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Katedra ochrany rostlin

Virus zakrslosti pšenice – metody pro navození rezistence obilnin, experimentální přenos a detekce v rostlině

doktorská disertační práce

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Úvod

Geminiviridae patří mezi nejdůležitější čeledi zahrnující původce hospodářsky významných virových chorob mnoha plodin včetně obilnin a zelenin. Zástupci z jednotlivých rodů této čeledi, zejména z rodu *Mastrevirus* u obilnin a *Begomovirus* u zelenin, představují patogeny s epidemickým výskytem způsobující vysoké výnosové ztráty pěstovaných plodin dosahující až 100 %. Významným patogenem obilnin z rodu *Mastrevirus* je v České republice především virus zakrslosti pšenice (*Wheat dwarf virus* – WDV), jenž lokálně může způsobit také až 100% ztráty. Disertační práce pojednává o současném stavu poznání tohoto viru, možném přístupu navození rezistence či tolerance vůči tomuto viru a možnostmi molekulárně biologických metod při přenosu viru na rostlinu či jeho následné detekci.

1 Literární přehled

1.1 Historické záznamy

První historický záznam o projevu rostlinného viru, přímo viru z čeledi Geminiviridae, je pravděpodobně ve sbírce japonské poezie datované do roku 752 n. l. Báseň, připisovaná japonské císařovně Koken, pojednává o podzimním žlutavém vzhledu rostlin během léta. Rostliny byly později identifikovány jako sadce (*Eupatorium*) (Saunders a kol. 2003), jež při napadení rostlinnými viry z čeledi Geminiviridae vykazují zmiňované nápadné symptomy. První vědecky doložené zmínky o projevech rostlinných virů z čeledi Geminiviridae jsou v záznamech amerického ministerstva zemědělství o výskytu vrcholové kadeřavosti řepy (beet curly top disease) v letech 1888-1899 (Bennett 1971) a popis proužkovitosti kukuřice (maize streak disease) Claude Fullerem v roce 1901 (Fuller 1901) způsobené příslušnými geminiviry.

1.2 Význam a ztráty způsobované rostlinnými viry z čeledi Geminiviridae

Rostlinné viry z čeledi Geminiviridae v současnosti působí velké ztráty na výnosech mnoha hospodářsky významných plodin (viz také Tabulka 1). Nárůst chorob způsobených touto skupinou rostlinných virů je zapříčiněn pravděpodobně souhrou více faktorů jako přenos pomocí hmyzích přenašečů, vůči nimž je poměrně obtížná ochrana porostu, nárůst odolnosti hmyzu vůči používaným insekticidním přípravkům, samotná expanze a migrace hmyzích přenašečů do dalších oblastí, možné klimatické změny vedoucí k teplejším zimám a delšímu přežívání samotných hmyzích přenašečů v porostu, rozšiřování zemědělsky využívaných ploch

a jejich vznik v nových oblastech, transport infikovaného rostlinného materiálu do nových oblastí, či v neposlední řadě možná rekombinace různých geminivirů při současném napadení rostlin (Vanderschuren a kol. 2007).

Viry z čeledi Geminiviridae v současnosti způsobují velké ztráty zemědělské produkce a při klimatických výkyvech a rostoucích ekonomických tlacích přispívají významnou měrou k destabilizaci regionů silně vázaných na postiženou plodinu. Devastující pandemie viru mozaiky kasavy (*Cassava mosaic virus*) ve východní a střední Africe v devadesátých letech 20. století znemožnila mnoha farmářům vypěstovat úrodu jejich tradičního zdroje obživy – kasavy (Strange a kol. 2005). Významné ztráty způsobuje virus žluté svinutky rajčete (*Tomato yellow leaf curl virus*) na rajčatech (téměř 100% ztráty úrody např. v Nikaragui (Rojas a kol. 2000)), virus zlaté mozaiky fazolu (*Bean golden mosaic virus*) na fazolích, virus kadeřavosti bavlníku (*Cotton leaf curl virus*) na bavlníku či virus proužkovitosti kukuřice (*Maize streak virus* – MSV) na obilninách, zejména kukuřici.

Tabulka 1. Hospodářsky závažné choroby způsobované viry z čeledi Geminiviridae (Vanderschuren a kol. 2007).

Choroba	Rod původce	Hostitelská plodina	Epidemická oblast	Ztráty na úrodě	Reference
proužkovitost kukuřice	*Mastrevirus*	kukuřice	sub-saharská Afrika	průměrně 20 %, až 100 %	Bosque-Perez (2000)
mozaika kasavy	*Begomovirus*	kasava	Afrika, Indie	celkově 15–24 %, až 90 %	Legg a kol. (2004), Patil a kol. (2005)
kadeřavost bavlníku	*Begomovirus*	bavlna	Pákistán	průměrně 30 %, až 80 %	Briddon a kol. (2000)
zlatá mozaika fazolu	*Begomovirus*	fazole	Florida, Střední a Jižní Amerika	10 % − 100 %	Blair a kol. (1995), Faria a kol. (1999)
žlutá mozaika	*Begomovirus*	luštěniny	Indie	10 % − 90 %	Varma a kol. (2003), Malathi a kol. (2005), Rouhibakhsh a kol. (2005)
žlutá svinutka rajčete	*Begomovirus*	rajčata	Evropa, Asie, Amerika, Austrálie	20 % − 80 %, až 100 %	Moffat (1999), Moriones a kol. (2000)

1.2.1 Virus zakrslosti pšenice ve světě a v ČR

V letech 1902, 1912, 1916 a 1918 byly ve Švédsku zaznamenány ztráty na obilninách způsobené chorobou nazývanou "slidsjuka" (Nilsson-Ehle 1918, Arenö 1999). Silně napadena byla především pšenice, kdy mnoho rostlin odumřelo. Henning (1918) a Tullgren (1918) popisovali infikované rostliny jako silněji odnožující, trpící zakrslostí a deformacemi, květenství zůstávalo úplně nebo částečně v naduřelé listové pochvě. Další epidemický výskyt ve Švédsku byl zaznamenán v roce 1942. Příčina tohoto onemocnění zůstává neznámá, nicméně

popsané symptomy silně připomínají zakrslost pšenice způsobovanou virem zakrslosti pšenice. Samotný virus zakrslosti pšenice byl poprvé popsán a charakterizován na konci padesátých let v tehdejším Československu (Vacke 1961) při zjišťování příčin vyšších ztrát na ozimé pšenici po předešlé zimě. Od té doby je virus postupně detekován v různých zemích střední a východní Evropy. V západní Evropě byly popsány epidemie výskytu tohoto viru až po roce 1989, kdy

Tabulka 2. První zaznamenané (publikované) výskyty viru zakrslosti pšenice v jednotlivých evropských a mimoevropských zemích.

Země	Rok	Reference	Země	Rok	Reference
Československo	1961	Vacke (1961)	Tunisko	2000	Najar a kol. (2000)
Rusko	1965	Pridanceva (1965)	Turecko	2003	Ilbagi a kol. (2003)
Švédsko	1970	Lindsten a kol. (1970)	Zambie	2004	Kapooria a kol. (2004)
Ukrajina	1975	Razvyazkina (1975)	Čína	2007	Xie a kol. (2007)
Bulharsko	1981	Stephanov a kol. (1981)	Sýrie	2011	Ekzayez a kol. (2011)
Maďarsko	1988	Gáborjányi a kol. (1988)	Írán	2011	Behjatnia a kol. (2011)
Francie	1990	Couzin a kol. (1990), della Giustina a kol. (1991), Lapierre a kol. (1991), Bendahmane a kol. (1995)			
Německo	1990	Huth a kol. (1993), Huth a kol. (1994)			
Itálie	1994	Conti (1994)			
Rumunsko	1995	Jilaveanu a kol. (1995)			
Polsko	2001	Jezewska (2001)			
Finsko	2005	Lemmetty a kol. (2005)			
Španělsko	2006	Achon a kol. (2006)			
Rakousko	2012	Habekuß a kol. (2012), Huss a kol. (2013)			
Spojené království	2012*	Schubert a kol. (2014)			

byl detekován a popsán poprvé ve Francii jako původce zcela nové choroby (Couzin a kol. 1990, della Giustina a kol. 1991, Lapierre a kol. 1991), a v Německu (Huth a kol. 1993, Huth a kol. 1994). Po roce 2000 byly zaznamenány jeho výskyty i v dalekých neevropských zemích (viz také Tabulka 2). Virová zakrslost pšenice působí závažné, lokálně až 100% (Sirlová a kol. 2005), ztráty na obilninách. Dobře dokumentované jsou jeho epidemie na polích obilnin např. ve Švédsku v devadesátých letech 20. století (Lindblad a kol. 2002b).

1.3 Přenos viru, seznam hostitelů, životní cyklus hmyzího přenašeče

1.3.1 Přenos geminivirů hmyzími přenašeči

Geminiviry jsou rostlinné viry obecně přenášené hmyzími přenašeči. Jednotlivé rody virů čeledi Geminiviridae jsou často vázány na hmyzí vektory z konkrétní čeledi. Viry z rodu *Mastrevirus*, kam patří i virus zakrslosti pšenice, jsou přenášeny vybranými druhy hmyzu z čeledi křískovitých (Cicadellidae). Některé viry z rodu *Mastrevirus* jsou přenášené i více zástupci křískovitých, např. virus proužkovitosti kukuřice (Storey 1928), u jiných virů je znám pouze jeden druh hmyzího přenašeče. U viru zakrslosti pšenice je všeobecně znám pouze přenos křískem polním (*Psammotettix alienus* Dahlbom 1850), ale Ekzayez a kol. (2011) uvádějí přenos detekovaný i jiným zástupcem křískovitých (*Psammotettix provincialis* Ribaut 1925). Virus je obecně přenášen cirkulativním, nepropagativním způsobem (Lindsten a kol. 1980, Lindsten a kol. 1991), předpokládá se ale i necirkulativní nepropagativní způsob přenosu (Wang a kol. 2014). V rostlině je pak přítomen v listech, kořenech (Lindsten a kol. 1980), stéble. Není přenosný dále osivem (Vacke 1961) ani mechanicky šťávou z infikovaných rostlin (Vacke 1961).

1.3.2 Hostitelské rostliny viru zakrslosti pšenice

Virus zakrslosti pšenice je rozlišován ve dvou kmenech, pšeničném (WDV-W) a ječném (WDV-B), mimo jiné také podle jejich preference hostitele, tzn. především pšenice seté (*Triticum aestivum L.*) respektive ječmenu setého (*Hordeum vulgare L.*), v hospodářském porostu (Lindsten a kol. 1991, Commandeur a kol. 1999, Koklu a kol. 2007). Pro oba dva kmeny byly potvrzeny společné rostlinné hostitele jak mezi hospodářsky významnými, tak mezi planými druhy z čeledi lipnicovitých (Poaceae): oves setý (*Avena sativa* L.), chundelka metlice (*Apera spica-venti* L.), oves hluchý (*Avena fatua* L.), lipnice roční (*Poa annua* L.), poháňka hřebenitá (*Cynosurus cristatus* L.), zaječí ocásek vejčitý (*Lagurus ovatus* L.). Nicméně ohledně pšenice a ječmene u daných kmenů pravděpodobně panuje vysoká míra

preference: Lindsten a kol. (1991) nezaznamenali žádný úspěšný přenos ječného kmene na pšenici, Commandeur a kol. (1999) detekovali ječný kmen v porostu pšenice, Mehner (2005) detekoval oba kmeny v infikovaném hospodářském porostu ječmene, Kundu a kol. (2009) detekovali pšeničný kmen i na ječmeni, Schubert a kol. (2007) detekovali pouze jednu z 328 symptomatických rostlin pšenice jako skutečně infikovanou ječným kmenem a naopak žádnou ze 113 symptomatických rostlin ječmene infikovanou pšeničným kmenem. Podobně Tobias a kol. (2011) detekovali ječný kmen pouze u jedné rostliny ječmene z cca dvouročního sledování porostu v Maďarsku a na Ukrajině. Při umělém přenosu pomocí agroinokulace infekčním klonem ječného kmenu se rostliny pšenice podařilo infikovat (Ramsell a kol. 2009). Wang a kol. (2008) detekovali v zasaženém porostu ječmene i pšeničný kmen, zasažené rostliny ovšem vykazovaly extrémní zakrslost. Občasně je tedy patrně možné detekovat ječný kmen i na rostlinách pšenice, podobně pšeničný kmen na ječmeni (Ramsell a kol. 2009), ovšem pravděpodobně velmi sporadicky. Tabulka 3 udává souhrnný seznam známých hostitelů viru zakrslosti pšenice s dohledanými informacemi o projevujících se příznacích nakažení.

Tabulka 3. Seznam známých hostitelů viru zakrslosti pšenice (Vacke 1972, Lindsten a kol. 1991, Arenö 1999, Mehner 2005, Kundu a kol. 2012-2015, Nygren a kol. 2015, Ripl a kol. 2015).

Hostitelské druhy vykazující příznaky	
oves setý (*Avena sativa* L.)	jílek vytrvalý (*Lolium perenne* L.)
oves hřebílkatý *(Avena strigosa* Schreb.)	jílek oddálený (*Lolium remotum* Schrk.)
Aegilops comosa Sibth. & Smith	jílek mámivý (*Lolium temulentum* L.)
mnohoštět válcovitý (*Aegilops cylindrical* Host)	lipnice roční (*Poa annua* L.)
Aegilops juvenalis Eig	žito seté (*Secale cereale* L.)
Aegilops mutica Boiss., *Amblyopyrum muticum* Boiss.	triticale (*Triticosecale rimpaui* Wittm. ex A. Camus)
Aegilops searsii Boiss.	pšenice setá (*Triticum aestivum* L.)
Aegilops sharonensis Eig	*Triticum boeticum* Boiss.
Aegilops speltoides Tausch	*Triticum dicoccoides* Koern. ex Schweinf.
mnohoštět Tauschův (*Aegilops tauschii* Coss.)	pšenice naduřelá dvouzrnka (*Triticum dicoccon* Schrank, *Triticum turgidum* subsp. *dicoccon* Schrank)

Aegilops triuncialis L.	pšenice naduřelá tvrdá (*Triticum durum* Desf., *Triticum turgidum* subsp. *durum* Desf.)
Aegilops umbellulata Zhuk.	pšenice jednozrnka (*Triticum monococcum* L.)
sveřep stoklasa *(Bromus secalinus* L.)	pšenice setá špalda (*Triticum spelta* L., *Triticum aestivum* subsp. *spelta* L.)
ječmen setý (*Hordeum vulgare* L.)	pšenice Timofejevova (*Triticum araraticum* Jakubz., *Triticum timopheevii* Zhuk.)
zaječí ocásek vejčitý (*Lagurus ovatus* L.)	*Triticum urartu* Thumanian ex Gandilyan
jílek mnohokvětý *(Lolium multiflorum* Lam.)	

Hostitelské druhy bez viditelných příznaků

sveřep bezbranný *(Bromus inermis* Leyss.)	chrastice rákosovitá (*Phalaris arundinacea* L.)
sveřep měkký (*Bromus hordeaceus* L., *Bromus mollis* L.)	lipnice luční (*Poa pratensis* L.)
srha laločnatá (*Dactylis glomerata* L.)	bér italský (*Setaria italica* L.)
ježatka kuří noha (*Echinochloa crus-galli* L.)	bér zelený (*Setaria viridis* L.)
pýr plazivý (*Elymus repens L.*, *Elytrigia repens* L.)	kukuřice setá (*Zea mays* L.)
kostřava luční (*Festuca pratensis* Huds.)	bojínek luční (*Phleum pratense* L.)

Další hostitelské druhy bez uvedení přítomnosti příznaků

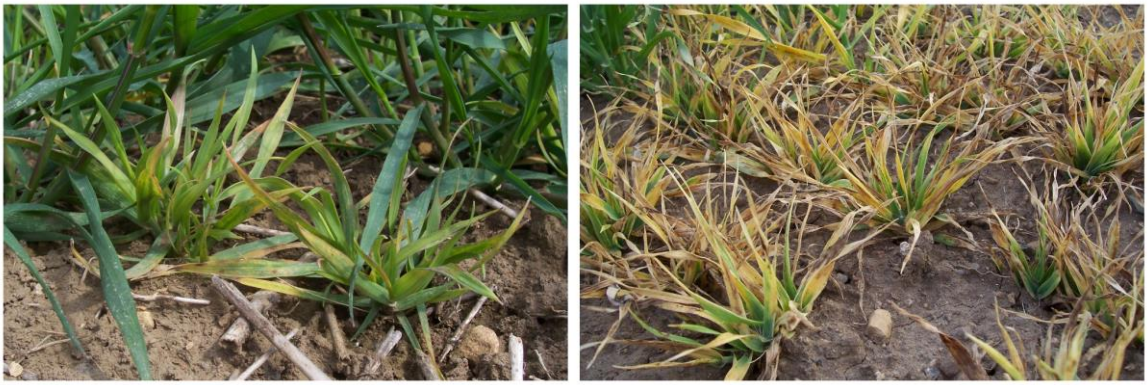
psineček obecný (*Agrostis* capillaris L.)	sveřep japonský (*Bromus japonicus* Thunb.)
psárka luční (*Alopecurus pratensis* L.)	sveřep jalový (*Bromus sterilis* L.)
chundelka metlice (*Apera spica-venti* L.)	sveřep střešní (*Bromus tectorum* L.)
oves hluchý (*Avena fatua* L., *Avena hybrida* Peterm.)	poháňka hřebenitá (*Cynosurus cristatus* L.)
oves jalový (*Avena sterilis* L.)	metlice trsnatá (*Deschampsia cespitosa* L.)
ovsík vyvýšený (*Arrhenatherum elatius* L.)	kostřava červená (*Festuca rubra* L.)

sveřep rolní (*Bromus arvensis* L.)	ječmen myší (*Hordeum murinum* L.)
sveřep luční (*Bromus commutatus* Schrad.)	

