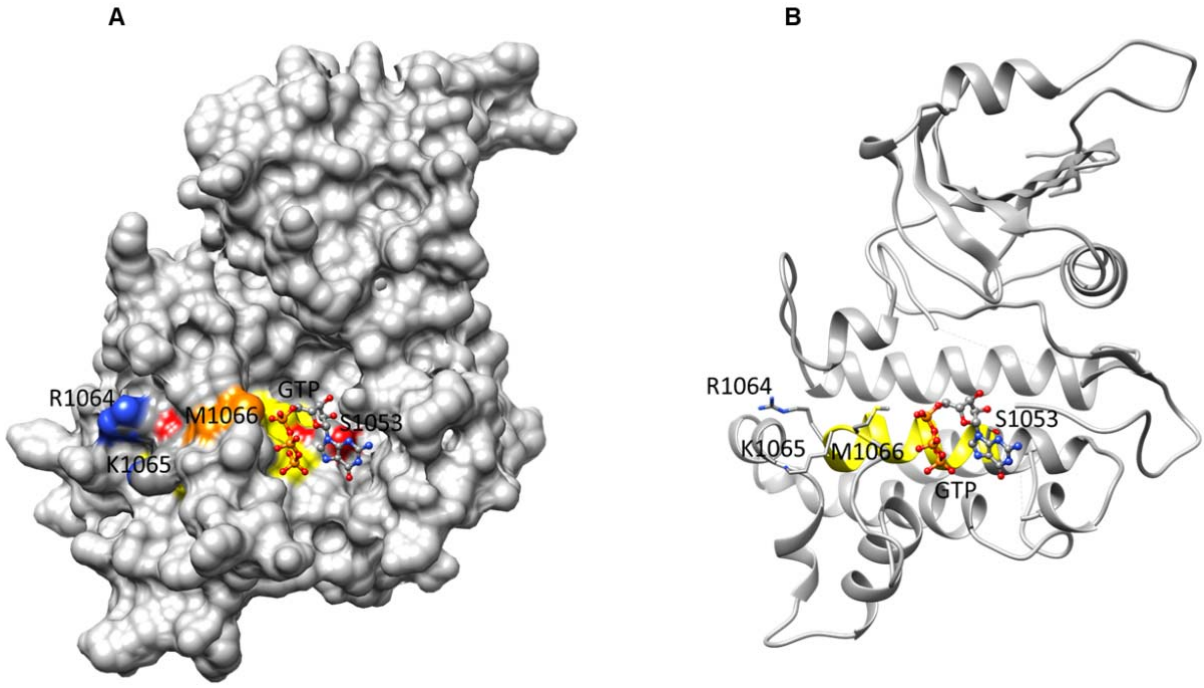


Figure 4. Docking of GTP at the GC center of BdPepR2 and interaction of GTP with key residues at catalytic center (yellow) is shown as (**A**) surface and (**B**) ribbon models, respectively. Functional residues at positions 1 (S1053, red) and 14 (M1066, orange) of the motif and neighboring R1064 (position 12) and K1065 (position 13) facing away from the GC center are shown.

To investigate whether [M] at position 14 in GC center of Bd PepR2 is preferable, two mutants with different GC centers were created: BdPepR2M1066A and BdPepR2M1066R. This substitution is based on previous findings that indicated that the conversion from defined [KR] into [AL] causes a decrease in GC activity [26]. In result LC–MS/MS quantification of cGMP levels (as illustrated in Figure 5A,C) demonstrated that the BdPepR2M1066A protein manifests a significant reduction in cGMP production (22.199 pmol mg protein^{-1} min^{-1} ($\pm$10.070)) in comparison to that of the wild-type BdPepR2 recombinant protein (72.1 pmol mg protein^{-1} min^{-1} ($\pm$9.48)). However, the BdPepR2M1066R protein exhibited only a slight reduction in cGMP production at 57.774 pmol mg protein^{-1} min^{-1} ($\pm$5.692) (as illustrated in Figure 5A,C) which is consistent with the notion that a positively charged residue [KR] is required to interact with the phosphate of GTP in catalytic centers identified by the GC motif, as observed in the *A. thaliana* homolog AtPepR1-GC and other GCs. However, our results showed that in the case of BdPepR2, the neutral nonpolar [M] residue can assume the role of [KR] in that position and even exhibiting stronger activity, which could be a unique feature of monocot GCs. Previously identified as key residues [KR] are polar and have positively charged side groups, whereas [M] is a nonpolar amino acid, but docking stimulation showed that [M] at position 14 is pointing into the catalytic center and that it may be able to interact with the phosphate of GTP. While the BdPepR2M1066A variant confirmed previous findings, mutation of amino acid at position 14 to alanine of the AtBRI1-GC catalytic center resulted in decreased cGMP production [26] or chemically similar leucine due to unsuccessful docking stimulation [17,27]. Additionally, we analyzed the AC activity of the two mutant proteins and it was lower than that of the BdPepR2 WT (data not shown). This indicates that the mutation of amino acid at position 14 does not affect AC activity. A 14-amino-acid-long GC catalytic domain search motif was first deduced from the alignment of designated catalytic domains from vertebrates, lower eukaryotes, and prokaryotes. As mentioned earlier, there are no data on the GC activity of proteins with [M] at position 14 at the catalytic center in either guanylyl or ACs in plants or in other eukaryotes and prokaryotes. However, our data on BdPepR2 GC in vitro activity and structural analysis indicate that an expansion of the search motif should be considered. Since the GC center in BdPepR2 does not fit the GC motif used for the identification of GCs in *A. thaliana* and other dicots, the GC prediction tool GCPred [28] did not identify this protein as a candidate GC. Thus, our results suggest that the catalytic center may be wider than previously believed, and more monocot GCs should be identified to improve existing predictive tools and expand our current understanding of plant GCs. To date, core motifs were developed, and the in vitro GC activity was also confirmed primarily for *A. thaliana*, as it is a very well-known model plant. Notably, *B. distachyon* belongs to monocotyledonous plants, whereas most GCs with confirmed activity are from *A. thaliana*, a dicotyledonous plant. To the best of our knowledge BdPepR2 is only the third active GC that contain a highly conserved 14-aa search center in the GC catalytic site reported from monocotyledonous plants, after HpPepR1 [13], and OsWAKL21.2 [14]. Determining whether the change from [KR] to [M] is unique to monocots warrants further research. Alignment of the BdPepR2 aa sequence with other predicted leucine-rich repeat receptor-like protein kinase PEPR1/2 proteins from the *Poaceae* family showed identity in all 14 amino acids in the GC catalytic domain (as illustrated in Figure S1). It is likely that other proteins similar to the *B. distachyon* GC search motif may also have catalytic activity, thereby implying that a significant number of GCs remain to be identified, especially in plants other than *A. thaliana* and dicots.

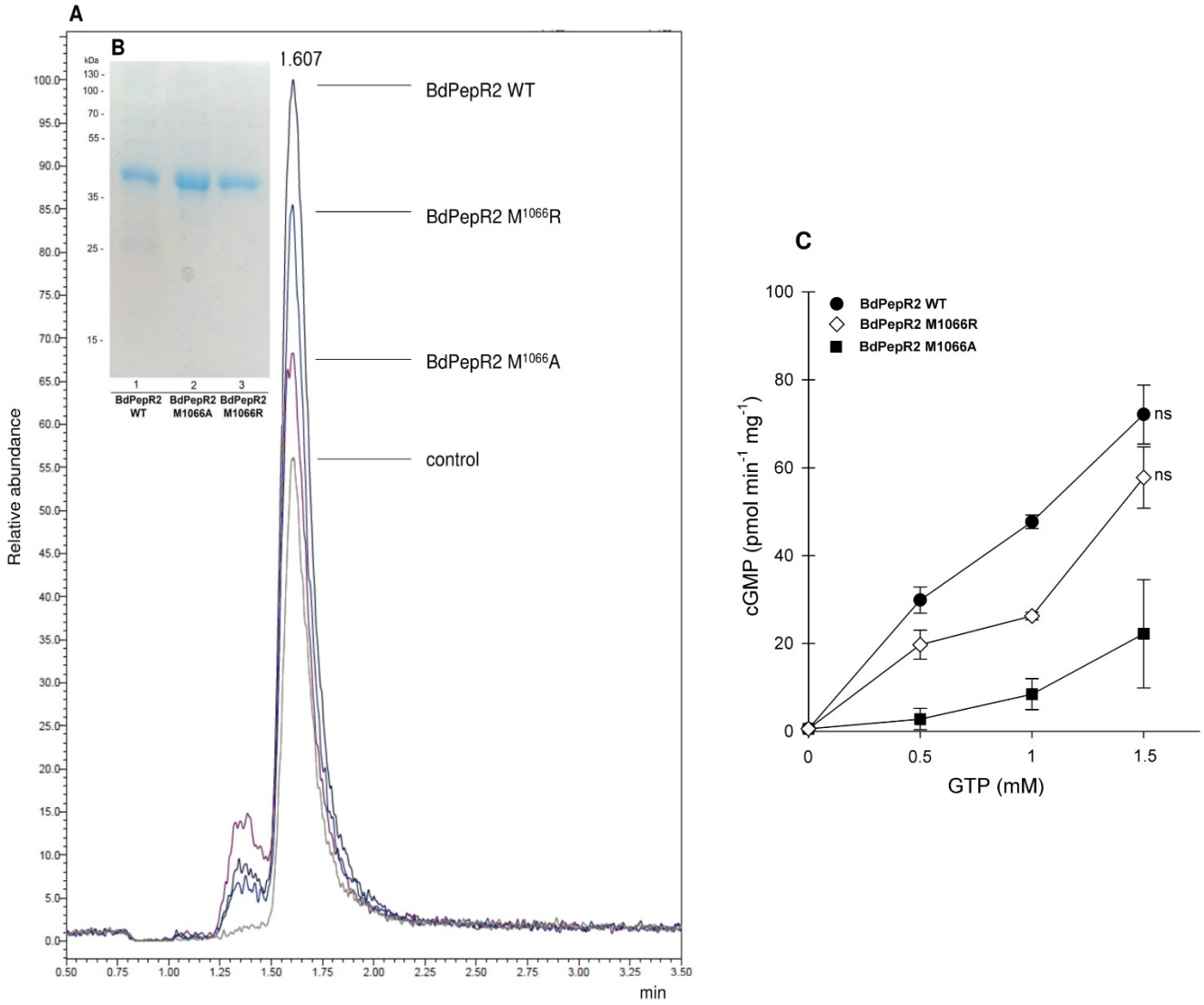


Figure 5. Detection of cGMP generated by BdPepR2 WT, BdPepR2 M1066A, and BdPepR2 M1066R by LC-MS/MS (**A**) Four superimposed ion chromatograms of cGMP generated by 5 µg recombinant BdPepR2 WT (black), BdPepR2 M1066R (blue), BdPepR2 M1066A (purple), and control (no protein, grey) after 15 min in the presence of 5 mM Mn^{2+}. (**B**) SDS-PAGE of BdPepR2 WT, BdPepR2 M1066R, BdPepR2 M1066A. (**C**) LC–MS/MS quantification of cGMP levels generated by BdPepR2 recombinant and mutant proteins with Met1066 substituted with Arg or Ala in presence of various GTP concentrations and 5 mM $MnCl_2$ after 15 min at 30 °C. Data are mean values (n = 3), and error bars show standard error of mean. Statistical analysis was performed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test. ns indicates not significantly different data.

2.4. BdPepR2 Kinase Activity

Using the Kinase-Glo plus luminescent kinase assay, we confirmed that BdPepR2 has serine/threonine kinase activity (as illustrated in Figure 6A). Moreover, we showed that all three proteins, BdPepR2, BdPepR2M1066A, and BdPepR2M1066R, have similar kinase activity. Both mutations had no effect on kinase activity, meaning that the amino acid at position 14 in GC center of Bd PepR2 does not affect the kinetic activity of protein, which suggests that the main function of this 14-aa-long region in the kinase domain is cGMP production. This is comparable to AtBRI1 [8] and AtPSKR1 [9] in which mutations in the GC catalytic center reduce cGMP generation but do not significantly change kinase activity. Moreover, we observe that kinase activity of BdPepR2, BdPepR2M1066A, and BdPepR2M1066R is suppressed by GTP and cGMP (as illustrated in Figure 6A). To check whether only cGMP or also GTP inhibits the kinase activity, we prepared an experiment

with different components of the reaction buffer. The magnesium ions alone or magnesium and manganese ions together (manganese is necessary cofactor for the GC activity of the BdPepR2) were added. Interestingly, we observe that only the product of GC activity, cGMP, statistically significantly suppress kinase activity of BdPepR2 in vitro, and under conditions where cGMP formation is inefficient (only magnesium), the kinase activity was as high as in the control (as illustrated in Figure 6B). A similar effect of suppressing kinase activity by cGMP was observed on the kinase activity of AtBRI1 [8] and AtPSKR1 [9]. This results confirm that in addition to a functional GC domain, the recombinant BdPepR2 also harbors a functional kinase domain, thus making it a twin-domain molecule; moreover, cGMP suppresses kinase activity.

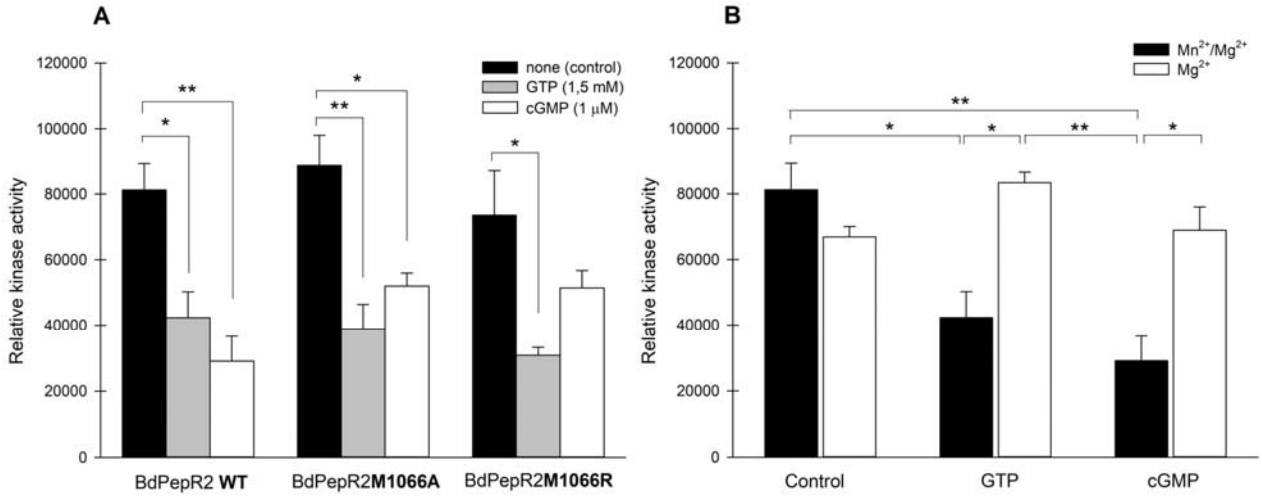


Figure 6. Determination of kinase activity of recombinant BdPepR2 by Kinase–Glo plus luminescent kinase assay. (**A**) Determination of kinase activity of recombinant BdPepR2 WT, BdPepR2 M1066A, and BdPepR2 M1066R and effect of cGMP or GTP on kinase activity. Reaction mixture contained 25 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$ and 5 mM $MgCl_2$, 1 mM DTT, 1 μg/μL Histone Type III-S, 100 μM ATP (control), supplemented with 1 μM cGMP or 1.5 mM GTP and 1 μg of purified protein (without GST-tag) in a final volume of 50 μL. (**B**) Determining effect of GTP and addition of cofactors on kinase activity of BdPepR2 WT. Reaction mixture contained 25 mM Tris/HCl buffer (pH 7.5), 5 mM $MgCl_2$ and/or 5 mM $MnCl_2$, 1 mM DTT, 1 μg/μL Histone Type III-S, 100 μM ATP (control), supplemented with 1 μM cGMP or 1.5 mM GTP and 1 μg of purified protein (without GST-tag) in a final volume of 50 μL. Data are mean values (n = 3), and error bars show standard error of the mean. Statistical analysis was performed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test (* $p < 0.05$; ** $p < 0.01$).