1.3.3 Projevy napadení virem zakrslosti pšenice

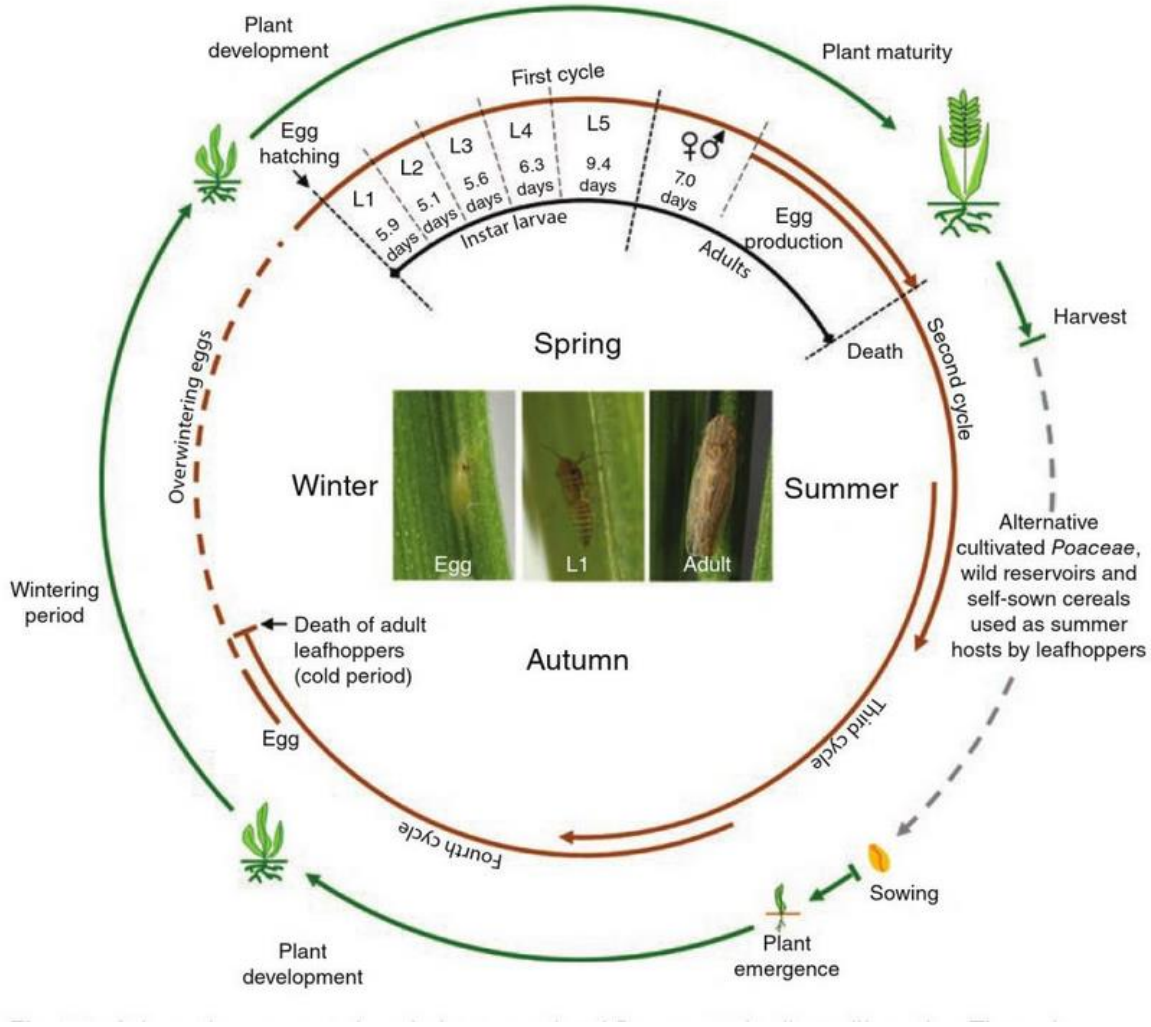
Mezi příznaky napadení patří především zakrslost (viz také Obrázek 1). Další příznaky jako žloutnutí, skvrnitost či proužkovitost listů, opožděné stádium metání či výrazné vadnutí se nevyskytují vždy, mohou se projevovat v závislosti na druhu či odrůdě (Vacke 1972, Ripl a kol. 2008). Ozimé oslabené rostliny často nepřežijí studené období zimy. Vacke (1972) udává, že mladé rostliny pšenice jsou výrazně náchylnější než dospělé rostliny. U rostlin pšenice, které chorobě podlehly, došlo k zastavení vývoje ve stadiu odnožování, v deštivých chladnějších periodách docházelo k jejich mírné regeneraci růstem nových odnoží následované jejich odumřením do fáze DC30 podle Zadoksovy makrofenologické stupnice pro obilniny (konec odnožování). Lindblad a kol. (2004) uvádějí příklad infekcí odrůdy pšenice, kde konstatují prudké snížení vnímavosti na inokulace virem po proběhnutí fáze DC30 Zadoksovy stupnice. Ripl a kol. (2008) dodávají, že nakažené rostliny z podzimního období, které občas překlenuly i tuto fázi, usychaly i vymetané v různém stupni zakrsnutí až ve stejné době jako rostliny zdravé.

Obrázek 1. Náchylné kultivary pšenice (vlevo) a ječmene (vpravo) s výraznými symptomy napadení virem zakrslosti pšenice (Cejnar a kol. 2019) – zakrslostí, žloutnutím, uvadáním.



1.3.4 Životní cyklus kříska polního

Obrázek 2. Schématická reprezentace životního cyklu kříska polního a ozimých obilnin (Abt a kol. 2015a). Vnější kruh zobrazuje hlavní stádia hospodářského cyklu ozimých obilnin. Vnitřní kruh zobrazuje potenciálně až 4 generace kříska polního. Za optimálních laboratorních podmínek (20 °C, 70-95% relativní vzdušná vlhkost, 18 h denní světlo) je délka životního cyklu kříska polního od vajíčka po úhyn dospělce 71 dní (Guglielmino a kol. 1997). Vajíčka nakladená na podzim přežívají na obilninách zimu a vyvíjejí se následující jaro. Pět larválních stádií (L1-L5) trvá podle (Manurung a kol. 2005) průměrně 5,9 (L1), 5,1 (L2), 5,6 (L3), 6,3 (L4) respektive 9,4 (L5) dnů. 7denní dospělci již jsou schopni se pářit. Křísek se během příznivých období vyskytuje v hospodářském porostu, nicméně během léta využívá také další divoké hostitele či obilné výdroly.



Životní cyklus kříska polního kulminuje typicky dvakrát do roka, na podzim při již zasetých ozimých odrůdách a na jaře, což přispívá k velkému rozšíření nemoci především v zemích s osevem jarního i ozimého hospodářského porostu, tzn. zejména v mírném klimatickém pásmu, kam spadají všichni nejvýznamnější producenti pšenice a ječmene. Podrobně byl cyklus studován např. v pracích Guglielmino a kol. (1997), Manurung a kol. (2005), Ripl a kol. (2008).

Cyklus (viz také Obrázek 2) začíná na podzim nakladením vajíček do mezofylu listů obilnin. Množství nakladených vajíček je ovlivněno mnoha parametry, včetně teploty či druhu

hostitelské rostliny. Samotné přezimování probíhá ve stádiu vajíček. Embryonální vývoj ve vajíčkách pak začíná se vzrůstem okolní denní teploty a prodloužením délky dne. Jako minimální práh vývoje larvy ve vajíčku se považuje 8 °C (Lindblad a kol. 2002a), viz také Ripl a kol. (2008). Vývoj ve vajíčkách trvá za optimálních laboratorních podmínek (20 °C, 70-95% relativní vzdušná vlhkost, 18 h světlo přes den) 18 dní a končí vylíhnutím nymfy. Nymfa postupně prochází pěti vývojovými stádii a za optimálních podmínek dokončí vývoj během 32 dní. Poslední vývojové stádium křiska polního, vývin samčích a samičích dospělců, v přírodních podmínkách nastává na začátku léta. U křiska polního nedochází k asexuální reprodukci, jak je tomu např. u mšic. Oplodnění a kladení prvních vajíček je viditelné nejdříve po 7 dnech stádia dospělce. Souhrnně, za optimálních podmínek celý životní cyklus od vajíčka k vajíčku může proběhnout během 58 dní, nicméně délka života dospělců je značně variabilní, od několika dnů po cca 10 týdnů (Ripl a kol. 2008). Z několika studií zabývajících se populační dynamikou křiska polního vychází, že hustota hmyzu může dosáhnout až 43 dospělců/m^2 (Manurung a kol. 2005) a poměr samčích a samičích jedinců se velmi blíží k 1. Klimatické podmínky v teplejších oblastech mírného pásma (Francie) umožňují během roku dokončení až čtyř generací křísků polních, zatímco podmínky ve studenějších oblastech (severní Evropa, severozápadní Čína) umožňují pouze dvě generace za rok (Schiemenz 1969). V podmínkách ČR pak dochází k vývoji tří generací, i když třetí generace je většinou jen částečná (Malenovský 2006). Larvy a dospělce lze najít na mladém hospodářském porostu na jaře a v létě na dozrávající úrodě. Dospělce je možné také najít na rostoucím ozimém porostu na podzim. Biologická aktivita dospělců se snižuje s klesajícími teplotami, při průměrné teplotě 15 °C je možné detekovat vysoký výskyt křísků polních, s teplotami pod 10 °C jejich pohyb v porostu téměř ustává (Lindblad a kol. 2002a), dospělci pak hynou s příchodem studeného zimního počasí. Dvoudenní teploty pod minus 5 °C křísky rovněž zahubí (Busch 2008).

Křísku polnímu vyhovuje obecně teplé a suché počasí. Chladné počasí v časném létě má negativní vliv na vývoj nymf, což se může projevit snížením populační hustoty následujících generací a přetrvat i do dalšího roku. Naopak teplý podzim prodlužuje dobu kladení vajíček a následný rozvoj populace v dalším roce. Dospělci i různá larvální stadia jsou schopna běžně přenášet virus zakrslosti pšenice (Lindsten a kol. 1991, Abt a kol. 2015b), u nymf byla prokázána o něco vyšší efektivita přenosu viru než u dospělců (Ghodoum Parizipour a kol. 2018). Uvedené faktory znatelně ovlivňují dynamiku šíření choroby. I s nízkou hustotou výskytu jedinců křiska polního se pak virus zakrslosti pšenice může rozšířit na mnoho rostlin, díky jeho přenosu larválními stádii a množství jimi navštívených rostlin. Pro celkové hospodářské ztráty na úrodě má velký význam, zda porost ozimých plodin byl infikován již na

podzim nebo ne (Roos a kol. 2011). Obecně je nicméně vhodná doba pro rozšíření infekce virem zakrslosti pšenice naštěstí poměrně úzká, což může být příčinou pouze sporadických výskytů velkých epidemií zamoření. Na podzim, kdy je křísek ještě aktivní a může přenášet virus do nově osetého porostu, je omezen klesající denní teplotou a na jaře, kde se v ozimém porostu šíří choroba zejména pomocí nymf, musí být teplota dostatečná pro jejich vývoj, avšak rostliny obilnin musí být ještě dostatečně mladé, než dospějí do rezistentnějších stádií vývoje.

Zajímavá studie chování kříska polního v porostu se současným výskytem mšic ukázala, že přítomnost mšic působí úhyn larev kříska polního (Alla a kol. 2001b) a pravděpodobně jako jejich obrannou reakci způsobuje vyšší počet jimi navštívených rostlin (Alla a kol. 2001a). Toto antagonistické chování mezi křískem polním a mšicemi (přenašeči žluté zakrslosti ječmene, *Barley yellow dwarf virus* – BYDV), dvěma druhy hmyzu běžně se vyskytujícím v porostu obilnin, tak přispívá k většímu rozšíření WDV v porostech silněji vystaveným i riziku inokulace viru žluté zakrslosti ječmene, dalším významným virovým patogenem obilnin v ČR a Evropě.

1.4 Molekulární podstata WDV

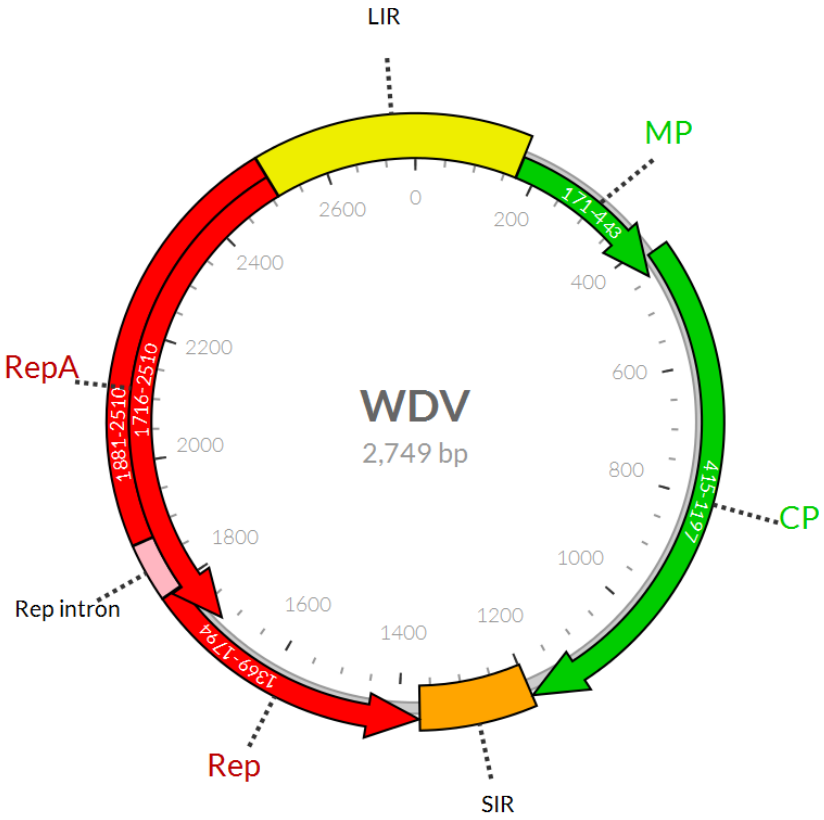
1.4.1 Molekulární podstata a možný původ geminivirů

Název celé čeledi je odvozeni od charakteristických (dvoj)kapsid kvasi-ikosahedrálního tvaru nesoucí jednovláknitou virovou DNA (ssDNA) s genomem okolo 2500-3000 párů bází. Někteří zástupci čeledi Geminiviridae mají genom cca dvojnásobné velikosti, ovšem rozdělený do dvou oddělených částí. Někdy jsou také doprovázené velmi různorodými cirkulárními ssDNA satelity, jež hrají klíčovou roli v dalším rozvoji choroby a ukazují na složitější provázanost geminivirů s ostatními rostlinnými viry.

Podle jedné z hypotéz mohly geminiviry vzniknout rekombinací plasmidu phytoplasmy, parazitické bakterie bez buněčné stěny, obsahující gen pro podporu replikace, s RNA rostlinným virem s genem pro vhodný obalový protein (Krupovic a kol. 2009).

Při replikaci geminiviry plně využívají rostlinné DNA polymerázy hostitele pro replikaci v rostlinných buňkách, které obsahují i mechanismy pro dodatečnou kontrolu a opravu chyb na rozdíl od RNA-dependentních RNA polymeráz replikujících RNA viry, nicméně studie zabývající se rozmanitostí různých rostlinných DNA virů nepotvrdily jejich nižší rozmanitost (Garcia-Arenal a kol. 2001). Proč tomu tak je zatím zůstává stále neobjasněno (viz také práce Martin a kol. (2011)).

Obrázek 3. Schéma organizace genomu viru zakrslosti pšenice. Nukleotidy číslované podle izolátu viru zakrslosti pšenice pšeničného kmene vyskytujícího se na území ČR (NCBI accession number FJ546188). MP – pohybový protein, CP – obalový protein, Rep – replikační protein, Rep A – replikační asociační protein, LIR – dlouhý mezigenový úsek obsahující replikační počátek, SIR – krátký mezigenový úsek.



Malý genom geminivirů vynucený pravděpodobně nutností šířit se do okolních rostlinných buněk i skrz plasmodesmata a nutnost zapojení replikačních enzymů rostlinných buněk vedou u geminivirů k silné manipulaci s replikačním cyklem rostlinných buněk a ovlivnění jejich metabolismu. Jako možný pozůstatek takovýchto násilných interakcí byla zaznamenána i přítomnost pozůstatků DNA sekvencí geminivirů v genomu jejich rostlinných hostitelů (Bejarano a kol. 1996, Lefeuvre a kol. 2011).

1.4.2 Fyzikálně chemické charakteristiky částic WDV

Viriony viru zakrslosti pšenice jsou částice s kapsidami oválného tvaru délky cca 30 nm a průměru cca 18 nm bez výrazného kapsomerového uspořádání patrného u mnoha ostatních geminivirů. Při extrakci se tvoří jedna až dvě sedimentující komponenty se sedimentačním koeficientem 70 S a případně 50 S. Druhá komponenta patrně souvisí s rozpadem dvojkapsid na samostatné kapsidy. Podíl absorbance A260/A280 vychází cca 1,3-1,4. Nukleová kyselina tvoří cca 20 % složení virionů (Lindsten 1991).