3. Materials and Methods

3.1. Construction of Expression Vectors

Total RNA was extracted from three-week-old *Brachypodium distachyon* Bd21 seedlings using a Universal RNA Purification Kit (EURx, Gdańsk, Poland). cDNA was synthesized using the NG dART RT kit (EURx, Gdańsk, Poland). A 942-bp fragment of BdPepR2 (NCBI accession number: XM_003571605.3; https://www.ncbi.nlm.nih.gov/nuccore; accessed on 27 March 2018) coding region corresponding to a 313-residue polypeptide [Met834→Lys1146] was amplified by PCR using specific primers (as illustrated in Table S1). Next, the PCR product was introduced into the linearized pGEX-6P-2 expression vector using In-Fusion cloning technology (In-Fusion HD Cloning Kit; Takara Bio USA, Inc., Mountain View, CA, USA). The *E. coli* BL21 strain, after being transformed with the resulting plasmid, was used to produce the glutathione S-transferase (GST)-tagged protein. BdPepR2 mutants (M1066A and M1066R) were generated using a QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). pGEX-6P-2:BdPepR2 was used as a template, and in each case, mutagenesis primers were designed through https://www.agilent.com/store/primerDesignProgram.jsp (accessed on 20 November 2020) the

relevant forward and reverse oligonucleotides are listed in Table S1). Each plasmid was verified through sequencing.

3.2. Expression and Purification of the Recombinant Proteins

For the expression of BdPepR2, BdPepR2M1066A, and BdPepR2M1066R recombinant proteins, the appropriate construct was transformed into One Shot BL21 (DE3)pLysS *E. coli* cells (Life Technologies, Carlsbad, CA, USA). Bacterial cells were grown in LB medium supplemented with 2% glucose at 37 °C. Fusion protein production was induced by adding IPTG at a final concentration of 1 mM and incubating the cells at 24 °C for 3 h in glass vessels connected to a BioFlo 120 bioprocess control station (Eppendorf, Hauppauge, NY, USA). The pH was maintained at 6.5 (±0.2), the dissolved oxygen parameter was set to 20%, and the agitation speed was 200 rpm. Bacteria were collected by centrifugation, and proteins were purified as previously described [13]. Proteins were released from the fusion protein by proteolytic cleavage of the protein with PreScission protease (GE Healthcare Europe GmbH, Freiburg, Germany) following the manufacturer's instructions. The homogeneity and purity of the protein fractions were analyzed with 10% (*v*/*v*) SDS/PAGE. Pure protein, without a GST tag, was used for the activity analyzes.

3.3. Determination of Guanylyl Cyclase Activity

The guanylyl cyclase activity was determined by estimating the rate of cGMP formation. For the enzymatic assay, the reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$ or/and 5 mM $MgCl_2$, GTP as a substrate (0.5–2 mM), and 5 µg of the purified protein in a final volume of 100 µL. After incubation at 30 °C for 15 min, the reaction was stopped at 100 °C for 10 min, and the samples were centrifuged at 16,000 *g* for 10 min. Moreover, ATP (1.5 mM) was used to analyze the specificity of the enzyme, and background cGMP levels were measured in tubes that contained the reaction mixture but no protein. The total cGMP concentration was determined in triplicate using liquid chromatography–tandem mass spectrometry (LC-MS/MS Nexera UHPLC and LCMS-8045 integrated system (Shimadzu Corporation, Kyoto, Japan)). The ionization source parameters were optimized in positive ESI mode using pure cGMP dissolved in HPLC-grade water (Sigma). Samples were separated using a reversed-phase C18 column (150 × 2.1 mm, 2.6 µm, Kinetex) in 10% methanol with 0.1% (*v*/*v*) formic acid (solvent A (water with 0.1% (*v*/*v*) formic acid), solvent B methanol with 0.1% (*v*/*v*) formic acid) at a flow rate of 0.3 mL/min. The interface voltage was set at 4.0 kV for positive (ES+) electrospray. Data acquisition and analysis were performed with the LabSolutions (Shimadzu Corporation, Kyoto, Japan) workstation for LCMS-8045. The enzyme activity was defined as the amount of cGMP produced by 1 mg of protein per min.

3.4. Determination of Proteins Kinase Activity

For quantification of BdPepR2 protein kinase activity, a Kinase-Glo plus luminescent kinase assay (V3771, Promega, Walldorf, Germany) was used according to the manufacturer's protocol. Briefly, purified truncated BdPepR2, BdPepR2 M1066A or BdPepR2 M1066R (1 µg) was added to a 50-µL reaction mixture (25 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$ or/and 5 mM $MnCl_2$, 1 mM DTT, 1 µg/µL Histone Type III-S, and 100 µM ATP) supplied with 1 µM cGMP or 1.5 mM GTP. Reactions were performed at 30 °C for 15 min and stopped by adding the Kinase-Glo reagent. After equilibrating the mixture at room temperature for 10 min, luminescence was monitored using a Synergy HT Multi-Mode Microplate Reader. The results are expressed in relative luminesce units; experiments were undertaken in triplicate.

3.5. Computational Modeling

Full-length amino acid sequences of BdPerR2 (NCBI accession number: XM_003571605.3; https://www.ncbi.nlm.nih.gov/protein/), AtPepR1 (NCBI accession number: OAP14914.1; https://www.ncbi.nlm.nih.gov/protein/), and AtPepR2 (NCBI accession number: OAP12577.1;

https://www.ncbi.nlm.nih.gov/protein/) were aligned using the Clustal Omega program. Domain predictions were performed using InterPro (https://www.ebi.ac.uk/interpro/) and LRRfinder (http://www.lrrfinder.com/lrrfinder.php). All web sites in this paragraph were accessed on 9 January 2021. The Met834→Lys1146 fragment of the BdPepR2 structure was modeled against the AtBRI1 template as described in [2] using MODELLER (ver. 9.25) software [29–31]. Docking simulations of GTP to the GC domain of BdPePR2 were performed using AutoDock Vina (ver. 1.1.2) [32]. All structures, binding poses, and images were analyzed and created using UCSF Chimera (ver. 1.10.1) [33].

4. Conclusions

The search for motifs and/or individual amino acids led to the analysis of the active centers of enzymes and the identification of new elements in the functional centers. Our successful change in amino acids provides strong validation of BdPepR2 activity. Thus, this result clarifies the next element in guanylyl cyclase activity and catalytic motif-site details. In this manuscript, we propose an explanation for the existence of other amino acids that are involved in cyclase activity. We identify that a motif with methionine, never before tested amino acid in cyclases, functions better than the conserved amino acids described previously; this motif binds to the phosphate acyl group and stabilizes the transition state from GTP to cGMP. So far, little is known about GCs in monocots plants. It is possible that the [M] at position 14 instead of [KR] is unique to monocots, and if so, more monocot candidates need to be examined to determine how the GCs evolved between monocots and dicots. Despite the importance of this discovery, it is still only one step in characterizing this group of plant proteins. Further studies will be necessary to compare the mechanisms governing the regulation of the enzyme, analyze their role in plant cells by transgenic plant production, and compare them to those of other GCs. Moreover, it would be important to define more closely the interactions among cyclase motif and kinase domain.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22126243/s1, Figure S1: The amino acid sequence alignment of predicted leucine-rich repeat receptor-like protein kinase PEPR1/2 proteins from the *Poaceae* family, Table S1: Sequence of the primers employed for PCR amplification in this study.

Author Contributions: Conceptualization, M.D., B.Ś.-B. and A.S.-J.; methodology, M.D. and B.Ś.-B.; formal analysis, M.D. and A.W.; investigation, M.D.; writing—original draft preparation, M.D.; writing—review & editing, B.Ś.-B., A.W., K.J. and A.S.-J.; visualization, K.J.; supervision, K.J. and A.S.-J.; funding acquisition, M.D., A.W. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by [National Science Centre (Poland) grant number 2018/29/N/NZ9/00812), and funds provided by Nicolaus Copernicus University (Toruń, Poland) for the Research Program of the Chair of Plant Physiology and Biotechnology. A.W. was supported by the National Natural Science Foundation of China (31850410470) and the Zhejiang Provincial Natural Science Foundation of China (LQ19C130001).

Data Availability Statement: Data is contained within the article or Supplementary Material.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Ashton, A.R. Guanylyl cyclase activity in plants? *Proc. Natl. Acad. Sci. USA* **2011**, *108*, E97–E98. [CrossRef] [PubMed]
2. Bojar, D.; Martinez, J.; Santiago, J.; Rybin, V.; Bayliss, R.; Hothorn, M. Crystal structures of the phosphorylated BRI1 kinase domain and implications for brassinosteroid signal initiation. *Plant J.* **2014**, *78*, 31–43. [CrossRef] [PubMed]
3. Gehring, C.; Turek, I.S. Cyclic nucleotide monophosphates and their cyclases in plant signaling. *Front. Plant. Sci.* **2017**, *8*, 1704. [CrossRef] [PubMed]
4. Turek, I.; Irving, H. Moonlighting Proteins Shine New Light on Molecular Signaling Niches. *Int. J. Mol. Sci.* **2021**, *22*, 1367. [CrossRef]

5. Hu, X.; Neill, S.J.; Tang, Z.; Cai, W. Nitric oxide mediates gravitropic bending in soybean roots. *Plant Physiol.* **2005**, *137*, 663–670. [CrossRef]
6. Szmidt-Jaworska, A.; Jaworski, K.; Kopcewicz, J. Involvement of cyclic GMP in phytochrome-controlled flowering of Pharbitis nil. *J. Plant Physiol.* **2008**, *165*, 858–867. [CrossRef]
7. Teng, Y.; Xu, W.; Ma, M. cGMP is required for seed germination in *Arabidopsis thaliana*. *J. Plant Physiol.* **2010**, *167*, 885–889. [CrossRef]
8. Kwezi, L.; Meier, S.; Mungur, L.; Ruzvidzo, O.; Irving, H.; Gehring, C. The *Arabidopsis thaliana* Brassinosteroid Receptor (AtBRI1) Contains a Domain that Functions as a Guanylyl Cyclase In Vitro. *PLoS ONE* **2007**, *2*, e449. [CrossRef]
9. Kwezi, L.; Ruzvidzo, O.; Wheeler, J.I.; Govender, K.; Iacuone, S.; Thompson, P.E.; Gehring, C.; Irving, H.R. The phytosulfokine (PSK) receptor is capable of guanylate cyclase activity and enabling cyclic GMP-dependent signaling in plants. *J. Biol. Chem.* **2011**, *286*, 22580–22588. [CrossRef]
10. Muleya, V.; Wheeler, J.I.; Ruzvidzo, O.; Freihat, L.; Manallack, D.T.; Gehring, C.; Irving, H.R. Calcium is the switch in the moonlighting dual function of the ligand-activated receptor kinase phytosulfokine receptor 1. *Cell Commun. Signal.* **2014**, *12*, 60. [CrossRef]
11. Wong, A.; Donaldson, L.; Portes, M.T.; Eppinger, J.; Feijó, J.A.; Gehring, C. *Arabidopsis* DIACYLGLYCEROL KINASE4 is involved in nitric oxide-dependent pollen tube guidance and fertilization. *Development* **2020**, *147*, dev183715. [CrossRef]
12. Qi, Z.; Verma, R.; Gehring, C.; Yamaguchi, Y.; Zhao, Y.; Ryan, C.A.; Berkowitz, G.A. Ca^{2+} signaling by plant *Arabidopsis thaliana* Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 21193–21198. [CrossRef]
13. Świeżawska, B.; Jaworski, K.; Duszyn, M.; Pawełek, A.; Szmidt-Jaworska, A. The Hippeastrum hybridum PepR1 gene (HpPepR1) encodes a functional guanylyl cyclase and is involved in early response to fungal infection. *J. Plant Physiol.* **2017**, *216*, 100–107. [CrossRef] [PubMed]
14. Malukani, K.K.; Ranjan, A.; Hota, S.J.; Patel, H.K.; Sonti, R.V. Dual Activities of Receptor-Like Kinase OsWAKL21.2 Induce Immune Responses. *Plant Physiol.* **2020**, *183*, 1345–1363. [CrossRef] [PubMed]
15. Rahman, H.; Wang, X.Y.; Xu, Y.P.; He, Y.H.; Cai, X.Z. Characterization of tomato protein kinases embedding guanylate cyclase catalytic center motif. *Sci. Rep.* **2020**, *10*, 4078. [CrossRef] [PubMed]
16. Ludidi, N.; Gehring, C. Identification of a novel protein with guanylyl cyclase activity in *Arabidopsis thaliana*. *J. Biol. Chem.* **2003**, *278*, 6490–6494. [CrossRef]
17. Wong, A.; Gehring, C.; Irving, H.R. Conserved functional motifs and homology modeling to predict hidden moonlighting functional sites. *Front. Bioeng. Biotechnol.* **2015**, *3*, 82. [CrossRef]
18. Wong, A.; Tian, X.; Gehring, C.; Marondedze, C. Discovery of Novel Functional Centers with Rationally Designed Amino Acid Motifs. *Comput. Struct. Biotechnol. J.* **2018**, *16*, 70–76. [CrossRef]
19. Chakraborty, S.; Nguyen, B.; Wasti, S.D.; Xu, G. Plant Leucine-Rich Repeat Receptor Kinase (LRR-RK): Structure, Ligand Perception, and Activation Mechanism. *Molecules* **2019**, *24*, 3081. [CrossRef]
20. Meier, S.; Ruzvidzo, O.; Morse, M.; Donaldson, L.; Kwezi, L.; Gehring, C. The Arabidopsis Wall Associated Kinase-Like 10 Gene Encodes a Functional Guanylyl Cyclase and Is Co-Expressed with Pathogen Defense Related Genes. *PLoS ONE* **2010**, *5*, e8904. [CrossRef]
21. Mulaudzi, T.; Ludidi, N.; Ruzvidzo, O.; Morse, M.; Hendricks, N.; Iwuoha, E.; Gehring, C. Identification of a novel Arabidopsis thaliana nitric oxide-binding molecule with guanylate cyclase activity in vitro. *FEBS Lett.* **2011**, *585*, 2693–2697. [CrossRef]
22. Turek, I.; Gehring, C. The plant natriuretic peptide receptor is a guanylyl cyclase and enables cGMP-dependent signaling. *Plant Mol. Biol.* **2016**, *91*, 275–286. [CrossRef]
23. Szmidt-Jaworska, A.; Jaworski, K.; Pawełek, A.; Kopcewicz, J. Molecular cloning and characterization of a guanylyl cyclase, PnGC-1: Involved in light signaling in Pharbitis nil. *J. Plant Growth Regul.* **2009**, *28*, 367–380. [CrossRef]
24. Świeżawska, B.; Jaworski, K.; Szewczuk, P.; Pawełek, A.; Szmidt-Jaworska, A. Identification of a Hippeastrum hybridum guanylyl cyclase responsive to wounding and pathogen infection. *J. Plant Physiol.* **2015**, *189*, 77–86. [CrossRef]
25. Rogers, N.M.; Seeger, F.; Garcin, E.D.; Roberts, D.D.; Isenberg, J.S. Regulation of soluble guanylate cyclase by matricellular thrombospondins: Implications for blood flow. *Front. Physiol.* **2014**, *5*, 134. [CrossRef] [PubMed]
26. Wheeler, J.I.; Wong, A.; Marondedze, C.; Groen, A.J.; Kwezi, L.; Freihat, L.; Vyas, J.; Raji, M.A.; Irving, H.R.; Gehring, C. The brassinosteroid receptor BRI1 can generate cGMP enabling cGMP-dependent downstream signaling. *Plant J.* **2017**, *91*, 590–600. [CrossRef]
27. Wong, A.; Gehring, C. The *Arabidopsis thaliana* proteome harbors undiscovered multi-domain molecules with functional guanylyl cyclase catalytic centers. *Cell Commun. Signal.* **2013**, *11*, 48. [CrossRef] [PubMed]
28. Xu, N.; Fu, D.; Li, S.; Wang, Y.; Wong, A. GCPred: A web tool for guanylyl cyclase functional centre prediction from amino acid sequence. *Bioinformatics* **2018**, *34*, 2134–2135. [CrossRef]
29. Al-Younis, I.; Wong, A.; Gehring, C. The *Arabidopsis thaliana* K+-uptake permease 7 (AtKUP7) contains a functional cytosolic adenylate cyclase catalytic centre. *FEBS Lett.* **2015**, *589*, 3848–3852. [CrossRef] [PubMed]
30. Al-Younis, I.; Wong, A.; Lemtiri-Chlieh, F.; Schmöckel, S.; Tester, M.; Gehring, C.; Donaldson, L. The *Arabidopsis thaliana* K^+—Uptake permease 5 (AtKUP5) contains a functional cytosolic adenylate cyclase essential for K^+ transport. *Front. Plant Sci.* **2018**, *9*. [CrossRef]

31. Šali, A.; Blundell, T.L. Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* **1993**, *234*, 779–815. [CrossRef] [PubMed]
32. Troot, O.; Olson, A.J. Software news and update AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461. [CrossRef]
33. Pettersen, E.F.; Goddard, T.D.; Huang, C.C.; Couch, G.S.; Greenblatt, D.M.; Meng, E.C.; Ferrin, T.E. UCSF Chimera—A visualization system for exploratory research and analysis. *J. Comput. Chem* **2004**, *25*, 1605–1612. [CrossRef] [PubMed]

```
Brachypodium      NVGLILYEYMPEGTLFELLHERKPQVALGWMARHQIALGVAQGLSYLHQDCVPMIVHRDV      992
Hordeum           GAGLILYEYMPEGTLFELLHGRRPQVALGWTARHQIALGLAQGLSYLHQDCVPMIVHRDV      1060
Triticum          GAGLILYEYMPEGTLFELLHGRKPQVALGWTVRHQIALGLAEGLSYLHQDCVPMIVHRDV      993
Oryza             SVGLILYEYMPEGTLFELLHRRKPHAALDWTVRHQIAFGVAQGLSYLHHDCVPMIVHRDV      976
Panicum           SIGLILCEYMPEGTLFDLFHQRKPQVALDWMTRHQIALGIAQGLSYLHHDCVPMIVHRDV      966
Setaria           SVGLILCEYMPKGTLFDLLHQRKPQVALDWMIRHQIALGVAQGLSYLHHDCVPMIVHRDV      1001
                  . **** ****:****:*:* *:*:.**.*  *****:*:*:******:***********

Brachypodium      KSSNILMDVELVPKLTDFGMGKIVGDEDSDATVSVIVGTLGYIAPEHGYSTRLSEKSDVY      1052
Hordeum           KSNNVLMDADMVPKLADFGMGKIVGDEDADATVSVIVGTLGYIAPEHGYSTRLTEKSDVY      1120
Triticum          KSSNVLMDADMVPKLADFGMGKIVGDEDADATVSVIVGTLGYIAPEHGYSTRLTEKSDVY      1053
Oryza             KSSNILMDTELVPKLTDFGMGKIVEDDDLDATVSVVVGTLGYIAPEHGYYTRLTEKSDVY      1036
Panicum           KSSNILMDSELVPKLTDFGMGKIVHDEDADATVSVIVGTLGYIAPEHGYSTRLTEKSDVY      1026
Setaria           KSSNILMDTELVPKLTDFGMGKIVHDEDANATVSAVIGTLGYIAPEHGYSTRLTEKSDVY      1061
                  **.*:*** ::****:******** *:* :****.::************ ***:******

Brachypodium      SYGVVLLELLCRKMPVDSAFGDGVDIVTWMRSNLKQADHC-SVMSCLDEEIVYWPEDEQA      1111
Hordeum           SYGVVLLELLCRKMAVDPAFGDGVDIVTWMRSNPKQADRRPAAMSCLDEEIVYWPEDEQA      1180
Triticum          SYGVVLLELLCRKMAVDPAFGDGVDIVTWMRSNLKQADRRPAAMSCLDEEIVYWPEDEQA      1113
Oryza             SYGVVLLELLCRKMPVDPAFGDSVDIVTWMRSNLTQADRR-VIMECLDEEIMYWPEDEQA      1095
Panicum           SYGVVLLELLCRKMPVDPSFGDGVDIATWMRTKLKQADHS-SIISLMDEEIMYWPEDEQE      1085
Setaria           SYGVVLLELLCRKMPVDPSFEDGVDIATWIRTKLKQADRC-SIIDLMDEEIMYWPEDDQE      1120
                  ************** ** :* *.***.**:*:: .***:    :. :****:*****:*

Brachypodium      KALHLLDLAISCTEVACQLRPSMREVVNVLVRMDK----    1146
Hordeum           RALDMLDLAISCTQFSFQSRPSMREVVNTLLRMDM----    1215
Triticum          KALDLLDLAISCTQAACQSRPSMREVVNTLVRMDMQGFP    1152
Oryza             KALDLLDLAMYCTQLACQSRPSMREVVNNLMRMDK----    1130
Panicum           KALDLLDLAVSCSQVACQSRPSMREVVNMLMKIEK----    1120
Setaria           KALDLLDLAVSCTQVACQSRPSMREVVNMLLKIEK----    1155
                  :**.:****: *:: : * ********* *::::
```

Figure S1. Alignment of the BdPepR2 aa sequence with other predicted leucine-rich repeat receptor-like protein kinase PEPR1/2 proteins from the *Poaceae* family.
XP_003571653.2 leucine-rich repeat receptor-like protein kinase PEPR2 [Brachypodium distachyon]
KAE8784640.1 Receptor-like protein kinase [Hordeum vulgare]
XP_037434473.1 leucine-rich repeat receptor-like protein kinase PEPR2 [Triticum dicoccoides]
XP_015612899.1 leucine-rich repeat receptor-like protein kinase PEPR2 [Oryza sativa Japonica Group]
XP_025797572.1 leucine-rich repeat receptor-like protein kinase PEPR1 isoform X1 [Panicum hallii]
XP_004983380.2 leucine-rich repeat receptor-like protein kinase PEPR1 [Setaria italica]

Table S1 SPECIFIC PRIMERS SEQUENCES

Sequence of the primers employed for PCR amplification in this study. The table includes oligonucleotide sequences employed for cloning (lower case letters indicate the *Sal*I and *Not*I restriction sites, respectively) and site-directed mutagenesis

Primer nomenclature	5'-3' oligonucleotide sequence
BdPepR2 (for cloning)	
BdPepR2 forward	GAATTCCCGGgtcgacAATGCCTGGGCGCCTATCG
BdPepR2 reverse	AGTCACGATgcggccgcTTACTTGTCCATTCTCACCAAGACA
BdTTM3 (for site-directed mutagenesis)	
BdPepR2 M1066A forward	TGCAGAATCCACAGGCGCCTTCCTGCACAGGAGC
BdPepR2 M1066A reverse	GCTCCTGTGCAGGAAGGCGCCTGTGGATTCTGCA
BdPepR2 M1066R forward	ATCCACAGGCCTCTTCCTGCACAGGAGCTC
BdPepR2 M1066R reverse	GAGCTCCTGTGCAGGAAGAGGCCTGTGGAT

Artykuł II

Duszyn, M.; Świeżawska-Boniecka, B.; Skorupa, M.; Jaworski, K.; Szmidt-Jaworska, A. BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate. Int. J. Mol. Sci. 2022, 23, 2674.

International Journal of
Molecular Sciences

MDPI

Article

BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate

Maria Duszyn [1,*], Brygida Świeżawska-Boniecka [1], Monika Skorupa [1,2], Krzysztof Jaworski [1] and Adriana Szmidt-Jaworska [1]

[1] Department of Plant Physiology and Biotechnology, Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University in Toruń, Lwowska St. 1, 87-100 Torun, Poland; swiezawska@umk.pl (B.Ś.-B.); monika_skorupa@umk.pl (M.S.); jaworski@umk.pl (K.J.); asjawors@umk.pl (A.S.-J.)
[2] Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University in Toruń, Wilenska St. 4, 87-100 Torun, Poland
* Correspondence: duszyn@umk.pl

Abstract: Guanosine 3′,5′-cyclic monophosphate (cGMP) is an important signaling molecule in plants. cGMP and guanylyl cyclases (GCs), enzymes that catalyze the synthesis of cGMP from GTP, are involved in several physiological processes and responses to environmental factors, including pathogen infections. Using in vitro analysis, we demonstrated that recombinant BdGUCD1 is a protein with high guanylyl cyclase activity and lower adenylyl cyclase activity. In *Brachypodium distachyon*, infection by *Fusarium pseudograminearum* leads to changes in *BdGUCD1* mRNA levels, as well as differences in endogenous cGMP levels. These observed changes may be related to alarm reactions induced by pathogen infection. As fluctuations in stress phytohormones after infection have been previously described, we performed experiments to determine the relationship between cyclic nucleotides and phytohormones. The results revealed that inhibition of cellular cGMP changes disrupts stress phytohormone content and responses to pathogen. The observations made here allow us to conclude that cGMP is an important element involved in the processes triggered as a result of infection and changes in its levels affect jasmonic acid. Therefore, stimuli-induced transient elevation of cGMP in plants may play beneficial roles in priming an optimized response, likely by triggering the mechanisms of feedback control.

Keywords: guanylyl cyclase; 3′,5′-cyclic guanosine monophosphate; cGMP; PepR2; *Brachypodium distachyon*; *Fusarium pseudograminearum*; biotic stress; phytohormones; jasmonate

Citation: Duszyn, M.; Świeżawska-Boniecka, B.; Skorupa, M.; Jaworski, K.; Szmidt-Jaworska, A. BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate. *Int. J. Mol. Sci.* **2022**, *23*, 2674. https://doi.org/10.3390/ijms23052674

Academic Editor: Setsuko Komatsu

Received: 2 February 2022
Accepted: 27 February 2022
Published: 28 February 2022

1. Introduction

Cyclic nucleotides (cNMPs), adenosine-3′,5′-cyclic monophosphate (cAMP), and guanosine-3′,5′-cyclic monophosphate (cGMP), are involved in signal transduction in all living cells [1,2]. New information regarding the characterization of new enzymes, adenylyl and guanylyl cyclases (ACs; GCs), synthesizing cNMPs and the signal transduction pathways in which they can participate is continuing to increase [3–8]. The role played by cNMPs in plants is currently poorly understood. To date, cGMP and GCs are known to be involved in several physiological processes and responses to environmental factors, including stressors. In recent years, examples of cyclic nucleotide and phytohormone interactions have been observed, e.g., cGMP is involved in plant hormone signaling and alters phosphorylation of *Arabidopsis thaliana* root proteins [9], several hormones fluctuate in response to changes in cGMP concentration in tomato [10], cGMP interacts with ABA-induced changes in H_2O_2 and NO and stimulates increased concentration of free Ca^{2+}, leading to changes in the signaling pathway that causes stomatal closure in *A. thaliana* [11], *Oryza sativa* cGMP-dependent protein kinase mediates NO-cGMP signaling in response to

GA [12], cGMP promotes ethylene production, and enhances the perception of ethylene [13]. While all of these findings collectively support diverse roles of cyclic nucleotides in plant development and environmental responses, the underlying molecular mechanisms are not well understood. Moreover, there is still much missing information and gaps in the understanding of the interactions between cGMP and hormones in response to pathogen infection. There is evidence that cGMP, GCs [4,5,14], and hormones are involved in the plant response to pathogen infection [15–18]. Therefore, it is worth exploring whether and how these molecules cooperate.

Fusarium crown rot (FCR) caused by the fungal pathogen *Fusarium pseudograminearum* is a disease that results in major yield and quality losses in many economically important plant species worldwide, including cereals. Although *Fusarium* utilizes multiple infection strategies, these fungi are considered to be hemibiotrophs capable of transitioning to necrotrophs depending on the specific environmental and metabolic stimuli. More specifically, infected seedlings can die before or after emergence, and typical disease symptoms of surviving seedlings include browning of the coleoptile, subcrown internode, lower leaf sheaths, and adjacent stems and nodal tissues. Browning can become evident within a few weeks after planting or at any time during plant development. Moreover, infected plants may develop white heads without or with shriveled grains [19].