1.4.3 Uspořádání genomu mastrevirů

Viry rodu *Mastrevirus*, včetně viru zakrslosti pšenice, mají ssDNA uloženu v rámci jedné cirkulární komponenty velikosti 2600 až 2800 párů bází. Genom obsahuje tři geny, z nichž se exprimují čtyři různé proteiny – čtvrtý protein vzniká na základě rozdílného sestřihu příslušného transkriptu (Dekker a kol. 1991, Palmer a kol. 1998, Boulton 2002). Geny jsou rozděleny do dvou skupin, na geny kódované komplementárně k virové ssDNA (complementary-sense – C-sense) a na geny kódované ve stejné orientaci jako virová ssDNA (virion-sense – V-sense) (viz také Obrázek 3). C-sense gen pro replikační protein (replication protein – Rep) a při jiném sestřihu RNA i pro replikační asociační protein (replication associated protein – Rep A) je přepisován do RNA z vláken se stejnou orientací jako původní virová ssDNA a zmíněné geny mají kodonovou orientaci shodnou s orientací komplementárního vlákna DNA vůči původní virové ssDNA. V-sense geny pro pohybový protein (movement protein – MP) a obalový protein (coat protein – CP) jsou přepisovány do RNA z vlákna komplemetárního k původní virové DNA a mají stejnou kodonovou orientaci jako původní virové ssDNA vlákno. Genom rovněž obsahuje dva nepřepisované úseky, dlouhý mezigenový úsek (long intergenic region – LIR) a krátký mezigenový úsek (short intergenic region – SIR), jež oddělují úseky C-sense a V-sense genů, viz také práce Ramsell (2007).

1.4.4 Přenos viru zakrslosti pšenice křískem polním

Wang a kol. (2014) ukázali, že po nasátí virových částic křískem polním se virus šíří také přímo do slinných žláz, kde sice takto vydrží pouze několik hodin, ovšem umožňuje bezprostředně další šíření necirkulativním způsobem, což není u dalších mastrevirů obvyklé. Standardně se virus šíří dále do zažívacího traktu, kde obalový protein virových částic vykazuje specifickou afinitu k vnitřnostem hmyzího přenašeče. Tato afinita také determinuje i druhy příslušného přenášejícího hmyzu (Briddon a kol. 1990, Hofer a kol. 1997). Virové částice následně prostupují přes hemolymfu zpět do slinných žláz. WDV je tak křískem polním přenášen zejména cirkulativně-nepropagativním způsobem (Lindsten a kol. 1980, Lindsten a kol. 1991), není znám úspěšný přenos přes vajíčko na potomstvo (Ripl a kol. 2008).

Dinant a kol. (2004) ukázali, že V-sense geny pro obalový a pohybový protein a tedy formování nových virových částic, mají vysokou expresní aktivitu zejména ve floému, odkud je také pravděpodobně nejvyšší šance, že budou virové částice opět nasáty hmyzím přenašečem. V rámci rozdílů mezi jednotlivými mastreviry je WDV částic v infikovaných rostlinách ve floému relativně málo na rozdíl např. od MSV (Markham a kol. 1989, Lindsten 1991).

Pro samotné nasátí viru z infikované rostliny stačí křísku polnímu někdy i pouze 5-10 minut (Vacke 1961, Mehner 2005). Infikovaní křísci mohou úspěšně přenést virus na zdravou rostlinu již během 15minutového sání (Ripl a kol. 2008). Křísek polní uchovává WDV v těle a přenáší jej po dobu až 50 dní (je uváděno i až 100 dní), tzn. prakticky po celý svůj život (Mehner 2005, Ripl a kol. 2008). Všechna larvální stádia jsou také schopná přenášet virus zakrslosti pšenice, avšak efektivita přenosu WDV u jednotlivých stadií kolísá (Mehner a kol. 2003).

1.4.5 Replikace a šíření viru na molekulární úrovni

Křísek polní, jenž při inokulaci saje šťávu z rostlin, cíleně napichuje přímo floémové pletivo (Tholt a kol. 2015) a umožňuje tak vstup virových částic do bezprostřední blízkosti buněk floému, kam tyto částice i cílí (Dinant a kol. 2004, Tholt a kol. 2018), patrně kvůli snadnějšímu transportu po rostlině i kvůli potenciálnímu dalšímu přenosu hmyzím přenašečem. Lindsten (1991) ukázal, že virové částice WDV ztrácejí infektivitu, pokud jsou vystaveny působení proteáz. Jediným virovým proteinem přítomným ve virové částici blízce příbuzného MSV při infikaci rostliny je jeho obalový protein (Mullineaux a kol. 1988). U MSV je rovněž známo, že jeho obalový protein, velmi podobný obalovému proteinu WDV, má zvýšenou afinitu k DNA (Liu a kol. 1997) a také obsahuje nukleární naváděcí sekvenci (Liu a kol. 1999a), která směřuje virus buněčným transportem přímo do buněčného jádra a umožní tak začít replikaci viru a expresi virových genů.

Pro virovou replikaci pomocí rostlinných polymeráz i pro transkripci V-sense genů je důležité vytvoření dvouvláknité DNA (dsDNA). U WDV i MSV byla prokázána přítomnost cca 80nukleotidového primeru nasednutého na příslušný úsek ssDNA v oblasti krátkého mezigenového úseku, SIR, poblíž struktury možného replikačního počátku komplementárního vlákna. *In vitro* experimenty prokázaly, že iniciace a syntéza komplementárního vlákna je možná z tohoto primeru (Donson a kol. 1984, Hayes a kol. 1988b).

Protilehlý dlouhý mezigenový úsek, LIR, obsahuje potenciální replikační počátek virového vlákna. Pravděpodobný replikační počátek tvoří vlásenku invertovanými opakujícími se sekvencemi (Arguello-Astorga a kol. 1994) a vrchol této vlásenky tvoří devítinukleotidová sekvence TAATATTAC (Macdowell a kol. 1985), mezi geminiviry velmi konzervovaná (Howarth a kol. 1986). Přilehlá oblast důležitá pro replikaci pak pokrývá přibližně 300 bazí uvnitř LIR (Sanz-Burgos a kol. 1998). Ačkoliv vytváření vlásenky na daném místě nebylo experimentálně potvrzeno, u sekvence poblíž příslušného místa bylo prokázáno prostorové ohnutí (Suarez-Lopez a kol. 1995). Podobně bylo prokázáno, že samotná sekvence potenciální vlásenky je důležitá pro efektivní replikaci, protože u WDV a MSV mutantů s porušenou

sekvencí se vytratila i schopnost replikace příslušných virových konstruktů (Hofer a kol. 1992, Schneider a kol. 1992). LIR region viru zakrslosti pšenice dále také obsahuje promotory včetně TATA-boxů (Arguello-Astorga a kol. 1994), jež řídí expresi C-sense i V-sense genů, tzn. obousměrně (Morris-Krsinich a kol. 1985, Dekker a kol. 1991). Protilehlý SIR region pak obsahuje příslušné ukončovací a polyadenylové sekvence pro C-sense i V-sense transkripty (Hayes a kol. 1988b).

Protože geminiviry nekódují vlastní polymerázy, jsou zcela závislé na DNA aparátu hostitele. Pro geminiviry byly popsány dva potenciální mechanismy replikace, jednak replikace pomocí replikace kruhových plasmidů (rolling circle replication – RCR, (Saunders a kol. 1991)) a jednak případně i rekombinant-dependentní rekombinace (recombination dependent recombination – RDR;(Jeske a kol. 2001)). Replikovatelné DNA meziprodukty, charakteristické pro RCR či RDR, byly vizualizovány pro begomovirus mozaiky mračňáku (*Abutilon mosaic virus* - AbMV) na 2D gelové elektroforéze z purifikované virové DNA. Konečným produktem obou mechanismů je kompletní genomová ssDNA, připravená na enkapsidaci do nově vzniklých virových částic. Ohledně replikace DNA nicméně stojí za pozornost, že Macdonald a kol. (1988) izolovali z napadených rostlin pšenice i virovou DNA, která kromě kompletní genomové DNA WDV obsahovala i cca poloviční DNA obsahující otevřené čtecí rámce pro C-sense geny, potenciální replikační počátek ssDNA vlákna i struktury možného replikačního počátku komplementárního vlákna a místo pro nasednutí 80nukleotidového primeru v oblasti SIR ukazující na možnost tvorby další intermediátní DNA v průběhu vývoje infekce.

1.4.5.1 Replikace a úloha proteinu Rep

Pro samotnou replikaci viru je z virových proteinů potřeba pouze jeho replikační protein (Rep), který má velmi konzervovanou sekvenci v rámci celé čeledi geminivirů. Otevřený čtecí rámec tohoto C-sense genu zahrnuje intron a bez jeho sestřihu z mRNA případně vzniká replikační asociační protein (Rep A). Oba proteiny sdílejí svojí počáteční sekvenci (5'-konec mRNA), ale díky přítomnosti intronu v jejich DNA se jejich koncová sekvence liší (3'-konce transkriptů) (Schalk a kol. 1989, Dekker a kol. 1991).

Rep protein se sekvenčně specificky váže na místa v LIR regionu lokalizovaná poblíž TATA-boxů asociovaných s příslušnými C- a V-sense promotory (Missich a kol. 2000), kde vytváří vysoce afinitní komplexy (Castellano a kol. 1999). Nukleoproteinový komplex WDV Rep proteinu a DNA z oblasti LIR byl pozorován i elektronovým mikroskopem (Sanz-Burgos a kol. 1998). Rep protein se rovněž sekvenčně specificky váže v oblasti LIR na sekvenci

potenciálního replikačního počátku viru zakrslosti pšenice, kde rozpoznává příslušnou devítinukleotidovou sekvenci na potenciálním vrcholu vlásenky (Heyraud-Nitschke a kol. 1995). WDV Rep zde spolu s DNA vytváří nízko afinitní komplex a také umožňuje její rozdělení či navázání při replikaci (Castellano a kol. 1999). Štěpné místo v DNA (potenciální počátek replikace) pro protein Rep bylo experimentálně nalezeno mezi sedmým a osmým nukleotidem (TAATATT↓AC) ve zmíněné devítinukleotidové sekvenci (Heyraud-Nitschke a kol. 1995).

Část na počátku Rep proteinu (N-konci) obsahuje postupně tři konzervované motivy běžné u prokaryotických proteinů pro iniciaci replikace pomocí RCR (Koonin a kol. 1992) a také NTP vaznou doménu (Gorbalenya a kol. 1989) potřebnou pro ATPázovou aktivitu a efektivní replikaci (alespoň u monopartitního begomoviru (Desbiez a kol. 1995)). Bylo prokázáno, že WDV Rep interaguje i s komplexem pšeničného replikačního faktoru C, jenž je důležitý pro zapojení proteinů hostitele do DNA syntézy (Luque a kol. 2002). V replikačním proteinu byly rovněž identifikovány domény s pravděpodobnou vazností na další transkripční faktory (*myb*-podobné transkripční faktory, (Hofer a kol. 1992)). Navíc Liu a kol. (2014) prokázali u WDV replikačního proteinu i schopnost potlačení případné RNA interference, obranného mechanismu rostlin vůči případné vysoké transkripci některých genů. U replikačního proteinu WDV Rep byla prokázána také tvorba cca osminásobných homo-oligomerů a také hetero-oligomerů (s WDV Rep A) (Missich a kol. 2000).

1.4.5.2 Úloha proteinu Rep A

U druhého z produktů C-sense čtecího rámce, proteinu Rep A nikdy nebyla u mastrevirů prokázána jeho fyzická přítomnost v infikovaných buňkách, nicméně většina (80 %) C-sense transkriptu ve WDV či MSV infikovaných buňkách je detekována jako nesestříhaná (Dekker a kol. 1991, Wright a kol. 1997) a Rep A i Rep proteiny byly izolovány při umělé bakteriální expresi (Missich a kol. 2000).

Protože proteiny WDV Rep a WDV Rep A sdílejí svoji počáteční sekvenci, domény nacházející se v počáteční části Rep proteinu (N-konci) jsou také přítomny i na Rep A proteinu (pro přehled u obdobného MSV viz také práce Shepherd a kol. (2007b)). Na Rep A proteinu podobně jako na Rep proteinu se nacházejí tedy i tři konzervované motivy běžné u prokaryotických proteinů pro iniciaci replikace pomocí RCR (Koonin a kol. 1992) a má schopnost rozdělení či navázání DNA, nicméně Rep A protein již není schopen iniciovat replikaci (Schalk a kol. 1989, Collin a kol. 1996), pravděpodobně pro chybějící doménu pro ATPázovou aktivitu nacházející se u proteinu Rep až v jeho odlišné části, u jeho C-konce (viz

také Desbiez a kol. (1995), Shepherd a kol. (2007b)). Rovněž tak domény s pravděpodobnou vazností na *myb*-podobné transkripční faktory u proteinu Rep A chybí. WDV Rep A tvoří, podobně jako WDV Rep, nukleoproteinové komplexy v oblasti LIR lokalizované poblíž TATA-boxů asociovaných s C- a V-sense promotory. Tyto nukleoproteinové komplexy jsou mírně odlišné od komplexů tvořených pomocí proteinu WDV Rep (Missich a kol. 2000).

U WDV Rep A proteinu podobně jako u WDV Rep proteinu byla rovněž prokázána schopnost potlačení případné RNA interference (Liu a kol. 2014). V koncové části WDV Rep A proteinu odlišné od WDV Rep proteinu byl identifikován vazný motiv, jež je schopen vázat tzv. GRAB proteiny (geminivirus Rep A-binding proteins - GRAB), které mají výraznou podobnost sekvence k proteinům s NAC doménou (Xie a kol. 1999), jež ovlivňují vývoj a stárnutí u rostlin, a které, pokud jsou exprimovány, inhibují replikaci WDV DNA v suspenzní kultuře pšeničných buněk.

Hofer (1992) (viz také Collin a kol. (1996)) zmiňuje, že WDV Rep A obsahuje nukleární naváděcí sekvenci, zatímco samotný WDV Rep ne.

Rep A protein je důležitý pro expresi V-sense genů WDV. Plasmidový konstrukt WDV bez možnosti exprimovat Rep A vykazoval o 90 % menší expresi z V-sense promotoru v pšeničných protoplastech (Hofer a kol. 1992, Collin a kol. 1996). U WDV Rep A i MSV Rep A byla prokázána aktivace exprese z V-sense promotoru i v kukuřičných buňkách (Munoz-Martin a kol. 2003).

Samostatnou kapitolou je interakce proteinu Rep A s lidskými a rostlinnými proteiny podobnými retinoblastomu (retinoblastoma-related proteins - RBR) přes identifikovaný LxCxE motiv aminokyselin a jemu příslušnou pRBR doménu v sekvenci DNA. Příslušný LxCxE motiv pro interakci Rep proteinu či Rep A proteinu s RBR proteiny je poměrně konzervovaný mezi mastreviry (Xie a kol. 1995). Interakce Rep A s proteiny RBR patrně nutí buněčný cyklus rostlinných buněk přejít do stavu podobného S-fázi pravděpodobně uvolněním transkripčního faktoru E2F z RBR (Gutierrez 2000), podobně jako u lidských nebo zvířecích buněk. Buňky v tomto stavu mají aktivovaný aparát pro syntézu DNA a jsou pravděpodobně lépe schopny replikovat virovou DNA. Dvě potenciální místa pro vázání E2F transkripčního faktoru byla detekována také v promotorech umístěných v LIR regionu. Interakce mezi jedním z těchto míst a lidským E2F byla potvrzena v práci Munoz-Martin a kol. (2003), kde byla také prokázána schopnost WDV Rep A zvýšit expresi reportérového genu navázaného na minimální 35S promotor doplněný trojitým opakováním jednoho z E2F-vazných motivů. WDV Rep A ve zmíněné studii 18krát zvyšoval expresi příslušného reportérového genu, WDV Rep A s poškozeným RBR motivem pouze cca 2krát.

WDV Rep i WDV Rep A mají příslušnou pRBR doménu a Collin a kol. (1996) popisují, že WDV Rep i WDV Rep A interagují s lidskými RBR. Nicméně Gutierrez a kol. (2004) upřesňují, že s rostlinnými RBR, jež se od lidských RBR liší na N-konci (Xie a kol. 1996), interaguje pouze WDV Rep A protein a nikoliv WDV Rep protein, stejně jako i u MSV (Horvath a kol. 1998) nebo u viru žluté zakrslosti fazolu (*Bean yellow dwarf virus* – BeYDV) (Liu a kol. 1999b).