The aim of our research was to determine the enzymatic activity of the new, putative guanylyl cyclase GUCD1 from *Brachypodium distachyon* and to analyze its involvement in changes in the level of cyclic nucleotides as a manifestation of the response to infection. Our preliminary studies have shown that cyclic nucleotide levels are altered following *B. distachyon* infection by *F. pseudograminarum*. This prompted us to determine the signaling elements involved in the inoculation and infection process in the above experimental setup. This study provides novel insights into the roles of cGMP signaling elements in cereal reactions to fungal pathogens. Furthermore, our results provide a basic framework for further research on cGMP signaling in biotic stress responses.

2. Results and Discussion

Much attention is currently focused on unraveling the molecular mechanisms involved in plant signal transduction cascades in the infection process. The steps between pathogen perception and the initiation of cellular defense response programs in plants remain only partially understood. The presented work supplements this knowledge with respect to cyclic nucleotides and purine nucleotide cyclases.

2.1. Sequence Analysis of BdGUCD1

The amino acid sequence of BdGUCD1 is referred to in the NCBI database as a Guanylyl Cyclase Domain Containing 1 (NCBI: XP_003568333.1). Analysis of predicted 268-aa amino acid sequences using the BlastP program revealed similarities in the BdGUCD1 protein compared to other monocots of putative guanylyl cyclases, with the highest identity score being with *H. vulgare* (XP_044978661.1; 90% identity), *P. miliaceum* (RLN00560.1; 85%), and *T. dicoccoides* (XP_037471862.1; 90%). In addition, the sequence shared homology with previously characterized GCs, such as ZmGC1 (ABD18446.1; 83%) [20], HpGC1 (ADJ94125.1; 64%) [5], AtGC1 (AAM51559.1; 55%) [21], and PnGC1 (ABG67691.2; 52%) [22].

There are two groups of GCs. The first, with canonical GC domains, often appears as a stand-alone protein. In the second, the cyclase domain is part of a larger, multidomain protein complex. This group includes so-called moonlight proteins, which in addition to the GC domain also have kinase domains. The GC domains of these proteins contain 14-amino acid active centers [21,23,24]. Determination of conserved residues within these predicted 14-amino acid catalytic centers led to the discovery of a number of candidate molecules with guanylyl cyclase activity. However, as mentioned earlier in the BdGUCD1 sequence, such motif was not found. It seems that such situation is no exception as neither the PnGC1 [22] nor HpGC1 [5] have such a motive. The lack of a motive while maintaining cyclase activity is very important information. The discovery may indicate that other

cyclases may exist in plant cells, without the previously sought 14-amino acid catalytic motif or with other domains responsible for cyclase activity.

2.2. In Vitro Analysis of the AC and GC Activity of BdGUCD1

The truncated 624-bp fragment of the *BdGUCD1* cDNA was cloned into the pGEX-6P-2 vector in frame with a glutathione S-transferase (GST) tag and expressed in *E. coli* BL21 cells as a GST-BdGUCD1 recombinant protein. The molecular mass of the 207-aa-long BdGUCD1 polypeptide was predicted in silico to be 29.78 kDa (while GST-BdGUCD1 is ~55 kDa), and the isoelectric point was predicted to be 4.97 (http://web.expasy.org/compute_pi; accessed on 17 December 2021). The recombinant BdGUCD1 protein was purified and used for GC enzymatic activity determination. Affinity chromatography enabled purification of the GST-BdGUCD1 fusion protein as a main 55 kDa band (Figure 1A). The preliminary experiments and obtained results showed that the foreignness of GST did not affect the enzymatic activity of the proteins tested.

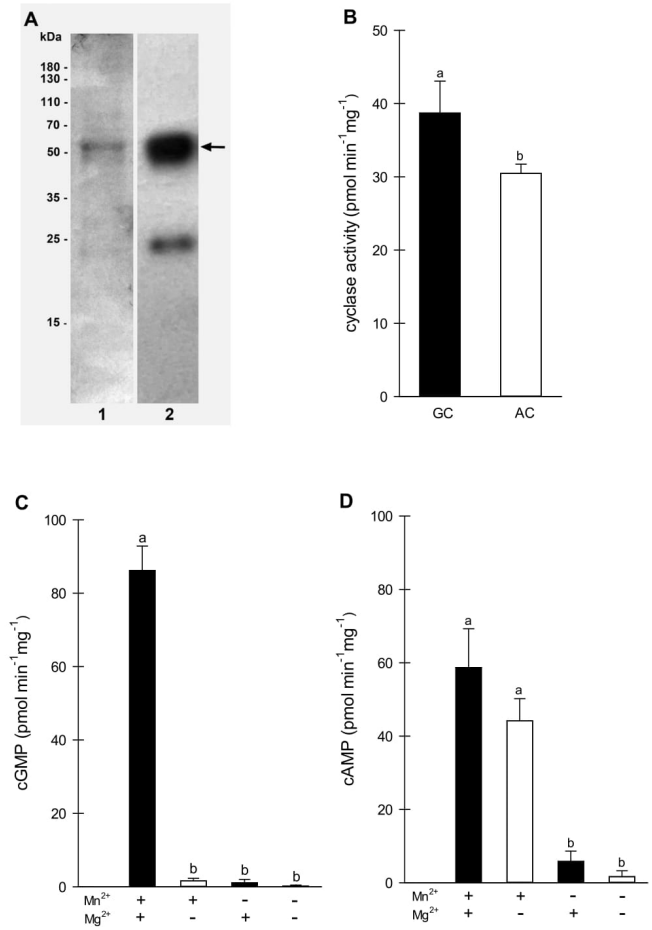


Figure 1. Enzymatic activity of recombinant BdGUCD1. (**A**) SDS–PAGE (1) and Western blot analysis using anti-GST antibodies (2) of BdGUCD1. The arrow indicates the position of the analyzed protein; (**B**) determination of BdGUCD1 substrate specificity. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM GTP, 1 mM ATP, and 5 µg of the purified protein in a final volume of 100 µL; (**C**) determination of BdGUCD1 cofactor specificity for guanylyl cyclase activity. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$ and/or 5 mM $MgCl_2$, 1 mM GTP, and 5 µg of purified protein in a final volume of 100 µL; (**D**) determination of BdGUCD1 cofactor specificity for adenylyl cyclase activity. The reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$ and/or 5 mM $MgCl_2$, 1 mM ATP, and 5 µg of purified protein in a final volume of 100 µL. Data are shown as mean values (n = 3), and error bars indicate the standard error of the mean. Different letters above the bars indicate significant differences at $p < 0.05$.

The purified intracellular domain of the BdGUCD1 protein was tested for its ability to convert GTP substrate to cGMP product (or ATP to cAMP) in the presence of magnesium and/or manganese ions as cofactors. For GC activity, the maximal BdGUCD1 activity was reached at 1 mM GTP 15 min after starting the reaction and the generated level of cGMP was 86.17 pmol mg protein^{-1} min^{-1} (±5.44) (Figure 1C). However, adenylyl cyclase activity was lower and reached 58.70 pmol cAMP mg protein^{-1} min^{-1} (±8.63) (Figure 1D). The values shown above apply to variants where one of the nucleotides (only GTP or only ATP) was present in the reaction mixture. However, lower AC activity was observed when both GTP and ATP were added to the reaction mix (Figure 1B). This indicates that BdGUCD1 is a protein with dual activity, but its substrate specificity for GTP is stronger. The kinetic parameters determined for GC activity of BdGUCD1 were a V_{max} of 149.9 fmol min^{-1} ug^{-1} protein and a K_m value of 0.73 mM and for AC activity V_{max} 76.74 fmol min^{-1} ug^{-1} protein and a K_m 0.32 mM. The BdGUCD1 GC activity was similar to the activity described for PnGC1 (78.1 pmol mg^{-1} min^{-1}) [22], BdPepR2 (72.1 pmol mg^{-1} min^{-1}) [25], and BdERL1 (70 pmol mg^{-1} min^{-1}) [26], but was higher compared to most other plant guanylyl cyclases. The different requirements for cofactors in the cyclase activity of BdGUCD1 are worth noting. Despite the lack of the 14-amino-acid catalytic motif characteristic of GC with the described amino acid responsible for magnesium or manganese binding, BdGUCD1 requires the presence of these ions for its activity. In the case of GC activity, both Mn^{2+} and Mg^{2+} ions are necessary (Figure 1C). However, for AC activity, only Mn^{2+} is needed (Figure 1D). This may be related to the regulation of BdGUCD1 activity depending on the ions present in the environment.

As mentioned previously, the 14-amino-acid catalytic motif was not found in the BdGUCD1 sequence. However, we have confirmed the high guanylyl and lower adenylyl activity. This may indicate the existence of a cyclase group with a different structure and catalytic domain. It is worth mentioning here that proteins with confirmed GC activity often have additional phosphodiesterase, AC, or kinase activity modulated by cyclic nucleotides or calcium, which presumably may affect GC activity and may be important for the various roles of these enzymes in plant cells.

2.3. Effect of F. pseudograminearum Treatment on BdGUCD1 and BdPepR2 Expression and Endogenous Levels of Cyclic Nucleotides

The available data in the literature indicate that cGMP and enzymes with in vitro confirmed GC activity are involved in the response to pathogen infection [4–7,27,28]. Active resistance is triggered by pathogens and involves biochemical and molecular responses. Pathogens produce elicitors called pathogen/microbe-associated molecular patterns (PAMPs/MAMPs), including peptides, metabolites, cell wall components, enzymes, and toxins, to suppress plant defense. Following pathogen attack, the damaged host produces damage-associated molecular patterns (DAMPs), including plant signal molecules. These elicitors or PAMPs/MAMPs/DAMPs are recognized by pattern recognition receptors (PRRs). Among the "moonlighting kinases with GC activity", some proteins act as receptors that recognize PAMPs/DAMPs and activate defense against pathogens. PRRs initiate downstream signaling, and then PRR-derived signals are transmitted via further phosphorylation cascades [29]. Among the *A. thaliana* DAMPs, there is a family of AtPep1–6 peptides. Most likely, each AtPep binds to the plant cell plasma membrane AtPepR1 receptor, which also belongs to an LRR-RLK with GC activity. It was speculated that the binding of AtPep to the AtPepR1 receptor causes local elevation of cGMP, which subsequently leads to the activation of a plasma membrane-localized cyclic nucleotide-gated, Ca^{2+}-conducting channel and CNGC-dependent cytosolic Ca^{2+} elevation [6].

To supplement the existing state of knowledge, we attempted to determine the role of *BdGUCD1*, *BdPepR2* [25], and cyclic nucleotides during the stress response. The endogenous levels of cGMP and cAMP and the quantitative expression levels of the *BdGUCD1* and *BdPepR2* genes were measured at four time points after *F. pseudograminearum* infection (1, 3, 5, and 7 dpi). In control 3-week-old *B. distachyon* plants, endogenous cGMP levels

oscillate at approximately 3 pmol g^{-1} fresh weight and double 24 hours after infection. Three days after infection, levels consecutively decreased, but remained statistically higher than that in the control variant and then decreased in subsequent days (Figure 2A). In contrast, the levels of cAMP remained unchanged after *F. pseudograminearum* infection compared to control variants throughout the entire 7-day experiment (Figure 2B). These data confirmed that only cGMP is involved in the plant's response to infection in agreement with previous observations when pathogen infection causes the accumulation of cGMP in *A. thaliana* plants and cGMP played a key role in local responses controlling the induction of systemic acquired resistance [30]. Furthermore, the accumulation of cGMP in *A. thaliana* was observed hours after contact with avirulent *Pseudomonas syringae* strains. Moreover, this observation can be linked to the immediate recognition of specific pathogen avirulent gene-encoded molecules by resistance genes. This early detection of the pathogen and activation of the defense response involves the production of a range of signaling molecules, such as reactive oxygen species, NO, JA, SA, ethylene, and transcriptional activation of defense-related genes [28]. Changes in endogenous cGMP levels were also observed in *Hippeastrum hybridum* after mechanical wounding and fungal infection [5] and in *A. thaliana* seedlings subjected to salt stress [31].

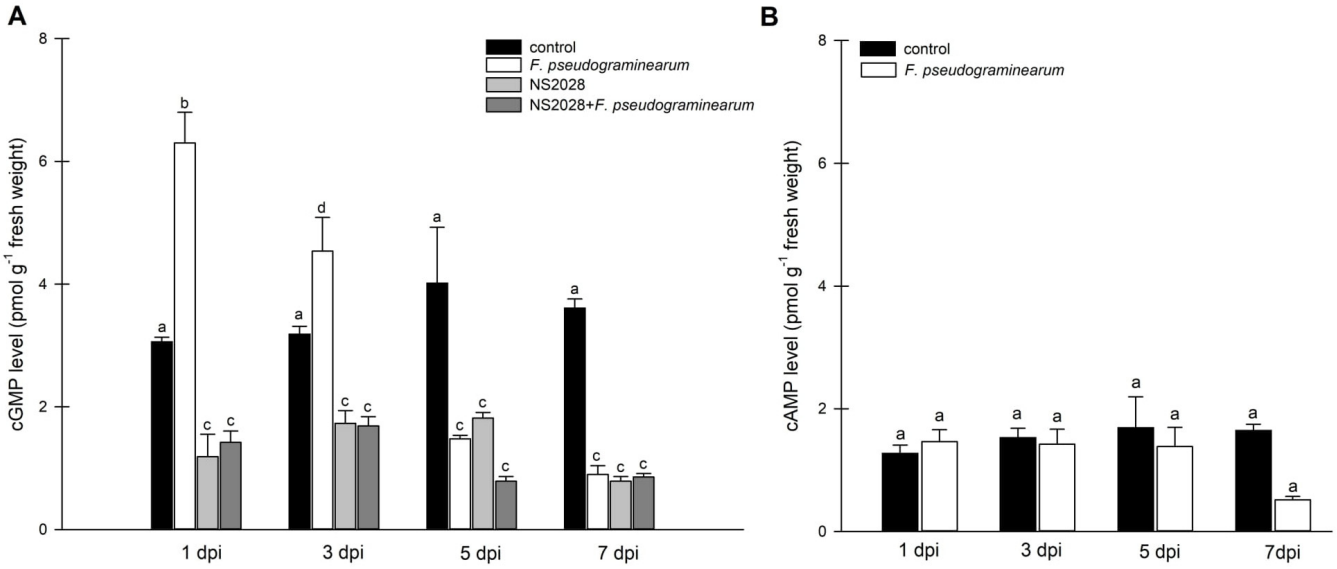


Figure 2. Determination of intracellular cyclic nucleotides levels in *B. distachyon* plants infected with *F. pseudograminearum*. (**A**) Time course of cGMP generation in B. distachyon in response to F. pseudograminearum infection and inhibitor (NS 2028) treatment; (**B**) time course of cAMP generation in *B. distachyon* in response to *F. pseudograminearum* infection. Control plants were not infected or treated with the inhibitor. The data are shown as mean values ($n = 3$), and error bars represent the standard error of the mean. Different letters above the bars indicate significant differences at $p < 0.05$ (ANOVA followed by Tukey's test).

Cellular cNMP levels in plants represent the pooled outcome of cyclase (and PDE) activities expressed by different genes of diverse biological functions. To examine the observed changes in cGMP to enzyme activity, we focused on expression analysis of the *BdGUCD1* and *BdPepR2* genes, which encode proteins with guanylyl cyclase and kinase activity, and determining their expression profile in response to *F. pseudograminearum* infection. BdPepR2 is a plasma membrane receptor of peptide signalling molecules with confirmed guanylyl cyclase activity [25], while BdGUCD1 is presumed to be a cytosolic protein. The data showed that during the 7-day test cycle after fungal infection expression of both genes fluctuated. The mRNA levels of *BdGUCD1* were significantly upregulated the day after infection, then decreased and peaked 7 days after infection (Figure 3A). With respect to *BdPepR2*, the highest expression levels with regard to the control were

observed 1 and 5 days after infection (Figure 3B). *F. pseudograminearum* caused changes in expression levels of both genes at the beginning (a rapid threefold increase was observed in both genes one day after infection) and later stages of the stress response (5 dpi in the case of *BdPepR2* and 7 dpi in *BdGUCG1*). These changes in mRNA levels are consistent with previous hypotheses, since the *BdPepR2* gene encodes a protein presumably acting as a receptor responsible for pathogen protein recognition. This significant induction of *BdPepR2* gene expression stimulated by *F. pseudograminearum* may suggest a role for the BdPepR2 protein in the activation of the plant defense response. Importantly, an increase in gene expression does not guarantee an increase in the corresponding protein abundance, because transcription is only one of the levels of regulation of protein expression. Of note, the profile of *BdPepR2* gene expression coincided with the peak in endogenous cGMP levels one day after *F. pseudograminearum* inoculation (Figure 2A). It has previously been shown that the accumulation of *HpPepR1* transcript was sharply increased after fungal infection, whereas mechanical wounding had no effect on the expression profile of the studied gene [4]. Together, these results may indicate participation of the cGMP-dependent pathway in alarm plant reactions induced by pathogen infection. We showed that BdPepR2 is proteins with guanylyl cyclase and kinase activity, which is modulated by cGMP [20]. So the dual activity of the BdPepR2 may explain the elevated transcript levels 5 days after infection when cGMP levels remain low. Among the group of "moonlighting kinases with GC activity", two other proteins, AtWALK−10 and AtPepR1, act as receptors that recognize PAMPs/DAMPs and activate defense against pathogens. Expression of AtWAKL10 was shown to be induced by both biotrophic and necrotrophic pathogens, or their elicitors, and it was suggested that this indicates the participation of AtWAKL10 in the response to a broad range of pathogens [28]. Among *A. thaliana* DAMPs is a family of AtPep1–6 peptides. Most likely, each AtPep binds to the plant cell plasma membrane AtPepR1 receptor, which also belongs to an LRR-RLK with GC activity. The authors suggested that the binding of AtPep to the AtPepR1 receptor induces local elevation of cGMP, subsequently leading to the activation of a plasma membrane-localized cyclic nucleotide-gated, Ca^{2+}-conducting channel and CNGC-dependent cytosolic Ca^{2+} elevation [6]. Moreover, two tomato homologues of *A. thaliana* PEPRs, SlGC17 and SlGC18, exhibited in vitro GC activity. Cosilencing of *SlGC17* and *SlGC18* genes significantly attenuated resistance to tobacco pathogens and reduced PAMP- and DAMP-triggered immunity by decreasing flg22-, chitin-, and AtPep1-elicited Ca^{2+} and H_2O_2 bursts [27]. These results indicate how important the role of moonlighting proteins and proteins with GC activity can be in plant responses to biotic stressors. This significant induction of both genes, *BdPepR2* and *BdGUCD1*, observed 1 day after infection may be an indication of the importance of cGMP in response to fungal infection.

2.4. Effect of F. pseudograminearum Treatment on Phytohormone Levels

Both salicylic acid (SA) and jasmonic acid (JA), so-called immunity hormones, are involved in stress reactions and basal and/or induced resistance against plant pathogens. Importantly, they do not act independently from each other, but rather form a network of interactions in response to pathogens with different lifestyles. Once the SA pathway is activated at the site of infection, a defense response is triggered in distal plant parts to protect undamaged tissues. Mutants insensitive to SA or defective in SA accumulation display enhanced susceptibility to pathogens. Moreover, an increase in SA levels in pathogen-exposed tissues leads to the induction of pathogenesis related (PR) genes [32]. Many of the *F. pseudograminearum*-responsive genes are altered by the toxin and by plant defense-related hormones, such as SA and JA [19]. In *A. thaliana*, SA and JA interact antagonistically, which can modulate defense gene expression in response to pathogen infection [33]. JA and SA signaling were found to co-induce broad-spectrum disease-response genes, co-repress genes related to photosynthesis, auxin, and gibberellin, and reallocate resources of growth towards defense [34]. However, the detailed relationships between these hormones and other molecules are still unknown and worth further investigation.

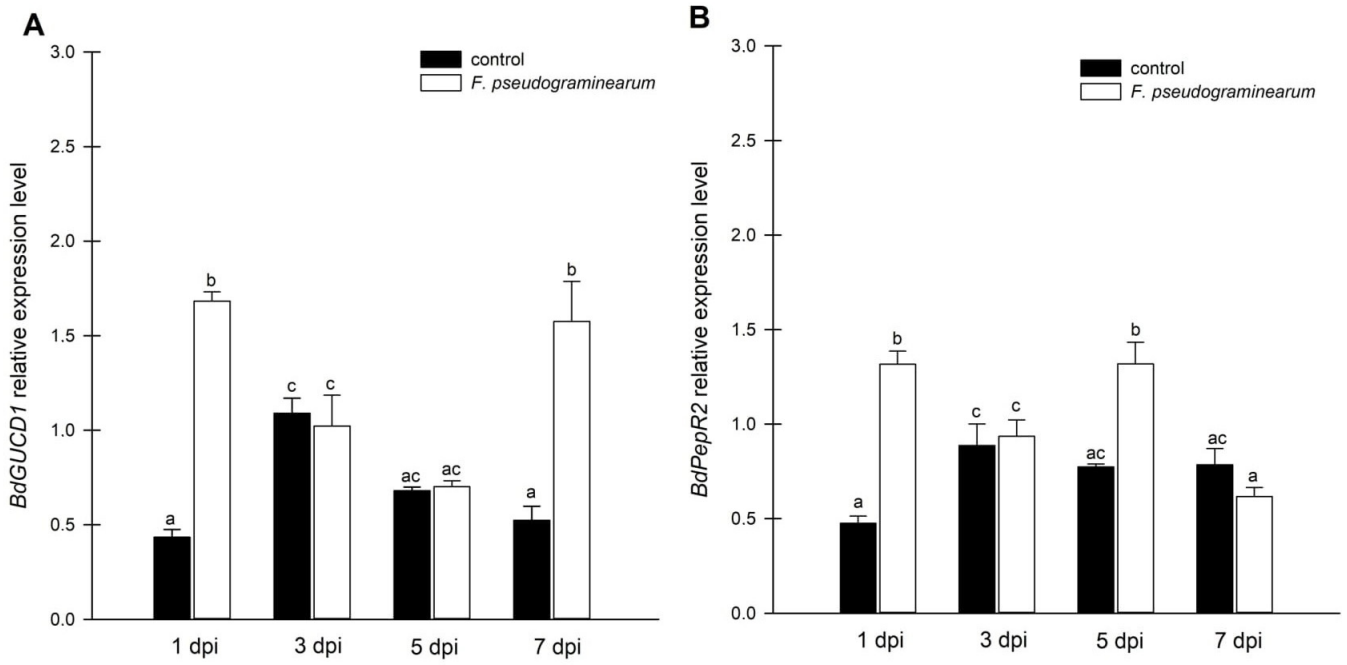


Figure 3. Expression analysis of guanylyl cyclase genes in *F. pseudograminearum*-infected *B. distachyon* plants. (**A**) mRNA levels of *BdGUCD1* in *B. distachyon* in response to *F. pseudograminearum* infection; (**B**) mRNA levels of BdPepR2 in *B. distachyon* in response to *F. pseudograminearum* infection. Control plants were not infected or treated with the inhibitor. Different letters above the bars indicate significant differences at $p < 0.05$ (ANOVA followed by Tukey's test).

To determine whether infection with the necrotrophic fungal pathogen *F. pseudograminearum* alters phytohormone levels in *B. distachyon*, we quantified SA, JA, and ABA levels using LC–MS/MS in a time course experiment. The results showed that JA levels increased at 1 dpi, but not at later time points. Additionally, SA levels rose at 1 dpi and a significant decrease compared to the control was noted (Figure 4). These results are consistent with a previous report, implying that JA acts as a primary responsive signal molecule during *F. pseudograminearum* infection in monocots [35,36]. Three days post germination, *B. distachyon* seedlings were treated with *F. pseudograminearum* induction of genes encoding plant hormones, and three defense-related phytohormones (JA, SA, and ABA) were observed at 3 and 7 dpi [37]. In the case of wheat, only SA levels were increased at 1 dpi, while JA levels were unchanged [38]. This confirms involvement of these phytohormones in the plant response to stress at various stages of development in monocots. However, all data concerning stress hormones were inconsistent and demonstrated induction of different hormones at different times in different plants. Changes in hormone levels can also be related to the stress response to cell damage during the first stages of responses. However, it is not possible to separate these stages, so it should be considered a larger process involving the response of plants to wounding and pathogen infection.

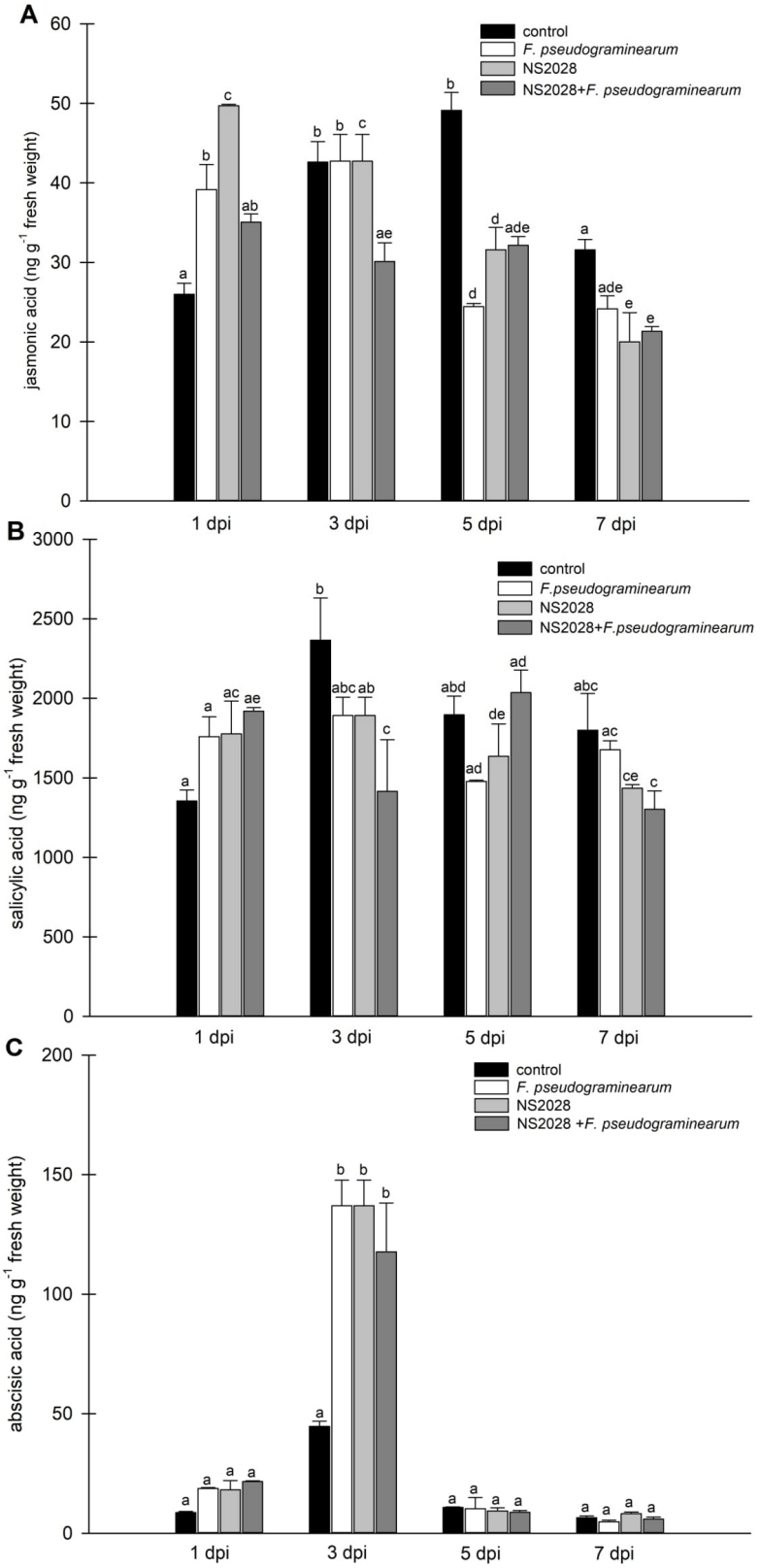


Figure 4. Determination of endogenous levels of phytohormones in *B. distachyon* infected with *F. pseudograminearum*. (**A**) Jasmonic acid; (**B**) salicylic acid; and (**C**) abscisic acid. Control plants were not infected or treated with the inhibitor. Data are shown as mean values (n = 3), and error bars indicate the standard error of the mean. Different letters above the bars indicate significant differences at $p < 0.05$ (ANOVA followed by Tukey's test).