Mutace v RBR-vazném motivu silně zredukovala replikaci v kultuře pšeničných protoplastů (Xie a kol. 1995), ale stejná mutace neměla efekt na replikaci BeYDV (Liu a kol. 1999b), což ukazuje, že různé druhy mastrevirů mohou mít odlišné strategie používané při replikaci v prostředí příslušného hostitele. Rovněž sekvence celého LIR regionu, jež obsahuje sekvence důležité pro replikaci, není nijak moc konzervována mezi jednotlivými mastreviry (Arguello-Astorga a kol. 1994). Za zmínku také stojí, že při experimentech v buňkách kukuřice WDV Rep A byl schopen aktivovat transkripci z WDV V-sense promotoru nezávisle na mutacích přítomných v pRBR doméně, ovšem aktivace transkripce z MSV V-sense promotoru pomocí WDV Rep A na tyto mutace byla citlivá (Munoz-Martin a kol. 2003). Shepherd a kol. (2005) prokázali, že u MSV mutantů s RBR-vazným motivem porušeným třemi mutacemi nedochází ke snížení replikace v kultuře suspenzních buněk podobně jako i u BeYDV a MSV byl dokonce schopen vyvolat systemickou infekci u svého hostitele, kukuřice seté, byť jen s velmi mírnými projevy infekce. V populaci mutovaného viru ovšem rychle převládl kmen se spontání jednonukleotidovou mutací, jež sice nevedla k obnovení RBR-vazné aktivity, ovšem fakt, že tento selekční tlak silně preferoval příslušnou mutaci, indikuje, že daný region může mít i další funkce než pouze vazbu na RBR proteiny. Mutace RBR-vazného motivu rovněž v MSV Rep A také ovlivnila lokalizaci viru v rostlině. Mutanti bez RBR vazné aktivity nemohli proniknout do buněk mezofylu, pravděpodobně pro vysokou koncentraci RBR v dospělých buňkách mezofylu (McGivern a kol. 2005).

WDV mutanti přímo s delecí WDV Rep intronu a tedy bez možnosti exprese WDV Rep A proteinu, se stále mohou replikovat v suspenzní kultuře buněk (Schalk a kol. 1989). Navíc, taková replikace měla dokonce vyšší výtěžek virové DNA (Collin a kol. 1996). Vyšší úroveň replikace bez příslušného Rep A proteinu byla pozorována také u MSV a BeYDV v suspenzní kultuře buněk, ovšem ani jeden z těchto dvou defektních mastrevirů nebyl schopen systemicky infikovat hostitelskou rostlinu (Liu a kol. 1998, Boulton 2002). Schopnost replikace Rep A-deficientních mutantů příslušných mastrevirů v suspenzních kulturách buněk ale ne v rostlinách pak může souviset s faktem, že buňky v suspenzní kultuře se aktivně dělí a tedy

Rep A protein není potřeba pro vyvolání stavu S-fáze (Liu a kol. 1998), případně také s prokázanou transaktivací transkripce V-sense genů, zodpovědných za transport viru.

Negativní regulace či efekt Rep A na množství virové DNA v suspenzních kulturách buněk je vysvětlován buď jako o něco efektivnější regulace mírně menšího genomu (bez intronu) či efektivnější replikace pokud C-sense transkripty generují pouze Rep protein (Liu a kol. 1998), nicméně je možné, že v situaci, kdy je naplno rozběhnuta transkripce Rep proteinu a může začít replikace virové DNA ve velkém měřítku, buněčná mašinerie už nestíhá provádět sestřih mRNA pro Rep protein, začne spontánně vznikat Rep A protein, který jednak posune buněčný cyklus do S-fáze a umožní tak samotnou replikaci virové DNA a také započne či zvýší transkripci i V-sense genů a podpoří tak vznik i obalového a pohybového proteinu. Negativní regulace tvorby Rep skrze menší počet C-sense transkriptů či i např. přes nějakou formu polofunkční hetero-oligomerizace s Rep proteinem je pouze vedlejší, ale ne nutně nechtěný, efekt.

1.4.5.3 Úloha proteinů CP a MP

Experimentálně není potvrzeno, v jaké formě je DNA transportována po buňce, zda stále jako celá virová částice, či je DNA nejprve zbavena obalů a až poté navázána na jednotlivé CP proteiny. U MSV obalový protein váže nespecificky ssDNA i dsDNA *in vitro* (Liu a kol. 1997) a je schopen transportovat ssDNA i dsDNA do jádra buňky, když byl vstříknut mikroinjekcí do kukuřičných či tabákových buněk (Liu a kol. 1999a). WDV CP je rovněž nezbytný pro vyvolání systémové infekce hostitele (Woolston a kol. 1989) a pro akumulaci ssDNA (Boulton a kol. 1989b), pravděpodobně pro přípravu nových virových částic.

Pohybový protein, WDV MP, plní pohybové funkce jak uvnitř jednotlivých buněk, tak mezi nimi. U blízce podobného MSV jeho MP byl zjištěn v blízkosti sekundárních plasmodesmat (Dickinson a kol. 1996), což nepřímo ukazuje na jeho roli při transportu virové DNA či DNA-proteinového komplexu mezi buňkami. Navázaný MSV MP a zelený fluorescenční protein (green fluorescent protein – GFP) byly schopné se dohromady pohybovat mezi buňkami daleko lépe než samotný GFP (Kotlizky a kol. 2000). V jednotlivých buňkách, absence MSV MP exprese nesnižovala replikaci viru nebo tvorbu nových virových částic (Boulton a kol. 1993), i když je MSV MP také nezbytný pro systemickou infekci hostitele (Boulton a kol. 1989b). MSV MP neváže DNA, ovšem bylo prokázáno vzájemné vázání MSV CP a MSV MP proteinů (Liu a kol. 2001a, Liu 2008). Mikroinjekční experimenty potvrdily, že MSV MP, pokud je vstříknut do buněk spolu s DNA MSV a fúzí proteinů CP a GFP, nukleární akumulace ssDNA i dsDNA je potlačena (Liu a kol. 2001a). MSV MP tak pravděpodobně

odvádí CP-DNA komplex či, pravděpodobněji (Liu a kol. 2001b), virovou částici z jádra na okraj buněk, kde může být dále transportován(a) přes plasmodesmata do sousedních buněk.

1.5 Zdroje resistence či tolerance vůči WDV

Běžně pěstované komerční odrůdy jarní i ozimé pšenice a ječmene jsou často velmi či středně náchylné (Vacke a kol. 2000, Vacke a kol. 2001, Bartoš a kol. 2002, Lindblad a kol. 2002b, Sirlová a kol. 2005), lze však zaznamenat i mírně náchylné odrůdy (Ripl a kol. 2013). Experimentálních kultivarů obilnin šlechtěných na vysokou odolnost vůči WDV je v literatuře uváděno rovněž minimum ((Benkovics a kol. 2010) – pšenice, (Habekuß a kol. 2009) – ječmen). Molekulárně genetické postupy pro navození rezistence u geminivirů jsou shrnuty např. v práci Shepherd a kol. (2009). Nicméně jejich aplikace přímo u rodu *Mastrevirus* se setkala s obtížemi. Potenciální navození rezistence založené na molekulárně genetické technice RNA-interference vůči obalovému proteinu WDV bylo patrně aktivně umlčováno replikačním proteinem WDV (Liu a kol. 2014). Až experimentální transgenní kultivar ječmene založený na technice RNA-interference vůči replikačnímu proteinu WDV vykazoval očekávané příznaky rezistence (Kis a kol. 2016). V literatuře je rovněž zmiňován i kultivar ječmene s vysokým potenciálem rezistence vůči WDV založeným na genetické technice CRISPR/Cas9 (Kis a kol. 2019). Nicméně rezistence či špatná interakce s hostitelem vázaná na přesnou nukleotidovou sekvenci může být velmi rychle překonána mutacemi v interferující sekvenci viru, jak je konkrétně známo i přímo u geminivirů (Shepherd a kol. 2005, Mehta a kol. 2019).

Adekvátní obranou vůči šíření viru v rámci konvenčního zemědělství stále zatím zůstává dodržování agronomických postupů, jež se snaží minimalizovat riziko přenosu viru na základě známého životního cyklu křísků polního a podmínek vhodných pro přenos viru, či obrana proti samotnému hmyzímu přenašeči (Ripl a kol. 2008, Abt a kol. 2015a).

2 Cíle a hypotézy

2.1 Hypotéza 1 – o možnosti navození rezistence obilnin pomocí transkripce či exprese některých úseků virové DNA

Hypotéza 1: Rezistence obilnin vůči viru zakrslosti pšenice může být navozena jejich transformací geny odvozenými od viru pomocí vhodných vektorů.

Cíl práce 1: Cílem bude připravit transformace schopné vektory se zabudovanou sekvencí genu, která do obilniny přinese požadovanou genetickou informaci. Zásadní otázkou bude výběr vhodného plasmidu a metody zabudování do genomu obilniny. V případně úspěšného vložení sekvence bude ověřován vliv vložené sekvence na vývoj rostlin a stupeň jejich rezistence vůči viru zakrslosti pšenice.

2.2 Hypotéza 2 – o zhodnocení možnosti přenosu viru na rostlinu molekulárně genetickými technikami

Hypotéza 2: Studium používaných transformačních metod rostlin umožňuje získané poznatky aplikovat i při zkoumání mechanismu přenosu viru zakrslosti pšenice do rostlin pomocí molekulárně-genetických metod. Používané transformační metody mohou být pravděpodobně upraveny k zajištění přenosu viru zakrslosti pšenice do rostlin.

Cíl práce 2: Získání a ověření postupů a metod pro přenos viru zakrslosti pšenice úpravou a optimalizací existujících molekulárně-genetických metod a ověření jejich účinnosti v porovnání s přenosem viru pomocí hmyzího přenašeče.

2.3 Hypotéza 3 – o možnosti detektce viru WDV dalšími metodami

Hypotéza 3: Současné cílené techniky jsou často využívány pro stanovení rozsahu napadení rostliny po konkrétní stanovené době. Tyto techniky mohou být dále optimalizovány pro případné studium časového vývoje množství viru v rostlině. Podobně, současný stav široce zaměřených molekulárně biologických technik v sobě skrývá, po případné optimalizaci, potenciál i pro detekci rostlinných virů.

Cíl práce 3: Cílem bude zaměřit se na existující detekční techniky a pomocí nich lépe popsat šíření vybraného obilného viru v rostlině. V případě nových technik se pak pokusit pomocí vybrané techniky detekovat přítomnost rostlinných virů v napadeném rostlinném materiálu.

3 Publikované práce

Cejnar, P., L. Ohnoutková, J. Ripl, T. Vlčko a J. K. Kundu (2018): *Two mutations in the truncated Rep gene RBR domain delayed the Wheat dwarf virus infection in transgenic barley plants*. Journal of Integrative Agriculture 17(11), pp. 2492-2500 (DOI: 10.1016/S2095-3119(18)62000-3).

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RESEARCH ARTICLE

Two mutations in the truncated Rep gene RBR domain delayed the *Wheat dwarf virus* infection in transgenic barley plants

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Abstract

Wheat dwarf virus (WDV), an important cereal pathogen, is closely related to *Maize streak virus* (MSV), a model virus of the *Mastrevirus* genus. Based on its similarity to known MSV resistance strategies, a truncated part of the WDV replication-associated (RepA) gene (WDVRepA215) and the WDV RepA gene with a mutated retinoblastoma-related protein (RBR) interaction domain (WDVRepA215RBR^{mut}) were cloned into the pIPKb002 expression vector and transformed into immature embryos of spring barley *cv*. Golden Promise plants through *Agrobacterium*-mediated transformation. A detailed study of T_1-generation plants infected by leafhoppers (*Psammotettix alienus*) fed on infection sources of variable strength was performed over a 5-week period encompassing the initial stages of virus infection. A DNA WDV TaqMan qPCR assay normalized using the DNA puroindoline-b SYBR Green qPCR assay for samples on a per week basis revealed an approximately 2-week delay in WDVRepA215RBR^{mut} plants to WDVRepA215 plants before significant increases in the WDV viral levels occurred. Both WDVRepA215 and WDVRepA215RBR^{mut} plants showed similar levels of transgenic transcripts over the screened period; however, the transgenic plants also showed increased numbers of infected plants compared to the control plants.

Keywords: *Wheat dwarf virus* (WDV), truncated Rep gene, RBR domain, qPCR screening

1. Introduction

Wheat dwarf virus (WDV), belonging to the genus *Mastrevirus* of the *Geminiviridae* family, is an important viral pathogen of cereal crops worldwide (Kundu *et al*. 2009; Nygren *et al*. 2015). It was first detected in the former Czechoslovakia (Vacke 1961). WDV is transmitted by a leafhopper, *Psammotettix alienus*, in a circulative, non-propagative manner by both the larval instars and the adult leafhoppers (Lindsten and Vacke 1991). WDV is a single-stranded DNA virus with a monopartite circular genome of approximately 2.7 kb that is divided into several functional regions: a short intergenic region containing the origin of replication and sites of potential promoters for viral genes, which are grouped into two open reading frames for virus movement and coat protein; a long intergenic region; and a complementary open reading frame that produces two other virus proteins through differential splicing — replication protein (Rep) and replication-associated protein (RepA), see also in Fig. 1. The Rep protein is responsible for replicating

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the virus DNA, and RepA is responsible for enhancing the production of coat proteins and movement proteins and also for interactions with plant cell cycle mechanisms. Coat protein is the only virus protein that is present in the virus capsid and is responsible for proper circulation of the virus within the leafhopper insect vector and proper intracellular movement. The movement protein is required for proper viral cell-to-cell movement (Boulton 2002).

For geminiviruses, several different molecular engineering resistance strategies exist, but only a few are known for the *Mastrevirus* genus (Shepherd *et al*. 2009; Kis *et al*. 2016; Zaidi *et al*. 2016). For *Maize streak virus* (MSV), a successful molecular engineering resistance strategy has already been performed (Shepherd *et al*. 2007a, b) based on the expression of the truncated MSV RepA protein in *Digitaria sanguinalis* and *Zea mays* plants. In MSV Rep and MSV RepA proteins, several structural and functional domains have been identified (Gutierrez 1999; Boulton 2002), and experiments with truncated MSV RepA proteins of different lengths expressed in transgenic *D. sanguinalis* plants produced different results. These results included the inhibition of plant growth, increased susceptibility to MSV or significantly decreased susceptibility to MSV (Shepherd *et al*. 2007b). An MSV RepA protein that was truncated to maintain only the first 219 amino acids but still contained functional DNA-binding domains (Koonin and Ilyina 1992), the RBR interaction domain (Xie *et al*. 1995) or the oligomerization domain (Horvath *et al*. 1998), was produced in the transgenic plants and reduced significantly the virus titres. Subsequently, a three-nucleotide mutation in the truncated MSV RepA protein gene sequence resulted in the complete inhibition of MSV in the tested plants (Shepherd *et al*. 2007b). This inhibition activity of MSV occurred even in response to infections caused by more divergent MSV isolates, but only to a reduced extent (Shepherd *et al*. 2014). For *Wheat dwarf virus*, the domains identified in the MSV Rep and RepA proteins were also identified in WDV either directly (Koonin and Ilyina 1992; Xie *et al*. 1995) or based on amino acid sequence homology between the MSV and WDV proteins (Missich *et al*. 2000). In this paper, we performed a functional analysis of the truncated RepA gene of WDV. Our result has shown that a two-point mutation in the RBR domain is able to delay the development of disease symptoms in the barley plants. The delayed disease symptoms also corresponded to the WDV titres detected by TaqMan qPCR.

2. Materials and methods

2.1. Construction of expression plasmids

Based on the amino acid sequence homology in the group of 123 WDV isolates obtained from the GenBank database, the MSV-Kom isolate (GenBank accession number AF003952; Shepherd *et al*. 2007b), an exact counterpart of the MSV RepA truncated 219-amino acid sequence, was identified as the first 215 amino acids in the WDV RepA protein and nucleotide sequences (ClustalX 2.1 Software; Larkin *et al*. 2007). Two expression vectors, pIPKb002+WDVRepA215 and pIPKb002+WDVRepA215RBR^{mut}, for the expression of the identified truncated WDV RepA sequence and the truncated WDV RepA sequence carrying a mutation in the RBR interaction domain, responsible for decreased susceptibility of maize plants to MSV, were manufactured. The identified 645-bp nucleotide sequence was amplified from the isolated DNA (GenElute™ Plant Genomic DNA Miniprep Kit, Sigma-Aldrich, USA) from greenhouse plants inoculated with leafhoppers transmitting the wild-type Czech WDV isolate (GenBank accession number FJ546189). The primer pair used (WDVRepA215CAfw+WDVRepA215-rv, see Table 1) also contained the CACC sequence for subsequent cloning and 6 nucleotides of the 3´ untranslated region (3´UTR) of the RepA gene sequence to maintain an exact analogue of the WDV RepA Kozak consensus sequence. An amplified and purified PCR product was cloned into the pENTR D-TOPO vector (pENTR™/D-TOPO® Cloning Kit, ThermoFisher Scientific, USA) according to the manufacturer's instructions. Using the LR clonase reaction (Gateway® LR Clonase® II Enzyme mix, ThermoFisher Scientific, USA), the donor sequence was transferred into the pIPKb002 vector (Himmelbach *et al*. 2007) under the *Z. mays* ubiquitin-1 (ZmUbi-1) promoter, along with the hygromycin phosphotransferase gene (hpt) controlled by another ZmUbi-1 promoter for selection in plants.

For the WDVRepA215 product carrying the mutation in the RBR interaction domain (Shepherd *et al*. 2007b), a WDVRepA215CAfw+WDVRepA215rv PCR product was purified and cloned into the pGEM-T Easy vector (Promega, USA), and a site-directed mutagenesis using the WDVRepRBRsdm-fw+WDVRepRBRsdm-rv primer pair was performed. The mutation was confirmed by digesting with *Afl*II. The extracted DNA of the pGEM-T Easy+WDVRepA215RBR^{mut} vector was used as a template for the creation of the pIPKb002+WDVRepA215RBR^{mut} plasmid as described previously. Both final vectors, pIPKb002+WDVRepA215 and pIPKb002+WDVRepA-215RBR^{mut} (see Fig. 1), were sequenced (GATC Biotech, Germany) to confirm the target sequence.