ABA appears to be a key regulator of defense against necrotrophs with both negative and positive contributions. Clarification of the nature of ABA function is further confounded by its interactions with other resistance pathways and the potential trade-offs resulting from the occurrence of abiotic stresses during infection [39]. Current knowledge indicates that ABA influences some responses of plant pathogen resistance (e.g., stomatal closure and red-ox homeostasis), although its effect may vary with a number of variables, such as type of tissue, age of the plant, pathogen type, and stage of the infection [40]. In our experiments, a significant change in ABA levels was observed up to three days after infection. ABA levels tripled on day three. Together with the infection progress (5–7 dpi), a dramatic drop in ABA concentration was noted. A similar relationship was recently observed in flax [41]. Upregulation of the terpenoid pathway and increased ABA content in flax upon *Fusarium* infection leads to activation of the early plant response (PR genes, cell wall remodeling, and redox status). In this system, levels of accumulated ABA strongly increased continuously during the first 36 h. Transgenic flax plants with an elevated ABA level showed increased resistance to fusariosis [41], which may be associated with higher expression of the chitinase gene. This was also shown in wheat, where in a more resistant cultivar, twofold higher levels of ABA were noted [42]. Therefore, we conclude that the elevated synthesis of ABA correlates with *B. distachyon* responses to *F. pseudograminarum* and is involved in the early response of the plant to infection. Isner et al. investigated potential interactions between phytohormones and cGMP signaling and showed that plant hormones (ABA, IAA, JA) evoke rapid and concentration-dependent changes in cellular cGMP. However, brassinosteroids and cytokinin did not affect the levels of cGMP [9]. There is some evidence of a direct connection or the lack of a direct connection between ABA and cGMP, so it appears to be whole plant, organ, or process dependent [11,43–46]. However, it can also be associated with the use of different methods to study the changes in the endogenous level of these molecules, which has become more accurate over time.

2.5. Impact of cGMP on Phytohormone Levels during Infection

The results presented above indicate the involvement of cyclic GMP in the response of *B. distachyon* to stress induced by *F. pseudograminearum* infection. Therefore, we also wanted to investigate whether any interactions between cGMP and hormones is observed during the response to stress. We administered the guanylate cyclase inhibitor (NS 2028) the day before infection and measured the levels of all molecules tested at the same time as previous variants. NS 2028 was previously used in the characterization of PnGC-1 [22] and plant natriuretic peptides [44]. Most importantly, endogenous levels of cGMP in the variants treated with NS 2028 were significantly lower than that in the control plants (Figure 2A). This confirms that NS 2028 blocked the activity of plant guanylate cyclases, resulting in low levels of cGMP. The inhibitor was not affected by the infection, guanylyl cyclase was still inhibited, and cGMP levels remained low.

Application of the inhibitor caused increased levels of JA compared to the control at the first analyzed time (1 dpi); at subsequent times, the values were similar or lower (Figure 4A). In the case of NS 2028, application of SA and ABA maintained a similar pattern, so the levels of these hormones changed similarly from day to day after administration of the inhibitor (and *F. pseudograminearum*) compared to the results discussed above (Figure 4B,C). In line with these previous findings, we showed that cellular cGMP reduction modulated the levels of jasmonic acid in plants. Based on the available data, it can be concluded that this is an indirect effect. Inhibition of cGMP levels may strengthen JA signal transduction-mediated production of defense elements, but this needs to be validated by further experiments.

In conclusion, these advances are very limited and represent the beginning of elucidating cyclic nucleotide signaling cascades in combination with hormones that require comprehensive and systemic research. The role for cGMP signaling in the action of phytohormones has previously been shown both in growth and development, as well as in responses to stress factors [47]. A strong link between brassinosteroids and cGMP has been described [48]. GMP has also been linked to auxins in the context of root development,

primarily due to its link with NO [49]. Studies on barley germination inferred a strong link between gibberellin (GA) and cGMP [43]. Isner et al. [9] showed that ABA induced cGMP in rice protoplasts seconds after treatment. Salt and osmotic stress are known to increase ABA levels and induce a rapid increase in cGMP levels in Arabidopsis seedlings [31]. cAMP was suggested to act upstream of salicylic acid (SA) during the plant defense process and induce PR1 expression [50] and it modulates the jasmonic acid (JA)-mediated signaling pathway [51]. The basis of the changes observed in these studies is not known, further complicated by the existence of a dependency network. While there are dependencies between these elements, we are only beginning to elucidate cyclic nucleotide signaling cascades in combination with hormones, which requires continued comprehensive and systemic research.

3. Materials and Methods

3.1. Construction of the Expression Vector, Expression and Purification of Recombinant Protein

Total RNA was extracted from 3-week-old *Brachypodium distachyon* Bd21 seedlings using a Universal RNA Purification Kit (EURx, Poland). cDNA was synthesized using the NG dART RT kit (EURx, Poland). A 624-bp fragment of the *BdGUCD1* (NCBI accession number: XM_003568285.3; https://www.ncbi.nlm.nih.gov/nuccore; accessed on 27 March 2018) coding region corresponding to a 207-residue polypeptide [Met^{62}→Leu^{268}] was amplified by PCR using specific primers (Table S1). Next, the PCR product was introduced into the linearized pGEX-6P-2 expression vector using In-Fusion cloning technology (In-Fusion HD Cloning Kit; Takara Bio USA, Inc., Mountain View, CA, USA). After being transformed with the resulting plasmid, the *E. coli* BL21 strain was used to produce the glutathione S-transferase (GST)-tagged protein. For the expression of BdGUCD1 recombinant protein, the construct was transformed into One Shot BL21 (DE3)pLysS *E. coli* cells (Life Technologies, Carlsbad, CA, USA). Bacterial cells were grown in LB medium supplemented with 2% glucose at 37 °C. Fusion protein production was induced by adding IPTG at a final concentration of 1 mM and incubating the cells at 22 °C for 3 h in glass vessels connected to a BioFlo 120 bioprocess control station (Eppendorf, Hauppauge, NY, USA). The pH was maintained at 6.5 (±0.2), the dissolved oxygen parameter was set to 20%, and the agitation speed was 200 rpm. Bacteria were collected by centrifugation and proteins were purified, as previously described [5]. The homogeneity and purity of the protein fractions were analyzed using 10% (*v*/*v*) SDS/PAGE and Western blotting analysis, as previously described [52].

3.2. Determination of Guanylyl and Adenylyl Cyclase Activity

Guanylyl and adenylyl cyclase activity was determined by estimating the rate of cGMP or cAMP formation. For the enzymatic assay, the reaction mixture contained 50 mM Tris/HCl buffer (pH 7.5), 5 mM $MnCl_2$ or/and 5 mM $MgCl_2$, GTP and/or ATP as a substrate (1 mM), and 5 µg of the purified protein in a final volume of 100 µL. After incubation at 30 °C for 15 min, the reaction was stopped at 100 °C for 10 min and the samples were centrifuged at 16,000× *g* for 10 min. Preliminary trials were also performed to determine the optimal reaction conditions (data not shown). Moreover, background cGMP levels were measured in tubes that contained the reaction mixture without protein. Total cGMP/cAMP concentration was determined in triplicate using liquid chromatography-tandem mass spectrometry (LC–MS/MS Nexera UHPLC and LCMS-8045 integrated system (Shimadzu Corporation, Kyoto, Japan)). The ionization source parameters were optimized in positive ESI mode using pure cGMP (or cAMP) dissolved in HPLC-grade water. Samples were separated using a reversed-phase C18 column (150 mm × 2.1 mm, 2.6 µm, Kinetex) in 10% methanol with 0.1% (*v*/*v*) formic acid (solvent A (water with 0.1% (*v*/*v*) formic acid), solvent B methanol with 0.1% (*v*/*v*) formic acid) at a flow rate of 0.3 mL/min. The interface voltage was set at 4.0 kV for positive (ES+) electrospray. Data acquisition and analysis were performed using the LabSolutions workstation for LCMS-8045. Enzyme activity was defined as the amount of cGMP or cAMP produced by 1 mg of protein per min.

3.3. Plant Material and Pathogen Inoculation

Brachypodium distachyon Bd21-1 was grown in a growth cabinet under long-day conditions (16/8 light/dark) at 24 °C. The *Fusarium pseudograminearum* isolate (laboratory code F0444) was obtained from GIORiN (Main Inspectorate of Plant Health and Seed Inspection; Toruń, Poland). Inoculum preparation was as described by [38]. Briefly, the inoculum was prepared using PDA plates inoculated for 10 days at 24 °C. Subsequently, spores were produced in mung bean broth by inoculation with agar plugs (0.5 mm) taken from the *F. pseudograminearum* plate culture and incubating on an orbital shaker at room temperature (15 rpm; ~22 °C) for 9 days. After filtration and centrifugation, spores were suspended in distilled water to a final concentration of 1×10^6 spores ml^{-1} (spores concentration was measured by visual counting with a hemocytometer). Three-week-old *B. distachyon* plants were treated with an *F. pseudograminearum* suspension. Some variants of the experiment were treated with 1 mM NS 2028 24 h prior to infection with *F. pseudograminearum*. The solution was precisely applied using a tiny brush on the leaves. Plant material was collected 1, 3, 5, and 7 dpi (days post-infection), immersed in liquid nitrogen, and stored at −80 °C. Frozen plant material was manually homogenized using a pre-cooled mortar and pestle with liquid nitrogen and stored in -80 °C for subsequent experiments, which were performed in triplicate.

3.4. Determination of Endogenous Levels of Phytohormones

Endogenous levels of abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) were measured using mass spectrometry combined with liquid chromatography (LC–MS). Phytohormones were extracted using a modified QuEChERS-based method [53]. Extraction buffer (80% acetonitrile, 5% formic acid (FA), 15% water, and 1 mM butylhydroxytoluol (BHT)) was added to a sample containing 100 mg of leaves homogenized in liquid nitrogen. In this step, internal standards were also added (10 ng/µL d_4SA; 4 ng/µL d_5JA; 2 ng/µL d_6ABA). The mixture was shaken overnight (at least 18 h; 200 rpm; 4 °C). After incubation, the salt mixture (magnesium sulfate anhydrous, sodium chloride) was added, and the mixture was then vigorously mixed for 1 min. The mixed samples were centrifuged at 10,000× *g* for 10 min to obtain the supernatant. The clean-up of the supernatant was performed by vigorously mixing the supernatant with sodium sulfate for 5 min, followed by centrifugation (10,000× *g* for 10 min). The supernatant was collected and dried using nitrogen gas. Samples were dissolved using 1 mL 1 M FA and subjected to solid phase extraction (SPE) using silica packed columns (Discovery® DSC-18 SPE Tube; Supleco). The columns were activated with 100% methanol and conditioned with 1 M FA. The applied samples were purified twice using 1 M FA. Elution was performed using 80% methanol (*v*/*v*). Each sample was lyophilized, suspended in 100 µl 35% methanol (*v*/*v*), and centrifuged. For phytohormone determination, LCMS-8045 tandem mass spectrometry (Shimadzu Corporation, Kyoto, Japan) was used. Chromatographic separation was performed on a Kinetex® 2.6 µm XB-C18 100 Å reversed-phase column (150 mm × 2.1 mm). Water with 0.1% formic acid (*v*/*v*) (A) and methanol with 0.1% formic acid (*v*/*v*) (B) were used as the mobile phase. Separation was performed on a linear gradient of 40–90% (*v*/*v*) methanol for 7 min at a flow rate of 0.3 mL/min at 30 °C. In mass spectrometry, the samples were subjected to negative electrospray ionization (ESI) and ions were fragmented by collision-induced dissociation (CID). The ionization voltage was −3 kV. Analysis of individual phytohormones was based on multiple reaction monitoring (MRM).

3.5. Determination of Endogenous Levels of Cyclic Nucleotides

Endogenous levels of cyclic nucleotides were measured using mass spectrometry combined with liquid chromatography (LC–MS). Cyclic nucleotides were extracted using a previously described method [54] for 100 mg of tissue.

3.6. Gene Expression Analysis

Total RNA was isolated from plant tissue using a GeneMATRIX Universal RNA Purification Kit (EURx) and digested using DNase I (Thermo Scientific) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 µg of total RNA using random hexamers and GoScript™ Reverse Transcriptase (Promega) following the manufacturer's instructions. The gene-specific primers and hydrolysis probes for qPCR were designed with ProbeFinder software from the Universal Probe Library Assay Design Center (Roche) and Primer3Plus software. The β-actin gene was used as a reference. To determine the PCR efficiencies, standard curves for both target and control genes were obtained using a series of cDNA dilutions as a template. The RT–qPCR was performed on a LightCycler® 480 using LightCycler 480 Probes Master following the manufacturer's protocol (Roche). Three independent biological replicates and three technical replicates were analyzed. Relative levels of gene expression were calculated according to the Pfaffl method [55]. A list of the PCR primers and probes used for the experiments is provided in Table S1.

3.7. Data Analysis

The statistical significance of differences between the mean values of the experimental variants of the analyzed parameters was determined using two-way ANOVA followed by Tukey's test in SigmaPlot 14.5 (Systat Software). Differences of $p < 0.05$ were considered significant. The mean and standard deviation were calculated. Error bars shown in all figures represent the standard deviation calculated from three repetitions of each experiment.

4. Conclusions

Under natural conditions, plants are exposed to attacks from a range of pathogens and pests that possess a variety of infection strategies. Cross-talk between various elements allows the plant to divert resources to the most appropriate defense mechanisms. Much attention is currently focused on unraveling the molecular mechanisms involved in plant signal transduction cascades during the infection process. The steps between pathogen perception and the initiation of cellular defense response programs in plants remain only partially understood. The work presented herein supplements this knowledge with respect to cyclic nucleotides and purine nucleotide cyclases. The observations made here allow us to conclude that cGMP is an element involved in the processes triggered as a result of infection and changes in its levels affect stress hormones, especially jasmonic acid. Therefore, stimuli-induced transient elevation of cGMP in plants may play beneficial roles in priming an optimized response, likely by triggering the mechanisms of feedback control.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23052674/s1.

Author Contributions: Conceptualization, M.D. and A.S.-J.; formal analysis, M.D. and M.S.; funding acquisition, M.D.; investigation, M.D., B.Ś.-B. and M.S.; methodology, M.D., B.Ś.-B. and M.S.; supervision, K.J. and A.S.-J.; visualization, K.J.; writing—original draft, M.D.; writing—review and editing, B.Ś.-B., M.S., K.J. and A.S.-J. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Science Centre (Poland) (grant number 2018/29/N/tNZ9/00812) and funds provided by Nicolaus Copernicus University (Toruń, Poland) for the Research Program of the Department of Plant Physiology and Biotechnology.

Data Availability Statement: Data are contained within the article or Supplementary Material.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

1. Pauline, S. Guanylyl cyclases across the tree of life. *Front. Biosci.* **2005**, *10*, 1485–1498. [CrossRef]
2. Świeżawska, B.; Duszyn, M.; Jaworski, K.; Szmidt-Jaworska, A. Downstream Targets of Cyclic Nucleotides in Plants. *Front. Plant Sci.* **2018**, *9*, 1428. [CrossRef]
3. Kwezi, L.; Meier, S.; Mungur, L.; Ruzvidzo, O.; Irving, H.; Gehring, C. The Arabidopsis thaliana Brassinosteroid Receptor (AtBRI1) Contains a Domain that Functions as a Guanylyl Cyclase In Vitro. *PLoS ONE* **2007**, *2*, e449. [CrossRef]
4. Świeżawska, B.; Jaworski, K.; Duszyn, M.; Pawełek, A.; Szmidt-Jaworska, A. The Hippeastrum hybridum PepR1 gene (HpPepR1) encodes a functional guanylyl cyclase and is involved in early response to fungal infection. *J. Plant Physiol.* **2017**, *216*, 100–107. [CrossRef]
5. Świeżawska, B.; Jaworski, K.; Szewczuk, P.; Pawełek, A.; Szmidt-Jaworska, A. Identification of a Hippeastrum hybridum guanylyl cyclase responsive to wounding and pathogen infection. *J. Plant Physiol.* **2015**, *189*, 77–86. [CrossRef]
6. Qi, Z.; Verma, R.; Gehring, C.; Yamaguchi, Y.; Zhao, Y.; Ryan, C.A.; Berkowitz, G.A. Ca^{2+} signaling by plant Arabidopsis thaliana Pep peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca^{2+} channels. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 21193–21198. [CrossRef]
7. Malukani, K.K.; Ranjan, A.; Hota, S.J.; Patel, H.K.; Sonti, R.V. Dual Activities of Receptor-Like Kinase OsWAKL21.2 Induce Immune Responses. *Plant Physiol.* **2020**, *183*, 1345–1363. [CrossRef]
8. Gehring, C.A.; Turek, I. Cyclic Nucleotide Monophosphates and Their Cyclases in Plant Signaling. *Front. Plant Sci.* **2017**, *8*, 1704. [CrossRef]
9. Isner, J.-C.; Nühse, T.; Maathuis, F.J.M. The cyclic nucleotide cGMP is involved in plant hormone signalling and alters phosphorylation of Arabidopsis thaliana root proteins. *J. Exp. Bot.* **2012**, *63*, 3199–3205. [CrossRef]
10. Miras-Moreno, B.; Zhang, L.; Senizza, B.; Lucini, L. A metabolomics insight into the Cyclic Nucleotide Monophosphate signaling cascade in tomato under non-stress and salinity conditions. *Plant Sci.* **2021**, *309*, 110955. [CrossRef]
11. Dubovskaya, L.V.; Bakakina, Y.S.; Kolesneva, E.V.; Sodel, D.L.; McAinsh, M.R.; Hetherington, A.M.; Volotovski, I.D. cGMP-dependent ABA-induced stomatal closure in the ABA-insensitive Arabidopsis mutantabi1-1. *New Phytol.* **2011**, *191*, 57–69. [CrossRef]
12. Shen, Q.; Zhan, X.; Yang, P.; Li, J.; Chen, J.; Tang, B.; Wang, X.; Hong, Y. Dual Activities of Plant cGMP-Dependent Protein Kinase and Its Roles in Gibberellin Signaling and Salt Stress. *Plant Cell* **2019**, *31*, 3073–3091. [CrossRef]
13. Li, J.; Jia, H.; Wang, J. cGMP and ethylene are involved in maintaining ion homeostasis under salt stress in Arabidopsis roots. *Plant Cell Rep.* **2014**, *33*, 447–459. [CrossRef] [PubMed]
14. Meier, S.; Ruzvidzo, O.; Morse, M.; Donaldson, L.; Kwezi, L.; Gehring, C.A. The Arabidopsis Wall Associated Kinase-Like 10 Gene Encodes a Functional Guanylyl Cyclase and Is Co-Expressed with Pathogen Defense Related Genes. *PLoS ONE* **2010**, *5*, e8904. [CrossRef] [PubMed]
15. Da Silva, M.N.; Carvalho, S.M.P.; Rodrigues, A.M.; Gómez-Cadenas, A.; António, C.; Vasconcelos, M.W. Defence-related pathways, phytohormones and primary metabolism are key players in kiwifruit plant tolerance to *Pseudomonas syringae* pv. actinidiae. *Plant Cell Environ.* **2021**, *45*, 528–541. [CrossRef]
16. Buhrow, L.M.; Liu, Z.; Cram, D.; Sharma, T.; Foroud, N.A.; Pan, Y.; Loewen, M.C. Wheat transcriptome profiling reveals abscisic and gibberellic acid treatments regulate early-stage phytohormone defense signaling, cell wall fortification, and metabolic switches following Fusarium graminearum-challenge. *BMC Genom.* **2021**, *22*, 798. [CrossRef]
17. Hemelíková, N.; Žukauskaitė, A.; Pospíšil, T.; Strnad, M.; Doležal, K.; Mik, V. Caged Phytohormones: From Chemical Inactivation to Controlled Physiological Response. *J. Agric. Food Chem.* **2021**, *69*, 12111–12125. [CrossRef]
18. Ngou, B.P.M.; Jones, J.D.; Ding, P. Plant immune networks. *Trends Plant Sci.* **2021**, *27*, 255–273. [CrossRef]
19. Kazan, K.; Gardiner, D.M. Fusarium crown rot caused byFusarium pseudograminearumin cereal crops: Recent progress and future prospects. *Mol. Plant Pathol.* **2018**, *19*, 1547–1562. [CrossRef]
20. Yuan, J.; Ali, M.L.; Taylor, J.; Liu, J.; Sun, G.; Liu, W.; Masilimany, P.; Gulati-Sakhuja, A.; Pauls, K.P. A guanylyl cyclase-like gene is associated with Gibberella ear rot resistance in maize (*Zea mays* L.). *Theor. Appl. Genet.* **2007**, *116*, 465–479. [CrossRef]
21. Ludidi, N.; Gehring, C. Identification of a Novel Protein with Guanylyl Cyclase Activity in Arabidopsis thaliana. *J. Biol. Chem.* **2003**, *278*, 6490–6494. [CrossRef]
22. Szmidt-Jaworska, A.; Jaworski, K.; Pawełek, A.; Kopcewicz, J. Molecular Cloning and Characterization of a Guanylyl Cyclase, PnGC-1, Involved in Light Signaling in Pharbitis nil. *J. Plant Growth Regul.* **2009**, *28*, 367–380. [CrossRef]
23. Wong, A.; Gehring, C.; Irving, H.R. Conserved Functional Motifs and Homology Modeling to Predict Hidden Moonlighting Functional Sites. *Front. Bioeng. Biotechnol.* **2015**, *3*, 82. [CrossRef]
24. Wong, A.; Tian, X.; Gehring, C.; Marondedze, C. Discovery of Novel Functional Centers with Rationally Designed Amino Acid Motifs. *Comput. Struct. Biotechnol. J.* **2018**, *16*, 70–76. [CrossRef]
25. Duszyn, M.; Świeżawska-Boniecka, B.; Wong, A.; Jaworski, K.; Szmidt-Jaworska, A. In Vitro Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach. *Int. J. Mol. Sci.* **2021**, *22*, 6243. [CrossRef] [PubMed]
26. Świeżawska-Boniecka, B.; Duszyn, M.; Hammer, K.; Wong, A.; Szmidt-Jaworska, A.; Jaworski, K. Brachypodium distachyon ERECTA-like1 protein kinase is a functional guanylyl cyclase. *Front. Biosci.* **2021**, *13*, 249. [CrossRef]

27. Rahman, H.; Wang, X.-Y.; Xu, Y.-P.; He, Y.-H.; Cai, X.-Z. Characterization of tomato protein kinases embedding guanylate cyclase catalytic center motif. *Sci. Rep.* **2020**, *10*, 1–16. [CrossRef] [PubMed]
28. Meier, S.; Madeo, L.; Ederli, L.; Donaldson, L.; Pasqualini, S.; Gehring, C. Deciphering cGMP signatures and cGMP-dependent pathways in plant defence. *Plant Signal. Behav.* **2009**, *4*, 307–309. [CrossRef]
29. Saijo, Y.; Loo, E.; Yasuda, S. Pattern recognition receptors and signaling in plant-microbe interactions. *Plant J.* **2018**, *93*, 592–613. [CrossRef]
30. Hussain, J.; Chen, J.; Locato, V.; Sabetta, W.; Behera, S.; Cimini, S.; Griggio, F.; Martínez-Jaime, S.; Graf, A.; Bouneb, M.; et al. Constitutive cyclic GMP accumulation in Arabidopsis thaliana compromises systemic acquired resistance induced by an avirulent pathogen by modulating local signals. *Sci. Rep.* **2016**, *6*, 36423. [CrossRef]
31. Donaldson, L.; Ludidi, N.; Knight, M.; Gehring, C.; Denby, K. Salt and osmotic stress cause rapid increases inArabidopsis thalianacGMP levels. *FEBS Lett.* **2004**, *569*, 317–320. [CrossRef]
32. Van Loon, L.; Rep, M.; Pieterse, C. Significance of Inducible Defense-related Proteins in Infected Plants. *Annu. Rev. Phytopathol.* **2006**, *44*, 135–162. [CrossRef] [PubMed]
33. Takahashi, H.; Kanayama, Y.; Zheng, M.S.; Kusano, T.; Hase, S.; Ikegami, M.; Shah, J. Antagonistic Interactions between the SA and JA Signaling Pathways in Arabidopsis Modulate Expression of Defense Genes and Gene-for-Gene Resistance to Cucumber Mosaic Virus. *Plant Cell Physiol.* **2004**, *45*, 803–809. [CrossRef] [PubMed]
34. Zhang, N.; Zhou, S.; Yang, D.; Fan, Z. Revealing Shared and Distinct Genes Responding to JA and SA Signaling in Arabidopsis by Meta-Analysis. *Front. Plant Sci.* **2020**, *11*, 908. [CrossRef]
35. Desmond, O.J.; Edgar, C.I.; Manners, J.M.; Maclean, D.J.; Schenk, P.; Kazan, K. Methyl jasmonate induced gene expression in wheat delays symptom development by the crown rot pathogen Fusarium pseudograminearum. *Physiol. Mol. Plant Pathol.* **2005**, *67*, 171–179. [CrossRef]
36. Desmond, O.J.; Manners, J.M.; Schenk, P.M.; Maclean, D.J.; Kazan, K. Gene expression analysis of the wheat response to infection by Fusarium pseudograminearum. *Physiol. Mol. Plant Pathol.* **2008**, *73*, 40–47. [CrossRef]
37. Powell, J.J.; Carere, J.; Sablok, G.; Fitzgerald, T.L.; Stiller, J.; Colgrave, M.L.; Gardiner, D.M.; Manners, J.M.; Vogel, J.P.; Henry, R.J.; et al. Transcriptome analysis of Brachypodium during fungal pathogen infection reveals both shared and distinct defense responses with wheat. *Sci. Rep.* **2017**, *7*, 1–14. [CrossRef]
38. Powell, J.J.; Carere, J.; Fitzgerald, T.L.; Stiller, J.; Covarelli, L.; Xu, Q.; Gubler, F.; Colgrave, M.L.; Gardiner, D.M.; Manners, J.M.; et al. The Fusarium crown rot pathogenFusarium pseudograminearumtriggers a suite of transcriptional and metabolic changes in bread wheat (*Triticum aestivum* L.). *Ann. Bot.* **2016**, *119*, 853–867. [CrossRef]
39. Ton, J.; Flors, V.; Mauch-Mani, B. The multifaceted role of ABA in disease resistance. *Trends Plant Sci.* **2009**, *14*, 310–317. [CrossRef]
40. Maksimov, I.V. Abscisic acid in the plants-pathogen interaction. *Russ. J. Plant Physiol.* **2009**, *56*, 742–752. [CrossRef]
41. Boba, A.; Kostyn, K.; Kozak, B.; Wojtasik, W.; Preisner, M.; Prescha, A.; Gola, E.M.; Lysh, D.; Dudek, B.; Szopa, J.; et al. Fusarium oxysporum infection activates the plastidial branch of the terpenoid biosynthesis pathway in flax, leading to increased ABA synthesis. *Planta* **2020**, *251*, 1–14. [CrossRef]
42. Buhrow, L.M.; Cram, D.; Tulpan, D.; Foroud, N.A.; Loewen, M.C. Exogenous Abscisic Acid and Gibberellic Acid Elicit Opposing Effects on Fusarium graminearum Infection in Wheat. *Phytopathology* **2016**, *106*, 986–996. [CrossRef]
43. Penson, S.P.; Schuurink, R.C.; Fath, A.; Gubler, F.; Jacobsen, J.V.; Jones, R.L. cGMP Is Required for Gibberellic Acid-Induced Gene Expression in Barley Aleurone. *Plant Cell* **1996**, *8*, 2325–2333. [CrossRef]
44. Wang, Y.H.; Gehring, C.; Cahill, D.M.; Irving, H.R.; Gehring, C. Plant natriuretic peptide active site determination and effects on cGMP and cell volume regulation. *Funct. Plant Biol.* **2007**, *34*, 645–653. [CrossRef]
45. Neill, S.J.; Desikan, R.; Clarke, A.; Hancock, J.T. Nitric Oxide Is a Novel Component of Abscisic Acid Signaling in Stomatal Guard Cells. *Plant Physiol.* **2002**, *128*, 13–16. [CrossRef]
46. Jiao, C.; Gu, Z. Cyclic GMP mediates abscisic acid-stimulated isoflavone synthesis in soybean sprouts. *Food Chem.* **2019**, *275*, 439–445. [CrossRef]
47. Isner, J.-C.; Maathuis, F.J.M. cGMP signalling in plants: From enigma to main stream. *Funct. Plant Biol.* **2018**, *45*, 93–101. [CrossRef]
48. Zhao, Y.; Qi, Z.; Berkowitz, G.A. Teaching an Old Hormone New Tricks: Cytosolic Ca^{2+} Elevation Involvement in Plant Brassinosteroid Signal Transduction Cascades. *Plant Physiol.* **2013**, *163*, 555–565. [CrossRef]
49. Pagnussat, G.C.; Lanteri, M.L.; Lamattina, L. Nitric Oxide and Cyclic GMP Are Messengers in the Indole Acetic Acid-Induced Adventitious Rooting Process. *Plant Physiol.* **2003**, *132*, 1241–1248. [CrossRef]
50. Jiang, J.; Fan, L.W.; Wu, W.H. Evidences for involvement of endogenous cAMP in Arabidopsis defense responses to Verticillium toxins. *Cell Res.* **2005**, *15*, 585–592. [CrossRef]
51. Lu, M.; Zhang, Y.; Tang, S.; Pan, J.; Yu, Y.; Han, J.; Li, Y.; Du, X.; Nan, Z.; Sun, Q. AtCNGC2 is involved in jasmonic acid-induced calcium mobilization. *J. Exp. Bot.* **2016**, *67*, 809–819. [CrossRef]
52. Świeżawska, B.; Duszyn, M.; Kwiatkowski, M.; Jaworski, K.; Pawełek, A.; Szmidt-Jaworska, A. Brachypodium distachyon triphosphate tunnel metalloenzyme 3 is both a triphosphatase and an adenylyl cyclase upregulated by mechanical wounding. *FEBS Lett.* **2020**, *594*, 1101–1111. [CrossRef]
53. Pu, C.-H.; Lin, S.-K.; Chuang, W.-C.; Shyu, T.-H. Modified QuEChERS method for 24 plant growth regulators in grapes using LC-MS/MS. *J. Food Drug Anal.* **2018**, *26*, 637–648. [CrossRef]

54. Van Damme, T.; Blancquaert, D.; Couturon, P.; Van Der Straeten, D.; Sandra, P.; Lynen, F. Wounding stress causes rapid increase in concentration of the naturally occurring 2′,3′-isomers of cyclic guanosine- and cyclic adenosine monophosphate (cGMP and cAMP) in plant tissues. *Phytochemistry* **2014**, *103*, 59–66. [CrossRef]
55. Pfaffl, M.W. Relative expression software tool (REST(C)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **2002**, *30*, 36e. [CrossRef]

Supplementary Methods

Table S1. Sequence of the primers employed for PCR amplification in this study. The table includes oligonucleotide sequences employed for cloning (lower case letters indicate the SalI and NotI restriction sites, respectively) and RT-qPCR.

	gene symbol	**primers sequence 5'->3'**	
1	*Bd*GUCD1 (for cloning)	F	ATTCCCGGgtcgacTATGGTGCTCAGGACGCTAG
		R	AGTCACGATgcggccgcTTACAGAGAGCTGTCTGGAAGTTTGC
2	*Bd*PepR2 (for RT-qPCR)	F	CGTGTACAGCTACGGAGTCG
		R	GCAGAATCCACAGGCATCTT
probe #128 (cat. no. 04693647001)			
3	*Bd*GUCD1 (for RT-qPCR)	F	GACATCGATAGAGTGGACGAACT
		R	GCACTGATGGATCTGCATTG
probe #151 (cat. no. 04694376001)			
4	*Bd*ACT1 (for RT-qPCR)	F	TGCTCCTCCTGAAAGGAAGT
		R	ACTCAGCCTTTGCAATCCAC
probe #120 (cat. no. 04693540001)			

Table S2. The qPCR efficiency values for the genes analyzed.