2.2. *Agrobacterium*-mediated transformation

The transformation of the expression vector sequences into spring barley *cv*. Golden Promise cereal plants was performed as described by Cejnar *et al*. (2017) based on a

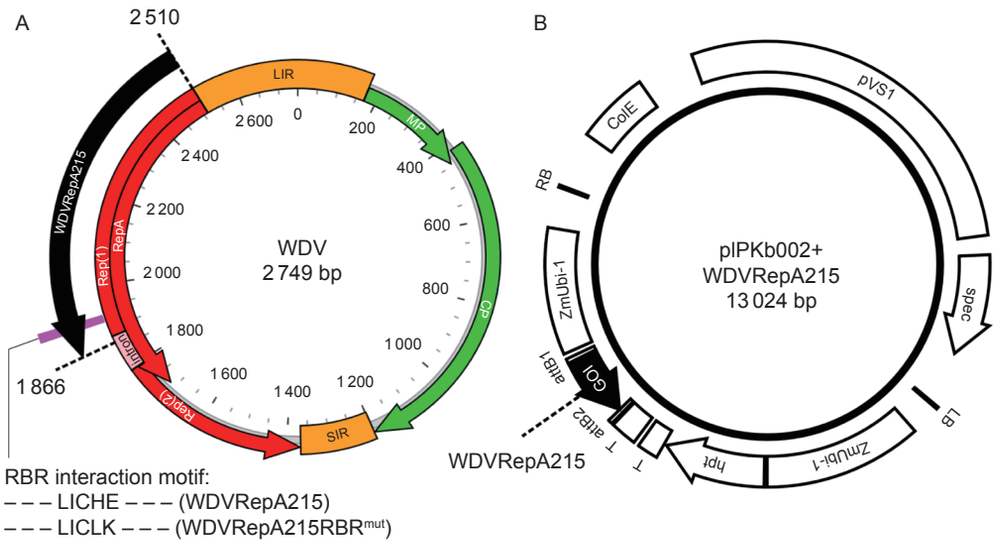

Fig. 1 *Wheat dwarf virus* (WDV) genome organization (WDV FJ546189 isolate) with the selected part of the replication-associated protein used (A) and organization of binary plasmid pIPKb002+WDVRepA215 (B). Rep, replication protein; RepA, replication-associated protein; MP, movement protein; CP, coat protein; SIR, short intergenic region; LIR, long intergenic region; RBR, retinoblastoma-related proteins interaction domain; ColE, ColE1 origin of replication; pVS1, pVS1 origin of replication; spec, streptomycin/spectinomycin adenyltransferase; LB and RB, left and right T-DNA border; ZmUbi-1, maize ubiquitin promoter followed by maize ubiquitin intron; hpt, hygromycin phosphotransferase; T, terminator; GOI, gene of interest; attB1 and attB2, LR clonase reaction sites.

Table 1 Primers and probes used in the study

Primer or probe name[1)]	Sequence (5′→3′)	Annealing temperature used (°C)
WDVRepA215fw	ATGGCCTCTTCATCTGCACC	63
WDVRepA215CAfw	CACCACTACCATGGCCTCTTCATCTGCACC	63
WDVRepA215rv	TGATTCGAGGCTTACGGAGT	63
WDVRepRBRsdm-fw	CTCATTTGCCTTAAGACCATTGAAAGCTGGAAAAATGAACATC	65
WDVRepRBRsdm-rv	TCAATGGTCTTAAGGCAAATGAGTGATTCGGTTGGAAACTCAG	65
F-Hyg/p204	GATTGCTGATCCCCATGTGT	62
R-Hyg/p204	GCTGCTCCATACAAGCCAAC	62
mRNApIPKbRepA-fw	CCCCTTCACCACTACCAT	57
mRNApIPKbRepA-rv	GTGGAGCTCTCTAACAGAAG	57
PinB-fw	TGCAAGGATTACGTGATG	59
PinB-rv	ACATTGTGGTGCTATCTG	59
UnivWDVfw	TCCGCGCTAGGACAGTCACT	60
UnivWDVrv	AAGATTGGCTCAAGGATATGACTCC	60
6FAM-WDVB-BHQ1 (TaqMan)	6-FAM-AGGCGAAGAATGATTCACCCT-BHQ-1	60

[1)] WDVRepRBRsdm-fw and WDVRepRBRsdm-rv mutated nucleotides in the site-directed mutagenesis primers are underlined.

previously published transformation protocol (Ohnoutkova *et al*. 2012). From 240 transformed embryos carrying the pIPKb002+WDVRepA215 vector, 4 independent transgenic plants were regenerated, and another 10 albino plants were obtained. Among 150 embryos transformed with the pIPKb002+WDVRepA215RBR^{mut} vector, six independent transgenic plants were regenerated, and no albino plants were obtained. DNA was isolated from the leaf tissue of the regenerated plants using the procedure reported by Edwards *et al*. (1991), and the presence of the genes of interest and the hpt selection gene were confirmed (WDVRepA215fw+WDVRepA215rv primer pair, F-Hyg/p204+R-Hyg/p204 primer pair).

2.3. Nucleic acid extraction protocols

RNA extraction from the barley plants was performed using TRIzol Reagent (TRIzol Reagent, ThermoFisher

Scientific, USA) applied on the leaf tissue ground in liquid nitrogen according to the manufacturer instructions. DNA extraction was performed using the procedure by Edwards *et al*. (1991) and applied on leaf tissue ground in liquid nitrogen. For screening experiment 2, a combined plant RNA+DNA extraction protocol was used, meaning that the TRIzol Reagent protocol for RNA extraction was followed by the DNA extraction after RNA extraction protocol (Triant and Whitehead 2009).

2.4. RT-qPCR RNA assays

For screening levels of transgenic WDVRepA215 and WDVRepA215RBR^{mut} transcripts in plants, regardless of the presence and levels of the corresponding viral transcripts, the pIPKbRepA215 AB SYBR RT-qPCR RNA assay with mRNApIPKbRepA-fw+mRNApIPKbRepA-rv primer pair was performed. The forward primer mRNApIPKbRepA-fw was designed to match the part of the 3´UTR originally included in the pIPKb002 vector, which differs in sequence from the corresponding 3´UTR in WDV Rep. The reverse primer, mRNApIPKbRepA-rv, was designed to recognize the initial part of the transcript to maintain a small 175-bp amplicon. For the normalization of the mRNA expression levels, the PINB AB SYBR RT-qPCR RNA assay with the PinB-fw+PinB-rv primer pair was performed. RT-qPCR RNA assays were performed on an Applied Biosystems 7300 Real-Time PCR System (ThermoFisher Scientific, USA) in a total volume of 12 µL containing 6 µL of reaction pre-mix (Power SYBR® Green RNA-to-CT™ 1-Step Kit, ThermoFisher Scientific, USA), 0.096 µL of RT Enzyme Mix (containing ArrayScript™ UP Reverse Transcriptase and RNase Inhibitor), 2 µL of sample, 0.4 µmol L^{-1} each primer and ddH_2O to the final volume. All the assays were performed in triplicate for the standard and tested samples. Thermal profiles, qPCR standards and detected efficiencies are shown in Table 2.

2.5. qPCR DNA assays

WDV DNA titre analysis was performed using a WDV Roche TaqMan qPCR DNA assay and was run on a Roche LightCycler® 480 Instrument II (Roche Applied Science, Germany) in a total volume of 12 µL containing 6 µL of reaction pre-mix (LightCycler® 480 Probes Master, Roche Applied Science, Germany), 2 µL of DNA sample, 0.4 µmol L^{-1} of each primer, 0.2 µmol L^{-1} of TaqMan probe and ddH_2O up to the final volume. For the normalization of the extracted DNA from the samples of different growth stages, PINB Roche SYBR qPCR DNA assay was performed. The assay was based on the detection of puroindoline-b copies (2 copies are present in wheat genome (Gautier *et al*. 2000), and 4 copies are hordoindoline-b1 and hordoindoline-b2 in barley genome (Galassi *et al*. 2012)) in extracted DNA. The assay was run on a Roche LightCycler® 480 Instrument II in a total volume of 12 µL containing 6 µL of reaction pre-mix (LightCycler® 480 SYBR Green I Master, Roche Applied Science, Germany), 2 µL of DNA sample, 0.4 µmol L^{-1} of each primer and ddH_2O up to the final volume. Thermal profiles for both qPCR DNA assays, qPCR standards and detected efficiencies are shown in Table 2. For the derivatives of the experimental and theoretical melting

Table 2 RT-qPCR RNA assays and qPCR DNA assays used in the study

Assay	pIPKbRepA215 AB SYBR RT-qPCR RNA assay	PINB AB SYBR RT-qPCR RNA assay	WDV Roche TaqMan qPCR DNA assay	PINB Roche SYBR qPCR DNA assay
Primer pair used, probe used	mRNApIPKbRepA-fw+ mRNApIPKbRepA-rv	PinB-fw+PinB-rv	UniWDVfw+UniWDVrv, TaqMan probe 6FAM-WDVB-BHQ1	PinB-fw+PinB-rv
qPCR thermal profile	48°C for 30 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 57°C for 60 s	48°C for 30 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 59°C for 60 s	95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 60 s	95°C for 10 min; 40 cycles of 95°C for 15 s and 59°C for 60 s
qPCR standard	The amplified product from the pIPKb002+WDVRepA215 template cloned into the pGEM-T Easy vector	The amplified product (using DNA extracted from wild-type barley *cv*. Golden Promise as template) was excised from gel and purified	The amplified product (using WDV FJ546193 isolate as template) was cloned into the pGEM-T Easy vector	The amplified product (using DNA extracted from wild-type barley *cv*. Golden Promise as template) was excised from gel and purified
qPCR efficiency (E=$10^{(-1/slope)}$; %)	97.31	106.77	99.49	105.32
R^2	0.9984	0.9938	0.9989	0.9981
The linear standard curve interval (copies; Taylor *et al.* 2010)	3.2×10^3–3.2×10^8	1.69×10^2–1.69×10^7	2.9×10^3–2.9×10^8	1.69×10^1–1.69×10^7

curves of PINB Roche SYBR qPCR DNA assay, see Appendix A.

2.6. Plant growth conditions and virus inoculation

All the plants were grown under controlled conditions (19/15°C, 16/8 h daylight/night, spectrum adjusted with high-pressure sodium bulbs) and sown in soil mixed with the substrate (2:1 autoclaved soil: autoclaved substrate ratio). In screening experiments 1–3, approximately twice as many seeds as required for testing were sown and allowed to grow under controlled conditions. At the 2-leaf stage (DC 12 by Zadoks, Zadoks *et al*. (1974)), approximately 5 days before inoculation, the first leaves were removed from all plants, and RNA was isolated and analysed using the pIPKbRepA215 and PINB AB SYBR RT-qPCR RNA assays. Among the plants that were positive for WDVRepA215 or WDVRepA215RBR^{mut} transcription, the plants with middle and high levels of transcription were selected. After pre-screening of the RNA of the tested plants, leafhoppers were allowed to feed on the selected WDV source of infection for 5 days prior to inoculation and then for 6 days on the tested plants (approximately DC12–13 by Zadoks). Each plant was exposed to 3 infected leafhoppers per plant.

2.7. Molecular per week screening of WDV-inoculated plants

For detailed screening of the changes in the virus titres on a molecular basis, the plants were sampled once per week, starting at the 2nd week after the start of inoculation. To reduce the damage to the plants during screening, each tested plant was sampled by cutting one leaf each week — the oldest leaf on the main shoot. This procedure allowed us to obtain samples of tissue associated with the plant phloem network, i.e., the tissue targeted by WDV (Dinant 2004), without interrupting the remaining plant phloem network of the stem and the other leaves. The screening of one leaf per plant per week allowed us to eliminate the effects of slight differences between the individual plants and differences in the progression of their infection. The controlled growing conditions for the barley plants were sufficient to produce at least one new leaf per week after approximately the 2-leaf-stage in both infected and uninfected plants.

2.8. Phenotype screening of WDV-inoculated plants

For WDV, a significant dwarfing of the WDV-infected plants is one of the main symptoms. However, when a leaf from the main shoot was cut each week for the molecular screening, the reported height of the whole plant could then be seriously affected. Here, two other parameters were measured: the height from the soil level (visible height) up to the axil of the first leaf of the main shoot and the visible height up to the highest axil of the leaf present on the whole plant. In detailed studies, the distance between these two parameters is reported.

3. Results

3.1. Evaluation of T_1 transgenic plants with WDVRepA215 and WDVRepA215RBR^{mut} transcripts shows a 2-week delay in significant increase of virus titre

Half of the tillers of the T_0 successfully regenerated transgenic WDVRepA215 and WDVRepA215RBR^{mut} barley plants from 4 different WDVRepA215 transgenic events (J1-1 to 4) and from 6 WDVRepA215RBR^{mut} transgenic events (K5-1 to 6) was moved to separate pots at the end of the tillering stage (DC 28–30 by Zadoks). To limit the potential acquired mature resistance (Lindblad and Sigvald 2004), the group was pre-screened for resistance to WDV. The leafhoppers were allowed to feed on a strong source of infection (FJ546189 and FJ546193 isolate mixture) for 5 days prior to inoculation and then for the subsequent 4 days on the inoculated plants, with approximately 15 leafhoppers per tiller group. The plants presented only mild symptoms of infection (decreased growth, more stunted and wilted leaves than in the non-inoculated control group), and their leaf tissue was sampled at 40 days following the start-of-inoculation (dpi). One tested plant died at approximately 30 dpi. The DNA was extracted and quantified by UV spectrophotometry, and the number of puroindoline-b reference gene DNA was also determined by the PINB Roche SYBR qPCR DNA assay. The WDV virus titres were detected by the WDV Roche TaqMan qPCR DNA assay and showed highly variable results, including plants with extremely high or extremely low virus titres. For the T_1 screening, only the transgenic events with extremely low virus titres were selected.

For the detailed study of WDV infection progress, an infection source of 98342 WDV/PINB (WDV isolate FJ546193) was selected. The screening experiment 1 included 3 inoculated plants from each promising transgenic event (J1-1, K5-1, K5-3), 3 inoculated wild-type plants (barley *cv*. Golden Promise) and 4 non-inoculated controls (one for each tested group, i.e., 16 tested plants in total). At the 3-leaf stage (DC13), the infected leafhoppers were allowed to feed on each plant for another 6 days. For the next 5 weeks, the plant phenotypes were screened, each week one leaf was cut to characterize the progression of WDV infection at the molecular level, and the DNA was extracted. Once every 2 weeks, the mRNA levels of WDVRepA215 or WDVRepA215RBR^{mut} were monitored

in the extracted RNA. Both the WDV titre levels and the phenotypic observations (see Fig. 2-IB and IC) confirmed that all transgenic plants were infected. However, in the inoculated control plants, only 1 of 3 control plants was infected. Significant increases in the virus titres during the screening period were visible for K5-1, K5-3 and the control plant, starting at approximately 4 weeks post-inoculation, and for J1-1 plants starting at approximately 2 weeks earlier at 2 weeks post-inoculation. The plants did not show any other significant differences after the end of screening when maintained in the controlled greenhouse (see Appendix B).

The biological replicate (screening experiment 2) from the previous study was run using a final (preselected) group of 3 inoculated plants of the T_1 J1-1 transgenic event, 3 inoculated plants of K5-1, 3 inoculated wild-type barley *cv*. Golden Promise controls and 3 barley *cv*.