	Gene	**Efficiency**
1	***Bd*PepR2**	1,983
2	***Bd*GUCD1**	1,977
3	***Bd*ACT1**	2,000

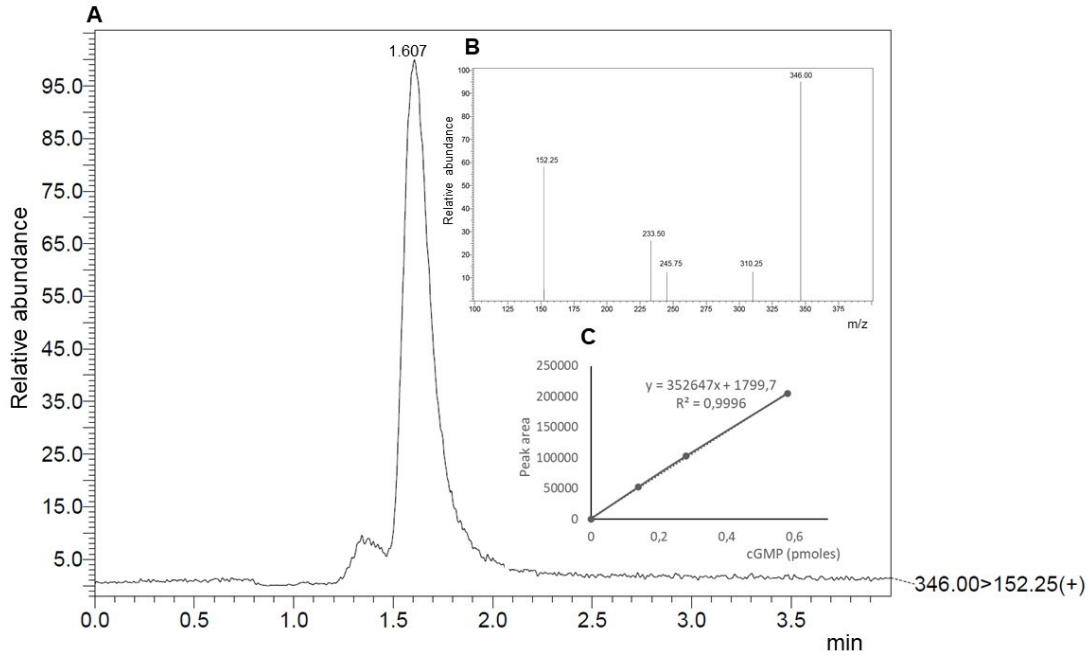


Figure S1. Detection of cGMP generated by BdGUCD1 by LC-MS/MS. (A) Determination of GC level by LC-MS/MS. Ion chromatogram of cGMP was generated from a reaction mixture containing 5 µg of purified protein (B) Inset showing parent cGMP ion at m/z 346.00 [M + H]+ and corresponding fragmented daughter ion at m/z 125.25 [M + H]+. Fragmented product ion was used for quantitation. (C) Inset showing the cGMP calibration curve performed with 0–0.58 pmoles of pure cGMP on the column.

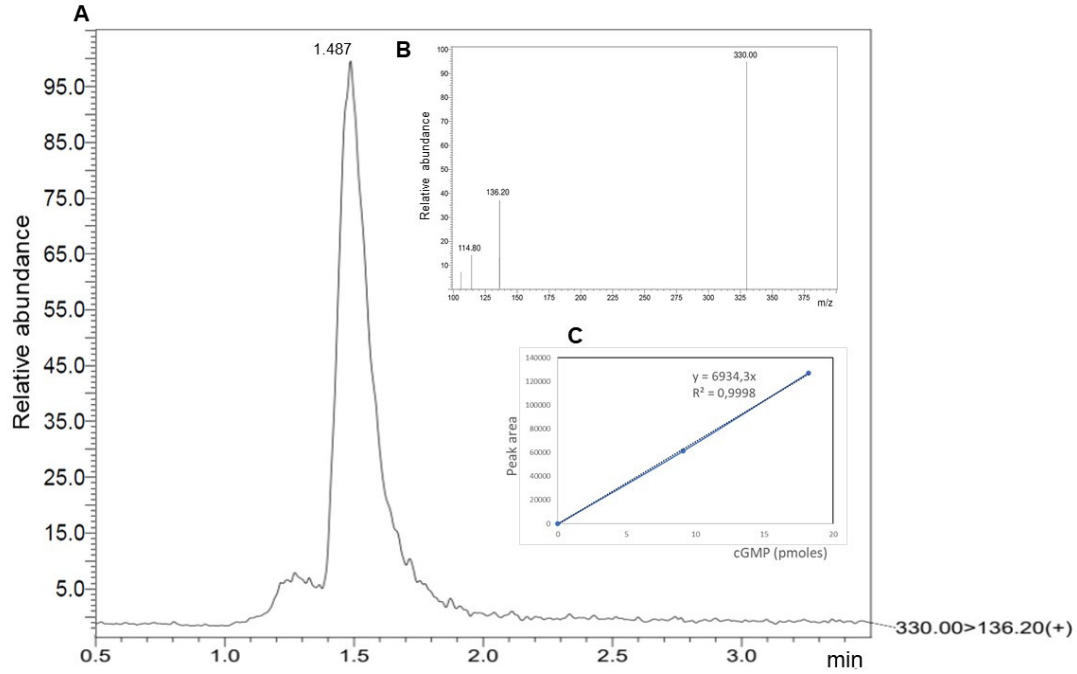


Figure S2. Detection of cAMP generated by BdGUCD1 by LC-MS/MS. (A) Determination of AC level by LC-MS/MS. Ion chromatogram of cAMP was generated from a reaction mixture containing 5 µg of purified protein or (B) Inset showing parent cAMP ion at m/z 330.00 [M + H]+ and corresponding fragmented daughter ion at m/z 136.20 [M + H]+. Fragmented product ion was used for quantitation. (C) Inset showing the cGMP calibration curve performed with 0–18.22 pmoles of pure cAMP on the column.

Oświadczenia współautorów publikacji

Toruń, 10.05.2022

Prof. Adriana Szmidt-Jaworska
Katedra Fizjologii Roślin i Biotechnologii
Wydział Nauk Biologicznych i Weterynaryjnych
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczam, że mój udział w realizacji pracy:

Duszyn, M.; Świeżawska-Boniecka, B.; Wong, A.; Jaworski, K.; **Szmidt-Jaworska, A.** "*In Vitro* Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach" Int. J. Mol. Sci. 2021, 22, 6243. (Artykuł I) polegał na współtworzeniu koncepcji pracy, analizie danych i korekcie manuskryptu pod względem merytorycznym i edytorskim. Swój udział oceniam na 10%.

Duszyn, M.; Świeżawska-Boniecka, B.; Skorupa, M.; Jaworski, K.; **Szmidt-Jaworska, A.** „BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate" Int. J. Mol. Sci. 2022, 23, 2674. (Artykuł II) polegał na współtworzeniu koncepcji pracy, analizie danych i korekcie manuskryptu pod względem merytorycznym i edytorskim. Swój udział oceniam na 10%.

Adriana Szmidt-Jaworska

prof. dr hab. Adriana Szmidt-Jaworska

Uniwersytet Mikołaja Kopernika w Toruniu
Wydział Nauk Biologicznych i Weterynaryjnych
Katedra Fizjologii Roślin i Biotechnologii
ul. Lwowska 1, 87-100 Toruń
tel. 56 611 44 55
(2289)

Toruń, 12.05.2022

dr Brygida Świeżawska-Boniecka
Katedra Fizjologii Roślin i Biotechnologii
Wydział Nauk Biologicznych i Weterynaryjnych
Uniwersytet Mikołaja Kopernika w Toruniu

OŚWIADCZENIE

Oświadczam, że mój udział w realizacji pracy:

Duszyn, M.; **Świeżawska-Boniecka, B.**; Wong, A.; Jaworski, K.; Szmidt-Jaworska, A. *In Vitro* Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach. Int. J. Mol. Sci. 2021, 22, 6243. (Artykuł I) polegał na współtworzeniu koncepcji pracy, udziale w realizacji doświadczeń, analizie i interpretacji otrzymanych wyników i korekcie manuskryptu pod względem merytorycznym. Swój udział oceniam na 20%.

Duszyn, M.; **Świeżawska-Boniecka, B.**; Skorupa, M.; Jaworski, K.; Szmidt-Jaworska, A. BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate. Int. J. Mol. Sci. 2022, 23, 2674. (Artykuł II) polegał na analizie otrzymanych wyników i korekcie manuskryptu pod względem merytorycznym. Swój udział oceniam na 10%.

Brygida Świeżawska-Boniecka

Aloysius Wong
Assistant Professor of Biology
College of Science and Technology
Wenzhou-Kean University
Room B419, General Education Hall
88 Daxue Road, Ouhai, Wenzhou
Zhejiang Province, China 325060
Email alwong@kean.edu; aloysiusw@wku.edu.cn
Office +86-577-5587-0494
Mobile +86-138-5771-3894 (China); +6011-1074-0588 (Malaysia)

温州肯恩大学
WENZHOU-KEAN UNIVERSITY

Wenzhou, Zhejiang May 9, 2022

To whom it may concern,

I hereby declare that my contribution to the article “In Vitro Characterization of Guanylyl Cyclase BdPepR2 from Brachypodium distachyon Identified through a Motif-Based Approach. Duszyn et al., *Int. J. Mol. Sci.* 2021, 22(12), 6243” was 10% (writing-review and editing).

Sincerely,

Aloysius Wong, PhD

Toruń, 10.05.2022

dr Monika Skorupa
Katedra Fizjologii Roślin i Biotechnologii
Wydział Nauk Biologicznych i Weterynaryjnych
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczam, że mój udział w realizacji pracy:

Duszyn, M.; Świeżawska-Boniecka, B.; **Skorupa, M.**; Jaworski, K.; Szmidt-Jaworska, A. „BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate" Int. J. Mol. Sci. 2022, 23, 2674. (Artykuł II) polegał na przeprowadzeniu eksperymentu, analizie i interpretacji wyników. Swój udział oceniam na 10 %.

Monika Skorupa

.....

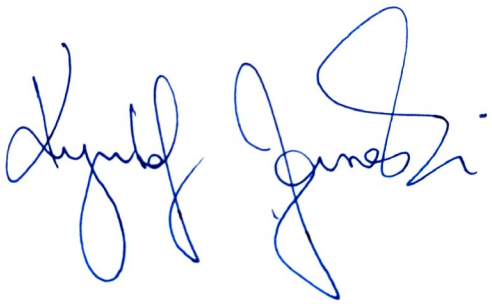
Toruń, 11.05.2022

dr hab. Krzysztof Jaworski, prof. UMK
Katedra Fizjologii Roślin i Biotechnologii
Wydział Nauk Biologicznych i Weterynaryjnych
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczam, że mój udział w realizacji pracy:

Duszyn, M.; Świeżawska-Boniecka, B.; Wong, A.; Jaworski, K.; Szmidt-Jaworska, A. "*In Vitro* Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach" Int. J. Mol. Sci. 2021, 22, 6243. (Artykuł I) polegał na współtworzeniu koncepcji pracy, analizie danych i korekcie manuskryptu pod względem merytorycznym i edytorskim. Swój udział oceniam na 10%.

Duszyn, M.; Świeżawska-Boniecka, B.; Skorupa, M.; Jaworski, K.; Szmidt-Jaworska, A. „BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate" Int. J. Mol. Sci. 2022, 23, 2674. (Artykuł II) polegał na analizie danych, współtworzeniu manuskryptu i korekcie pod względem merytorycznym i edytorskim. Swój udział oceniam na 5%.



Toruń, 10.05.2022

mgr Maria Duszyn
Katedra Fizjologii Roślin i Biotechnologii
Wydział Nauk Biologicznych i Weterynaryjnych
Uniwersytet Mikołaja Kopernika w Toruniu

Oświadczam, że mój udział w realizacji pracy:

Duszyn, M.; Świeżawska-Boniecka, B.; Wong, A.; Jaworski, K.; Szmidt-Jaworska, A. *In Vitro* Characterization of Guanylyl Cyclase BdPepR2 from *Brachypodium distachyon* Identified through a Motif-Based Approach. Int. J. Mol. Sci. 2021, 22, 6243. (Artykuł I) polegał na przygotowaniu koncepcji pracy, udziale w realizacji doświadczeń, analizie i interpretacji otrzymanych wyników i przygotowaniu manuskryptu. Swój udział oceniam na 50%.

Duszyn, M.; Świeżawska-Boniecka, B.; Skorupa, M.; Jaworski, K.; Szmidt-Jaworska, A. BdGUCD1 and Cyclic GMP Are Required for Responses of *Brachypodium distachyon* to *Fusarium pseudograminearum* in the Mechanism Involving Jasmonate. Int. J. Mol. Sci. 2022, 23, 2674. (Artykuł II) polegał na przygotowaniu koncepcji pracy, udziale w realizacji doświadczeń, analizie i interpretacji otrzymanych wyników i przygotowaniu manuskryptu. Swój udział oceniam na 65%.

Maria Duszyn