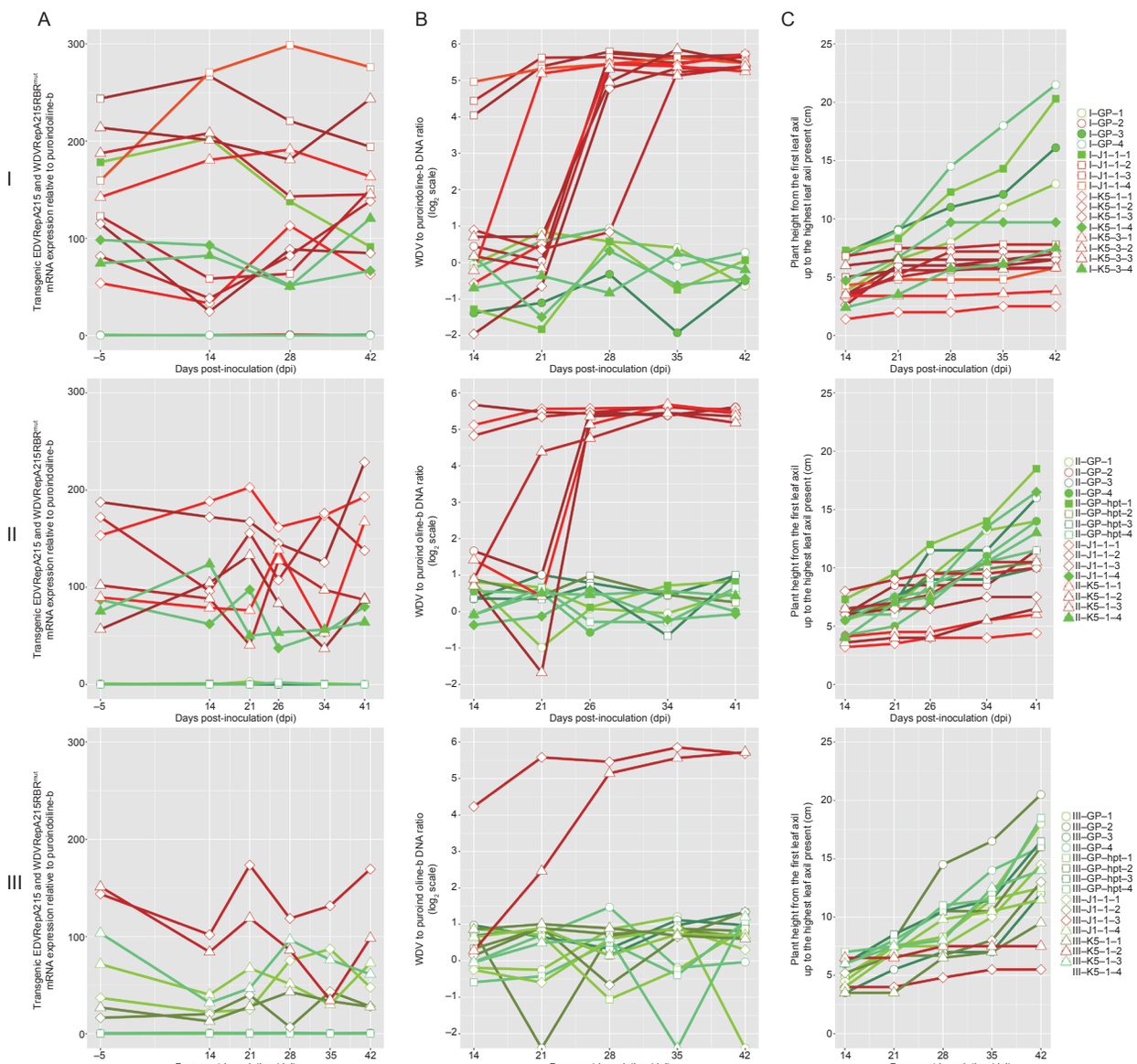

Fig. 2 Detailed screening of J1 (WDVRepA215), K5 (WDVRepA215RBRmut), GP (wild-type control) and GP-hpt (control hpt$^+$) plants in the first 6 weeks after *Wheat dwarf virus* (WDV) inoculation in screening experiments 1 (I), 2 (II) and 3 (III, extra-low strength of infection source experiment): inoculated plants (white filled shapes) and uninoculated control plants (green filled shapes); plants in which WDV was detected (red lines) or not detected (green lines) at 6 weeks after inoculation. A, mRNA levels of WDVRepA215 and WDVRepA215RBRmut transgenic transcripts as determined by the pIPKbRepA215 AB SYBR RT-qPCR RNA and PINB AB SYBR RT-qPCR RNA assays. B, WDV DNA titres detected by the WDV Roche TaqMan DNA qPCR assay normalized to the amount of DNA determined by the PINB Roche SYBR DNA qPCR assay. C, height of the plants measured from the axil of the first leaf of the main shoot up to the highest axil of the leaf present on the whole plant.

Golden Promise controls with only the hpt selection gene and without the WDVRepA215 or WDVRepA215RBR^{mut} transgene (hpt^{+} control plants, artefacts of screening for positive transformants from previous *Agrobacterium* transformations). In each group, one plant was included as an a non-inoculated control. The selected source of infection was determined to be approximately the same strength (78 315 WDV/PINB, WDV isolate FJ546180) as applied previously, and the inoculation conditions were also identical. However, during the screening in this experiment, a combined RNA+DNA extraction protocol was applied. The results are shown in Fig. 2-B and C. All the J1-1- and K5-1-inoculated plants, none of the inoculated hpt^{+} control plants and one of the wild-type barley *cv*. Golden Promise plants were infected. At the molecular level, all the J1-inoculated plants exhibited earlier significant increases in WDV titres (2 weeks post-inoculation) compared to the K5-1 plants and an infected wild-type plant (approximately 4 weeks post-inoculation). Based on the levels of mRNA transcripts in screening experiment 1 (Fig. 2-IA), the levels of mRNA transcripts in the weekly screening experiment 2 (Fig. 2-IIA) confirmed the stable levels of transgenic transcripts in both the WDV-infected and -uninfected plants.

3.2. WDVRepA215 and WDVRepA215RBR^{mut} plants are susceptible to infection even from extra-low-strength infection sources

To confirm whether the transgenic insert affected the susceptibility of the plant to WDV through susceptibility to the reduced virus inoculation dose, screening experiment 3 was performed with an extremely weak source of infection of 24 855 WDV/PINB (a wild-type plant 2 weeks after inoculation was used as a new source of infection, WDV isolate FJ546193). The final tested group of plants included 3 inoculated plants of J1-1, 4 inoculated plants of K5-1, 4 inoculated plants of barley *cv*. Golden Promise wild-type and 4 inoculated hpt^{+} control plants. There were 15 inoculated plants in total. In this experiment, no non-inoculated plants were added. The DNA and RNA from the leaf tissue were extracted, and the results of the DNA screening for the WDV, phenotype screening and the levels of RNA transcripts from the sampled period up to the 6th week post-inoculation are shown in Fig. 2 (IIIA–IIIC). With an extremely weak source of infection, one J1-1 and one K5-1 plants were infected, and no control plants (either wild-type or with antibiotic selection only) were infected. Even in this experiment, an approximately 2-week shift in the start of the detectable increase in the WDV titre was observed between J1-1- and K5-1-infected plants.

3.3. Stability of WDVRepA215, WDVRepA215RBR^{mut} and barley puroindoline-b mRNA levels during the first 6 weeks of barley plant growth

As a reference gene for testing the mRNA levels in each of the first 6 weeks of barley plant growth, the puroindoline-b gene was selected. The small number of copies present in the wheat and barley genomes decreases the possibility of many different promoters. For validation of the stability of the transcription of puroindoline-b genes in barley at different growth stages and with or without WDV infection, the data from detailed studies (screening experiments 1–3) were collected, and a two-way ANOVA was performed (number of puroindoline-b copies per ng of extracted RNA in samples) in R Software (v 3.4). The results did not show any significant dependence on the week of sampling ($Pr(>F)=0.55$), the WDV infection present ($Pr(>F)=0.37$) or both factors ($Pr(>F)=0.15$). This result allowed the use of the levels of the puroindoline-b gene transcripts as reference values for normalization of the mRNA transcript levels. We must note that, for example, the β-tubulin 6 gene (Jarosova and Kundu 2010) did not pass this two-way ANOVA test and showed a significant dependence on the week of sampling (Cejnar P, unpublished data). All the transgenic plants in screening experiments 1–3 confirmed the stable levels of transgenic transcripts in both the WDV-infected and uninfected plants (Fig. 2-IA, IIA, and IIIA).

4. Discussion

The different timing in the progression of virus infection between the transgenic plants — the plants with WDVRepA215 transcripts compared with the plants with WDVRepA215RBR^{mut} transcripts, i.e., with two-point mutations in the RBR interaction domain — was confirmed in three detailed screening experiments. The tested two-point mutation in the WDV RBR interaction domain was not lethal for WDV infection in barley, whereas the closely related MSV virus in maize, even for WDV, the conserved sequence of the RBR interaction domain, possibly played a supporting role in the mechanism of infection through its potential interaction with host RBR proteins. Even if it does not halt the infection, disruption of this mechanism is still likely detectable as a source of the delay observed in the WDV K5 plants compared with the WDV J1 plants, in which the regressed timing of infection in the K5 plants was very similar to the timing observed in the wild-type plants.

All three screening experiments also support the hypothesis that the transgenic insert of the first 215 amino acid codons of the WDV DNA sequence (FJ546189 isolate) enhance the susceptibility of the barley plant to the wild-type WDV infection, in contrast to the closely related MSV (see

Shepherd *et al*. 2007a, 2014). However, even in maize plants with their increased resistance to MSV, enhanced susceptibility was detected with shorter transgenic inserts of the MSV Rep gene. In maize, the increased susceptibility of plants translating shortened MSV Rep proteins was likely due to the DNA binding domains that are potentially responsible for enhanced replication, which could also be the cause of WDV in barley, in which these domains were also detected in the WDV sequence (Koonin and Ilyina 1992). For MSV, the corresponding resistance strategy significantly reduced the levels of tested virus isolates, with as little as 60.6% amino acid identity (Shepherd *et al*. 2014), thus validating the robustness of the amino acid substitutions. Here, all the experiments were performed with wild-type WDV isolates, which together showed at least 88% amino acid identity, and none of them showed any different symptoms or altered disease progression in the experiments. The detailed screening studies showed that the virus titres in inoculated transgenic and non-transgenic plants of the barley *cv*. Golden Promise at the 5th or 6th week post-inoculation reached very similar virus titres; thus, accumulation of the virus in the tested leaf tissues was likely not affected by either the WDVRepA215 or WDVRepA215RBR^{mut} products. The detailed studies with the lower strength infection sources showed the enhanced susceptibility of the transgenic plants to infection through the reduced levels of initial virus doses required for infection compared with the wild-type plants, thus affecting the general probability of infection of the plant. The potential mechanism of such a resistance breakdown behaviour could be either the effect of different virus-host interactions that evolved through the specialization of mastreviruses to their preferred hosts (WDV for barley, MSV for maize) or due to other mutations that occurred as a side effect of any other reaction to some other virus-host interaction evolutionary event (see also Shepherd *et al*. 2005; Guiu-Aragones *et al*. 2015; Pinel-Galzi *et al*. 2016).

5. Conclusion

The detailed screening experiments showed the progression of WDV during the first 6 weeks of infection in wild-type barley and WDVRepA215 or WDVRepA215RBR^{mut} barley plants. The significant increase in the WDV titres in response to tested two-point mutations in the tested transgene RBR domain region were delayed by approximately 2-week intervals as a probable result of the potentially disrupted infection mechanism. However, neither the transgene nor the included mutation were lethal for virus infection. The enhanced susceptibility of the transgenic plants to WDV compared with the wild-type plants was identified as having increased susceptibility to sources of low infection strength.

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Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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Wheat dwarf virus infectious clones allow to infect wheat and *Triticum monococcum* plants

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Abstract: We constructed *Wheat dwarf virus* (WDV) infectious clones in the bacterial plasmids pUC18 and pIPKb002 and tested their ability to inoculate plants using Bio-Rad Helios Gene Gun biolistic inoculation method and *Agrobacterium tumefaciens* agroinoculation method, and we then compared them with the natural inoculation method via viruliferous *P. alienus*. Infected plants were generated using both infectious clones, whereas the agroinoculation method was able to produce strong systemic infection in all three tested cultivars of wheat and *Triticum monococcum*, comparable to plants inoculated by viruliferous *P. alienus*. Infection was confirmed by DAS-ELISA, and WDV titres were quantified using qPCR. The levels of remaining bacterial plasmid DNA were also confirmed to be zero.

Keywords: WDV; *Triticum aestivum* L.; virus infectious clone; agroinoculation; biolistic inoculation; leafhopper; qPCR detection

Wheat dwarf virus (WDV), from the genus *Mastrevirus* (family *Geminiviridae*), is a pathogen affecting cereal crops that is transmitted by the leafhopper *Psammotettix alienus* (Dahlbom, 1850). It was described for the first time in former Czechoslovakia (Vacke 1961), and it has spread throughout Europe, Africa and Asia. The virus affects wheat, barley, oat, and some wild grasses (Vacke 1972; Lindsten & Vacke 1991). Infected plants are dwarfish, with many tillers, and shrunken or even lacking grains (Figure 1), which leads to a dramatically lower yield. It is one of the most dangerous cereal viral pathogens causing considerable commercial losses, especially in countries cultivating winter crops (Lindblad & Waern 2002; Širlová *et al.* 2005). WDV is transmitted by a leafhopper species, *P. alienus*, in a circulative, non-propagative manner (Lindsten *et al.* 1980; Lindsten & Vacke 1991). Two main strains are known – the wheat-adapted strain (WDV-W), affecting at least wheat, oat, rye, and some wild grasses, and the barley-adapted strain (WDV-B), affecting at least barley and oat (Lindsten & Vacke 1991). Oat and some wild grasses were identified as hosts of both strains (Lindsten & Vacke 1991; Vacke & Cibulka 1999; Ripl & Kundu 2015). There are also reports that WDV-W can infect barley and WDV-B can infect wheat in the field (Schubert *et al.* 2007; Kundu *et al.* 2009; Tobias *et al.* 2011) or using infectious clones (Ramsell *et al.* 2009).

Similar to other mastreviruses, WDV is a single-stranded DNA (ssDNA) virus with four coding proteins: replication protein (Rep), replication-associated protein (RepA), coat protein (CP), and movement protein (MP). The proteins are encoded both in a virion-sense orientation (CP, MP) in relation to ssDNA from the virion and in a complementary sense orien-

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Figure 1. Wheat (left) and barley (right) susceptible cultivars infected by *Wheat dwarf virus* in the field (Photo: J. Ripl)

tation (Rep, RepA) (Dekker *et al.* 1991) (Figure 2). The searching for cultivars with resistance to WDV is made difficult due to the complex ecology and life cycle of the virus, which is tightly bound to its natural insect vector and lacks mechanical transmissibility. No commercially available cultivars of wheat or barley resistant to this virus are known, and only a few experimental barley cultivars have been reported in the scientific literature (Habekuss *et al.* 2009; Kis *et al.* 2016). For partial resistance or tolerance to WDV, only a few cultivars are reported (Vacke & Cibulka 2000; Širlová *et al.* 2005; Benkovics *et al.* 2010). An important role may also be played by the proven natural resistance of some WDV proteins to gene-silencing mechanisms (Liu *et al.* 2014).

Many laboratory workflows for the preparation of infectious clones of WDV have been previously described (Hayes *et al.* 1988; Woolston *et al.* 1988; Bendahmane *et al.* 1995; Boulton 2008; Ramsell *et al.* 2009) together with different methodologies for age and part of the plant selected for inoculation (Hayes *et al.* 1988; Dale *et al.* 1989; Chen & Dale 1992; Ramsell *et al.* 2009), selected host cultivar (Woolston *et al.* 1988; Benkovics *et al.* 2010) or used agrobacterium strain (Marks *et al.* 1989). The different reached agroinoculation efficiencies (Dale

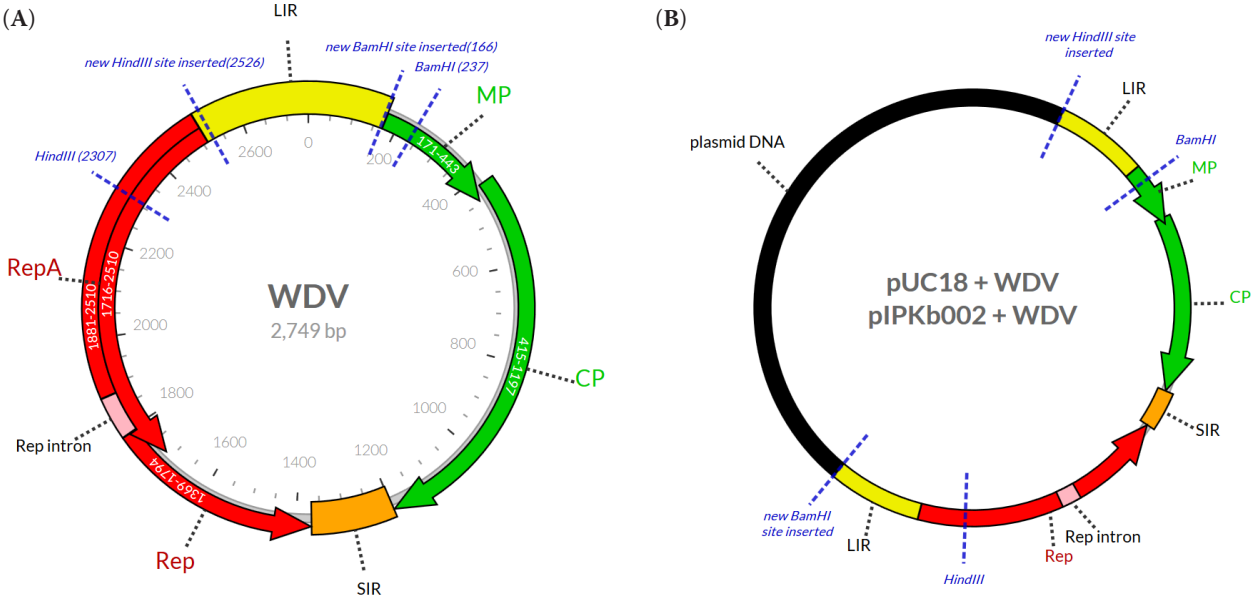

Figure 2. The structure of WDV genome and constructed WDV infectious clones: (**A**) structure and position of *Wheat dwarf virus* open reading frames and their orientation in the circular ssDNA WDV genome: LIR – long intergenic region with replication origin hairpin, MP – movement protein gene, CP – coat protein gene, RepA – replication-associated protein gene, Rep – replication protein gene, SIR – short intergenic region and (**B**) structure of pUC18 + WDV and pIPKb002 + WDV infectious clones

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et al. 1989; Bendahmane *et al.* 1995; Ramsell *et al.* 2009; Benkovics *et al.* 2010) are then also closely dependent on selected evaluation schemes for virus detection and quantification. The DNA of an infectious clone should contain a hairpin sequence of the circular DNA replication origin at the beginning and at the end, as this leads to the preferential release of viral DNA (Redinbaugh 2003; Ramsell *et al.* 2009). The infectious clone should also contain a replication origin for plasmid replication in bacteria (e.g., *E. coli*) and genes for plasmid selection. If used for agroinoculation, the plasmid must contain a replication origin targeted at replication in agrobacteria. However, a successful inoculation with an infectious clone in a prokaryotic bacterial plasmid was only achieved with the biolistic transformation of wheat (Jones & Shewry 2009) or even, with the closely related virus, *Maize streak virus* (MSV), via mechanical inoculation (Redinbaugh 2003). Following up on this finding in the literature, we constructed a WDV infectious clone in the bacterial plasmid pUC18, as well as in the plasmid pIPKb002, also containing the replication origin for replication in bacteria genera, and tested their ability to infect plants.

MATERIAL AND METHODS

Construction of infectious clones pUC18+WDV and pIPKb002+WDV. An infectious WDV clone was carefully constructed to contain a copy of a replication origin at the beginning and at the end but not overlapping the other regions outside. A 472 bp selected portion of the WDV genome of Czech wheat isolate (Kundu *et al.* 2009; WDV-W NCBI accession number FJ546188) was amplified by PCR from the beginning of circular replication origin hairpin sequence, adding a *HindIII* restriction site, following up to the existing *BamHI* restriction site (HindIIIREPcutF/WDVBamHIrv primer pair; Table 1). This product was then ligated into the target vector pUC18 (Takara Bio, Shiga, Japan) that had initially been extended with a *HindIII-KpnI* extender (a1fw/a1rw pair – Table 1), allowing for a blunt-ended digestion with the *EcoRV* restriction enzyme. The subsequent 2684 bp section of the WDV genome from the existing *BamHI* restriction site up to the end including the next copy of the circular replication origin hairpin sequence was amplified by PCR, appending a new *BamHI* restriction site to the end (WDVBamHIfw/BamHIMPcutR primer pair; Table 1). This product was then inserted into the pGEM-T Easy vector (Promega, Madison, USA), followed by redigestion with the *BamHI* restriction enzyme. This almost full copy of WDV was then inserted into the pUC18 target vector with the HindIIIREPcutF/WDVBamHIrv part from the previous reaction also being redigested with the *BamHI* restriction enzyme (Figure 2).

Table 1. Primers used for construction of WDV infectious clones derived from the WDV genome (NCBI accession number FJ546188, primers 1–4). The newly added restriction sites are underlined. Primers 5–6, a1fw, a1rv are synthesised extenders for the *HindIII-KpnI* region of pUC18 plasmid to allow blunt end cloning using *EcoRV* restriction digestion

Primer	Sequence (5'→3')
HindIIIREPcutF	AAGCTTTCCGGCAGGTCCTTAGCGAAA
WDVBamHIrv	GGATCCGGGATTGGAAGGGGTC
WDVBamHIfw	GGATCCTCCGACTACGCCTGGC
BamHIMPcutR	GGATCCTGGGCTACCACGCACTTCCT
a1fw	AGCTTCTGTTCGATATCTAGTACGGTAC
a1rv	CGTACTAGATATCGAACAGA

For the creation of an infectious clone in the pIPKb002 target vector (IPK, Gatersleben, Germany; Himmelbach *et al.* 2007), the WDV infectious clone was first constructed in the pENTR D-TOPO vector (Invitrogen, Waltham, USA) in the same way as for pUC18+WDV and then shuffled into the target vector using the LR-Clonase reaction (Invitrogen, USA). Both infectious clones were sequenced (GATC Biotech, Konstanz, Germany) to confirm their sequence and orientation.

Plant material. Experiments were performed on the *Triticum aestivum* L. winter wheat cultivars Alana and Svitava, as well as *Triticum monococcum* L. (accession No. 01C0106429), obtained from GenBank Prague-Ruzyně, Crop Research Institute, Czech Republic. The plants were grown in quantities of 4–5 plants per pot in autoclaved soil and were cultivated in a 16/8 h, 22/18°C day/night regime. On the inoculation day, the plants were kept in dark until inoculation. After inoculation, the plants were left to grow in the same conditions for the next 6 weeks. Leaf material from each tested plant was then disrupted in liquid nitrogen, and the samples were stored at –80°C until tested (up to 7 days).

WDV biolistic inoculation. The biolistic inoculation of the plants was performed using a Bio-Rad

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Helios Gene Gun (Bio-Rad Laboratories, Hercules, USA), and the optimal parameters for wheat were detected using control pIPKb002+GUS plasmid and subsequent GUS staining. Finally, 0.6 µm gold particles (Bio-Rad Laboratories, USA) with DNA of the selected infectious clone were prepared with 0.5 µg of DNA per shot and 0.5 µg of gold projectiles per shot.

Each tested plant from a given growth stage was shot twice from each bullet cartridge, two cartridges per plant. Two shots from one cartridge were targeted at the top of the leaf, and two shots from another cartridge were targeted at the base of the seedling. The shooting pressure was 150–180 psi (pounds per sq. inch, i.e. 1034–1241 kPa). All the shots were made with the diffraction screen included.

WDV agroinoculation. The selected pIPKb002+WDV clone was transferred together with the pSoup helper plasmid into *Agrobacterium tumefaciens* (AGL1 strain). The stock culture was cultivated in LB medium + streptomycin (50 µg/l) + rifampicin (50 µg/l) + tetracycline (5 µg/l), at 28°C with shaking at 150 rpm, for 40–48 h, and then centrifuged for 3 min (10°C, 1 200 *g*); the supernatant was removed from the pellet, and 1 ml of LB was added. The culture was centrifuged again for 2 min (10°C, 1 200 *g*), and then the pellet was diluted in 3 ml of sterile distilled water. Approximately 5-day-old plants were injected with a Hamilton 1801 RN 10 µl syringe (Hamilton Company, Reno, USA), 3 times at the base of the seedling (10 µl), twice vertically and one time horizontally, as described in Boulton (2008). The plants were growing in 25/25°C regime for the next two days and then switched to the regular 22/18°C regime.

WDV inoculation by Psammotettix alienus. The plants were inoculated at Zadoks stage DC12 or DC13 (Zadoks *et al.* 1974). Five days before inoculation, leafhoppers were left feeding on the wheat plants of cv. Ludwig infected with WDV-W (NCBI accession No. FJ546188) with significantly developed WDV infection. On the inoculation day, 10 leafhoppers were moved to each pot and left there for the next 5 days. Then, the leafhoppers were removed, and the plants were left to grow for the following 6 weeks.

Quantification of WDV titres. Plant DNA was isolated by adding 0.5 ml of extraction buffer (1 M guanidine thiocyanate, 20 mM Na_2H_2EDTA, 0.1 M MOPS, pH 4.6, 0.2% mercaptoethanol) to 50–100 mg of sampled tissue that had been disrupted and homogenised in liquid nitrogen. The solution was incubated for 30 min in a 60°C water bath with occasional vortexing followed by phenol-chloroform-isoamyl alcohol extraction (25 : 24 : 1; Affymetrix, Santa Clara, USA), chloroform extraction, isopropanol, and sodium acetate precipitation and two-step 70% ethanol purification. The qPCR for WDV detection was run on a 7300 Real-Time PCR System (Applied Biosystems by ThermoFisher Scientific, USA) using 6 µl of the Applied Biosystems™ PowerSYBR Green RNA-to-C_T™ 1-Step Kit without the reverse-transcriptase component, the U2WDV-fw/U2WDV-rv primer pair (in final concentration of 0.4 µM; Table 2), and 1 µl of tested sample and then filled with distilled deionised water up to a 12 µl reaction volume. The temperature parameters were set to 95°C for 10 min, 40 cycles of 95°C for15 s, 65°C for 1 min and, then, for the evaluation of a dissociation curve, 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 seconds. For all samples, the mean detected WDV concentration was calculated based on the testing of triplicate samples and subsequently normalised using the DNA sample concentration detected spectrophotometrically.

The amount of potentially remaining infectious clone plasmid vector DNA alone was quantified using forward and reverse primers (spec3RT-fw/spec3RT-rv; Table 2) targeted at a 178 bp amplicon in the spectinomycin/streptomycin resistance gene in pIPKb002 plasmid or using forward and reverse primers (betalac2-fw/betalac2-rv; Table 2) targeted at a 117 bp amplicon of the beta-lactamase gene in pUC18 or pGEM-T Easy plasmid. All other conditions were the same as for qPCR for WDV detection.

Table 2. Primers used for the detection of WDV and bacterial plasmids by qPCR

Primer	Sequence (5'→3')	Origin of the sequence
U2WDV-fw	CAGAGCCGAAACAGGCAAT	WDV coat protein gene
U2WDV-rv	GGTTCACGGTCCACTTCCTT	WDV coat protein gene
spec3RT-fw	GCAGTAACCGGCAAAATCGC	pIPKb001 spectinomycin/streptomycin resistance gene
spec3RT-rv	CGCCTTTCACGTAGTGGACA	pIPKb001 spectinomycin/streptomycin resistance gene
betalac2-fw	GCAACTTTATCCGCCTCCATC	pUC18 and pGEM-T Easy beta-lactamase gene
betalac2-rv	TGACACCACGATGCCTGTAG	pUC18 and pGEM-T Easy beta-lactamase gene

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As a qPCR standard, pGEM-T Easy plasmid was used with all detected sequences included (U2WDV-fw/U2WDV-rv primer pair amplicon inserted and ligated directly into the pGEM-T Easy plasmid and the spec3RT-fw/spec3RT-rv primer pair amplicon inserted into the plasmid *Sal I* restriction digestion site with primers containing the *Sal I* restriction digestion sequence GTCGAC; for the betalac2-fw/betalac2-rv differential qPCR, the amplicon is already included in the beta-lactamase gene sequence).

The efficiency factor of qPCR was 99.61% or better for U2WDV primer pair and spec3RT primer pair using the qPCR double standard, and the efficiency factor of qPCR for the betalac2 primer pair was 98.91%. qPCR was able to correctly detect the number of copies at least in the range of 6.68×10^1–6.68×10^8. All the samples with WDV infection detected by qPCR were also tested by DAS-ELISA (rabbit polyclonal antibodies to WDV, alkaline phosphatase-conjugated antibodies; SEDIAG, Bretenière, France) for the presence of viral proteins according to the manufacturer's instructions.

RESULTS

WDV infectious clone constructs. Two WDV-W infectious clones (pUC18+WDV and pIPKb002+WDV) for initiating WDV infection in plants were constructed. Both infectious clones contain the 1.1 length of WDV genomic sequence starting from the long intergenic region with the replication origin hairpin, going through all viral open reading frames up to the next added copy of the long intergenic region, with a terminating replication origin hairpin. The pUC18+WDV infectious clone also contains the *Escherichia coli* origin of replication pMB1 (ColE1 and pBR322) and the *E. coli* beta-lactamase/ampicillin resistance gene taken from the original pUC18 plasmid. The pIPKb002+WDV infectious clone also contained an *E. coli* ColE1 origin of replication, *Pseudomonas* pVS1 origin of replication for replication in other bacteria genera such as *Agrobacterium tumefaciens*, hygromycin phosphotransferase under the maize ubiquitin promoter for conferring hygromycin resistance in plants and streptomycin/spectinomycin adenyltransferase for streptomycin/spectinomycin resistance, where all added genes originated from pIPKb002 plasmid. For construction details see Materials and Methods and Figure 2.

Efficacy of WDV infectious clone using different inoculation methods. Four hundred sixty-one plants were agroinoculated in 11 experiments to allow for the optimisation of agroinoculation parameters and the testing of the agroinoculation method. No significant differences were detected when *Agrobacterium* for the inoculum was cultivated on the plate or in solution with all necessary antibiotics, only with the antibiotic for the agroinoculation plasmid (streptomycin) or completely without antibiotics. Using all of these different protocols, plants with WDV infection were detected by both qPCR and by ELISA in only 1–3 cases out of 25–35 tested samples, and another 1–4 WDV infected plants were detected only by qPCR. For wheat cv. Svitava and *Triticum monococcum,* 3 ELISA and qPCR positive cases out of 25 were reached, and for cv. Alana, only 1 ELISA and qPCR WDV infection out of 25 and 1 ELISA and qPCR WDV infection out of 35 inoculated plants were detected. This ratio was not improved even when agrobacterium was induced in induction buffer (acetosyringone 150 mM, 10 mM $MgCl_2$ in sterile distilled water) for 4 h in dark at room temperature just before the inoculation. If the plants were inoculated later than at Zadoks DC12 (approx. two weeks old), the inoculation did not lead to any positive results, and neither did the agroinoculation of young plants where the injection of the inoculation solution was replaced by the submersion of leaves into the inoculation solution with leaf tips injured with a razor blade (both 0 out of 16 tested plants). The visual control of agroinoculated plants does not allow clear discrimination of positively infected plants. Leaf yellowing, mottling or stunting of plants were often caused only by the agroinoculation treatment protocol. While dwarfing was detectable, the infected plants were higher than some wilting agrobacterium-treated controls.

Two hundred nine wheat plants were inoculated by biolistic inoculation in 15 different experiments with infectious clones placed in pUC18 or pIPKb002 plasmids. During the optimisation of the biolistic inoculation method using GUS staining, an optimal pressure for the shot was detected as 150–200 psi with the smallest golden particles (0.6 μm) and the youngest plants possible (Zadoks stage DC10–11). Shot particles with a larger diameter (1.0 μm) caused visible damage to target tissues. It was necessary to fix the Helios Gene Gun device in an additional rack to ensure that the target part of the plant was hit. We also tried to test older plants; however, with plants older than 7 days (Zadoks stage DC12–14), no positive results were obtained (0/16, pUC18 + WDV

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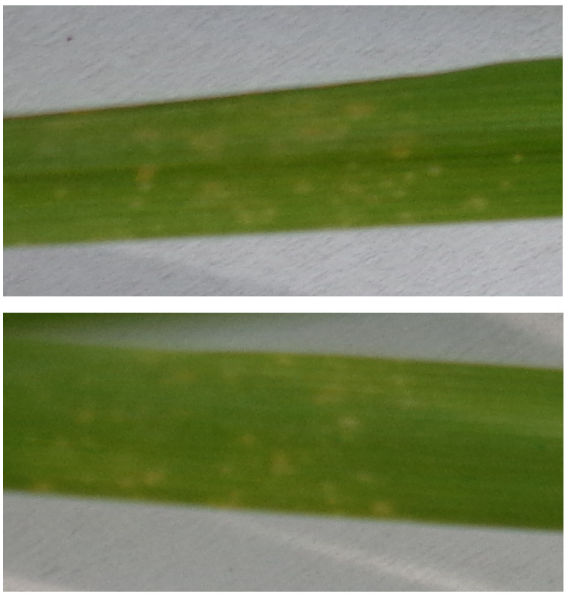

Figure 3. Yellowing spots appearing 2 days after successful inoculation of *Triticum aestivum* cv. Alana, 180 psi helium shot pressure, 0.6 µm gold particles (0.5 µg/shot), and 0.5 µg of WDV infectious clone DNA per shot

for 10, 14, and 21 days old plants). Using the biolistic inoculation method, we were able to get ELISA and qPCR-positive plants for both plasmids tested (for pUC18+WDV 1 positive case per 12 inoculated plants and 2 positive cases per 35 inoculated plants, for pIPKb002 1 positive case per 35 inoculated plants). In all infected plants, the amount of remaining DNA was quantified as at most 275 copies/ng of extracted DNA, i.e., several orders of magnitude less than the detected amounts of WDV DNA. Plants successfully inoculated with 0.6 µm gold particles showed yellowing spots in the shot area several days after inoculation in comparison with treated controls (Figure 3). However, for 1.0 µm or larger gold particles very tiny spots also appeared for treated controls.

Table 3. The best results obtained for different inoculation methods. The number of WDV infected plants out of all tested plants and the efficiency are depicted for the experiment with the highest reached efficiency

Method	Winter wheat cv.		*Triticum monococcum*
	Svitava	Alana	
Inoculation by *Psammotettix alienus*	10/10 (100%)	10/10 (100%)	10/10 (100%)
Agroinoculation	3/25 (12%)	1/25 (4%)	3/25 (12%)
Biolistic inoculation	1/12 (8.33%)	1/12 (8.33%)	0/35 (0%)

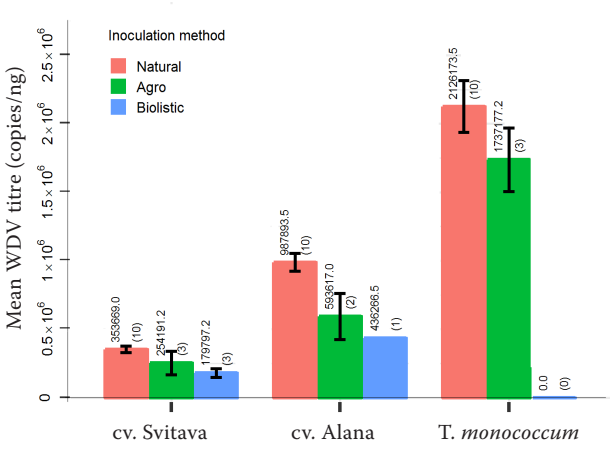

Figure 4. The mean WDV titres for each inoculation method and tested cultivar together with the interval of plus minus one standard error of the mean (SEM). The numbers in parentheses show the total number of infected plants positively detected by both qPCR and DAS-ELISA for the given method and cultivar

As a control experiment, 10 plants of each tested cultivar were inoculated by the leafhopper. Successfully inoculated plants were obtained with 100% efficiency of transmission (Table 3). All plants with qPCR detected WDV infection were also confirmed as infected by DAS-ELISA. The WDV titres detected for each inoculation method and tested cultivar are depicted in Figure 4. The high efficiency of the natural inoculation method allowed for us to estimate mean WDV titres for wheat cvs Svitava and Alana and for *Triticum monococcum* and to compare them with those obtained by agroinoculation and biolistic inoculation using the WDV infectious clone.

DISCUSSION

Wheat dwarf virus (WDV) is a leafhopper-transmitted virus, and the availability of the *P. alienus* virus vector is the only means of plant inoculation. The creation of an infectious clone of an insect-transmitted virus and its ability to infect plants independently of the insect vector may be a powerful tool for plant-virus interaction studies. Two infectious clones of WDV from a Czech wheat isolate have been described in this paper. These infectious clones, pUC18+WDV and pIPKb002+WDV, are able to infect wheat and *T. monococcum* plants. Two inoculation methods (biolistic and agroinoculation) were used, and the agroinoculation with an infectious WDV clone was

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demonstrated as an effective tool for virus inoculation in our hands. The success of agroinoculation with infectious *Maize streak virus* (MSV), a closely related mastrevirus, was described earlier in maize plants (Grimsley *et al.* 1987; Boulton *et al.* 1989). The agroinoculation of plants with pIPKb002+WDV using the *A. tumefaciens* AGL1 strain confirmed the suitability of the constructed infectious clone to initiate a WDV infection in plants. Promising results were obtained from the inoculation by the *A. tumefaciens* AGL1 strain regardless of the induction by acetosyringone prior to the agroinoculation or the agrobacterium cultivation method. The inoculation by *A. tumefaciens* AGL1 obtained from cultivation on plates gave similar results to those obtained with the culture of agrobacterium in a liquid medium. The reached agroinoculation efficiency for selected cultivars (4–12%) is similar to other reported results for wheat or barley (Bendahmane *et al.* 1995; Ramsell *et al.* 2009), however, the higher reached agroinoculation efficiencies were also reported using different agrobacterium strains (Marks *et al.* 1989; Benkovics *et al.* 2010). For MSV, comparison studies of its infectious clones show that the additional promoter sequence present (similarly to the used pIPKb002 plasmid) could increase the agroinoculation efficiency as well as the selected virus DNA orientation in the infectious clone plasmid (Martin & Rybicki, 2000). However, the similarity of WDV and MSV could be compromised, while the MSV infectious clone is known to be mechanically transmissible to its preferential host – maize (Redinbaugh 2003), and extremely high agroinoculation efficiencies were reported for maize (Grimsley *et al.* 1987, 1988).

Successful biolistic transformations of wheat or barley highlight the use of immature embryos or induced calluses cultivated on agar (Jones & Shewry 2009) or very young plant tissues for which the Bio-Rad Helios Gene Gun device is less convenient than, for example, the Bio-Rad Helios PDS1000/He with a vacuum chamber. Despite this, when the optimal parameters were chosen, WDV-infected plants were obtained using any of the two constructed infectious clones; however, the efficiency of transformation using the biolistic methodology was lower than the efficiency for agroinoculation. Our results demonstrate that a phloem-targeted virus such as WDV (Dinant *et al.* 2004; Tholt *et al.* 2018) can be successfully inoculated by a leafhopper free system using an infectious virus clone either by agroinoculation or in a biolistic manner. Many earlier reports have also shown that agroinoculation is a very effective system for delivery of infectious viral DNA into host cells (Grimsley *et al.* 1986) resulting in the induction of disease symptoms in plants (Czosnek *et al.* 1993; Kheyrpour *et al.* 1994). Similarly, biolistic inoculations with infectious viral DNA have a good efficiency to develop the disease in plants (Lapidot *et al.* 2007). In monocot plants such as cereals, tissue biolistic inoculation may be less effective (Helloco-Kervarrec *et al.* 2002), which correlates with our results. Quantitative analysis in our study has shown that in agroinoculated plants, the WDV titre is much higher than that of biolistic inoculation and is similar to plants naturally WDV inoculated by leafhopper.

CONCLUSION

We constructed two different infectious clones, in the bacterial plasmid pUC18 with a prokaryotic *E. coli* replication origin only and in the plasmid pIPKb002 having also the replication origin for *E. coli* and *A. tumefaciens*. We were able to obtain infected plants using both the constructed infectious clones either by biolistic inoculation or, for the pIPKb002+WDV infectious clone, by the agroinoculation method. Successfully infected plants then showed similar titres of WDV compared to plants inoculated by insect transmission.

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Article

Efficient Confirmation of Plant Viral Proteins and Identification of Specific Viral Strains by nanoLC-ESI-Q-TOF Using Single-Leaf-Tissue Samples

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Abstract: Plant viruses are important pathogens that cause significant crop losses. A plant protein extraction protocol that combines crushing the tissue by a pestle in liquid nitrogen with subsequent crushing by a roller-ball crusher in urea solution, followed by RuBisCO depletion, reduction, alkylation, protein digestion, and ZipTip purification allowed us to substantially simplify the sample preparation by removing any other precipitation steps and to detect viral proteins from samples, even with less than 0.2 g of leaf tissue, by a medium resolution nanoLC-ESI-Q-TOF. The presence of capsid proteins or polyproteins of fourteen important viruses from seven different families (Geminiviridae, Luteoviridae, Bromoviridae, Caulimoviridae, Virgaviridae, Potyviridae, and Secoviridae) isolated from ten different economically important plant hosts was confirmed through many identified pathogen-specific peptides from a protein database of host proteins and potential pathogen proteins assembled separately for each host and based on existing online plant virus pathogen databases. The presented extraction protocol, combined with a medium resolution LC-MS/MS, represents a cost-efficient virus protein confirmation method that proved to be effective at identifying virus strains (as demonstrated for PPV, WDV) and distinct disease species of BYDV, as well as putative new viral protein sequences from single-plant-leaf tissue samples. Data are available via ProteomeXchange with identifier PXD022456.

Keywords: protein extraction protocol; LC-MS/MS; virus detection; viral proteins detection

1. Introduction

Plant viruses are important pathogens of many agricultural crops worldwide. Streaking epidemics of plant virus diseases have caused significant crop losses [1] with potential social impact [2,3]. New viruses or divergent viral strains and isolates have frequently been identified in recent years. Highly specific molecular detection techniques, like polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), quantitative PCR (qPCR), or loop-mediated isothermal amplification (LAMP), are focused on selected regions which could not be conserved enough among all strains, resulting in false-negative results. Similarly, serological methods, like enzyme-linked immunosorbent assays (ELISA), are often targeted to specific epitopes. In the case of new hosts infected by the existing pathogens, or even pathogens that have undergone evolutionary pressure, there is an increased need for alternative cost-effective detection techniques to provide tools for independent confirmation of the presence of virus pathogens.

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique is well established as a wide-screen protein identification technique. However, in comparison to other wide-screen identification techniques like nucleic acids next-generation sequencing [4,5], its detection threshold, given by sufficient MS/MS protein fragment identification required, is high. When applied to the widely used *Escherichia coli* bacteria samples or HeLa human cell samples, the medium resolution LC-MS/MS [6] based instruments can identify several hundred or about a thousand proteins [7,8]. After optimization of the protein extraction protocol or using the depletion of the most abundant proteins, the total count of identified proteins could be increased by another few hundred proteins. Changing the technology to a high resolution LC-MS/MS could result in several thousands of identified proteins [9]. Subsequently, when state-of-the-art sample coverage is needed, the fractionation of samples and a long LC column of LC-MS/MS lead to many thousands of identified proteins [10,11] in Orbitrap or a quadrupole—time of flight (Q-TOF) mass spectrometers.

Mass spectrometry techniques have been used successfully for the detection of viral proteins [12,13], and especially for plant viruses [14]. At first, an extraction of virion particles or pure viral proteins was used and their subsequent identification was carried out by matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) or electrospray ionization (ESI). Later, the detection of viral proteins in total protein extracts was described in studies of differential changes in healthy and infected plant proteome [15,16], based mostly on 2D gel electrophoresis accompanied by MALDI-TOF and later directly on LC-MS/MS [16,17]. However, the initial amount of sampled tissue is often mentioned as a whole plant or a mixture of materials obtained from several plants.

To optimize protein extraction protocols, attention was focused on the precipitation steps (trichloroacetic acid-acetone, formic acid-acetonitrile, etc. were often used). Such steps add another source of variability to the results, which could lead to the failure to identify important proteins [18]. To eliminate this negative influence, increased weight of sampled material is required. Even though there are precipitation techniques reaching up to 100% efficiency [19,20] for small weight leaf samples (approximately 50–200 mg), we developed an extraction protocol without a chemical precipitation step. For plant samples, the protein extraction protocol must handle the disruption of cell walls (freeze-thaw cycle, mechanical disruption in liquid nitrogen, the addition of detergent, denaturation by heating, mechanical crushing in extraction buffer, etc.), together with the inhibition of any protein degradation process (adding a chaotropic agent or protease inhibitors) [20,21]. This is often followed by a selected contaminant removal process [19,22]. For LC-MS analysis, these steps are followed by the reduction of disulfide bonds, alkylation of free cysteine residues, and enzyme digestion to fragment proteins into peptides for identification purposes [23,24]. The proper use of all of these techniques must avoid incompatibility of the added agents with any subsequent enzyme digestion, and thus, these additional compounds must be often removed either by precipitation, micropipette-tip solid phase extraction, or at least by dilution to compatible concentrations. Steps leading to any side-effect modifications [25,26] can also significantly decrease the number of identified peptides. To overcome the limited LC column total protein capacity anticipating identification of low abundant proteins,

the samples could be fractionated; however, this significantly increases the amount of LC-MS/MS analyzed samples. If only one sample should be kept for analysis, the most abundant proteins unrelated to the study, like RuBisCO (Ribulose-1, 5-bisphosphate carboxylase/oxygenase) for plants, should be depleted to increase the coverage. Two efficient RuBisCO depletion methods are commonly used [27–29]: precipitation with protamine sulfate or with phytate in the presence of Ca^{2+} ions.

To successfully identify proteins in plant samples, mass spectrometry techniques compare the detected peaks of mass spectra with a database of in silico digested proteins potentially present in the samples [30,31]. Thus, for the identification of virus pathogens, a database of potentially present viral proteins should be assembled first. Host protein databases must also be used when in vivo infection or plant-microbe interactions are studied. However, with increasing numbers of virus or host protein sequences included, the false discovery rate (FDR), maintaining the credibility of identified proteins above random identification by chance, could eliminate many more identified sequences [32,33]. To keep the ratio of false-positive results low, only recognized peptides longer than any certain threshold are used for search, and here, the set of potential virus pathogens tested in each sample is limited to only those occurring at a given plant genus, using available online databases like Plant Viruses Online [34] or Descriptions of Plant Viruses [35,36]. For successful identification, two unique detected peptide fragments, together with their MS/MS fragmentation spectra matching the supposed sequence with an above-the-threshold score, are considered a confirmed presence for the tested protein, provided that FDR filtering techniques are also employed on both the peptide identification and the protein identification level [37,38].

In this work, we present an optimized plant protein extraction protocol (see Figure 1 for a scheme) enabling the extraction of host and virus proteins and subsequently confirming the presence of viral proteins, starting from as little as a single leaf of a plant with strong symptoms of infection. Such a protocol combined with at least medium resolution nanoLC-ESI-Q-TOF could be an efficient virus confirmation method for plants diagnosed by other low-threshold detection methods and left to develop strong symptoms. The small amount required for a protocol sample means that the plant need not be sacrificed, and can subsequently be used for further experiments. We tested the suitability of the protocol on many different plant species: economically important hosts, both monocots and dicots, such as barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), tobacco (*Nicotiana tabacum*, *Nicotiana benthamiana*), plum (*Prunus domestica*), apricot (*Prunus armeniaca*), common bean (*Phaseolus vulgaris*), goosefoot (*Chenopodium amaranticolor*), sorghum (*Sorghum bicolor*), and maize (*Zea mays*). Each tested sample was experimentally inoculated by a virus, and the range of viruses tested includes both the DNA and RNA viruses (seven different virus families): Wheat dwarf virus (WDV) from Geminiviridae, Barley yellow dwarf virus (BYDV/BYDV-PAV) from Luteoviridae, Brome mosaic virus (BMV) and Tomato aspermy virus (TAV) from Bromoviridae, Cauliflower mosaic virus (CaMV) from Caulimoviridae, Tobacco mosaic virus (TMV) and Turnip vein clearing virus (TVCV) from Virgaviridae, Plum pox virus (PPV), Turnip mosaic virus (TuMV), Bean common mosaic virus (BCMV), Sorghum mosaic virus (SrMV), Sugarcane mosaic virus (SCMV) all from Potyviridae, Tobacco ringspot virus (TRSV) and Broad Bean Wilt Virus 2 (BBWV-2) from Secoviridae, see also Figure 2 and Supplementary Table S1.

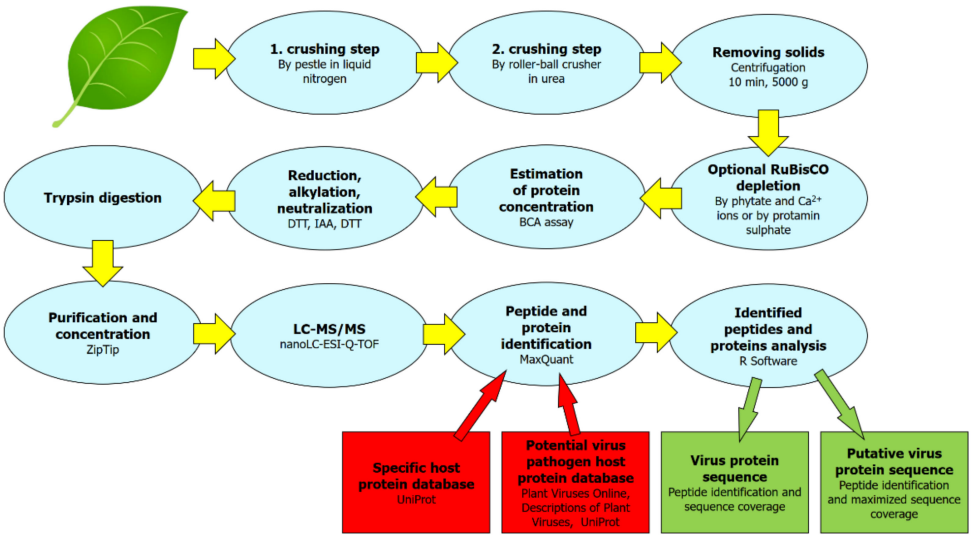

Figure 1. Sample processing scheme used in this work for efficient confirmation of viral proteins from plant leaf tissue.

Figure 2. Virus-positive sampled plants: (**a**) barley cv. Doreen—plant with strong dwarfism (WDV) and a control, (**b**) wheat (WDV) cv. Fielder (**c**) Chinese cabbage (CaMV) (**d**) Chinese cabbage (TVCV) (**e**) plum (PPV) (**f**) apricot (PPV) (**g**) tobacco (PPV) (**h**) tobacco (TMV) (**i**) tobacco (TuMV) (**j**) common bean (BCMV) (**k**) goosefoot (BBWV-2).

2. Results

2.1. Two Crushing Steps Improve Identification of Plant Proteins

To increase the protocol efficiency, two different crushing steps were included: crushing the material in liquid nitrogen by a pestle (crushing step 1, known from nucleic acids extraction methods) and crushing by a hand roller-ball crusher in a thick-walled plastic bag with a urea-based preservation solution (crushing step 2, where roller-ball crusher is often used in DAS-ELISA or similar methods). To confirm the efficiency of both steps, a reference material (plants of winter wheat, cv. Ludwig) was cultivated in the greenhouse and their leaves were cut by scissors to small pieces, put in one bag

and stored at −80 °C. An extraction protocol (without any RuBisCO depletion steps) was applied either (1) only with crushing step 1, where the preservation solution from crushing step 2 was also applied, (2) only with crushing step 2, or (3) with both crushing steps, having six technical replicates per each group (18 in total). The samples were weighed after disruption in liquid nitrogen, just before adding the preservation solution. Figure 3a shows detailed characteristics of the extracted samples using UHPLC Dionex Ultimate 3000 RSLCnano connected to a mass spectrometer ESI-Q-TOF Bruker Maxis Impact for LC-MS/MS analysis. The disruption with a hand roller-ball crusher improves the number of identified peptides, number of identified proteins, and sample coverage, in comparison to disruption in liquid nitrogen only. For both crushing steps applied, there is another increase in these characteristics. The protein concentration of the extracted samples shows the highest correlation with the initial sample weight (Pearson's correlation coefficient of 0.71), i.e., almost twice as high as with any other listed quantity. However, a significant increase in protein concentration can also be seen when both crushing steps are applied instead of only crushing step 1 (both groups having the same sample weight mean). To reduce the effect of the initial sample weight on the amount of extracted proteins, we can use the yield for subsequent comparisons; then, a significant increase is visible when both crushing steps are applied instead of only one.

In the second experiment, the effect of RuBisCO depletion steps was studied. Several plants of winter barley, cv. Doreen, cultivated in the greenhouse were cut to small pieces, stored at −80 °C, and the mixture was used as the reference material. Both crushing steps were applied and the solids were removed. Before optional depletion steps in the protocol, the supernatant of all the samples was merged to keep the same initial protein concentrations and then aliquoted to 18 Eppendorf tubes. For three groups (each having six technical replicates) the rest of the protocol, either (1) without RuBisCO depletion step (omitting the Optional RuBisCO depletion step), or (2) with RuBisCO depletion by phytate and Ca^{2+} ions (Optional RuBisCO depletion step, variant A), or (3) with RuBisCO depletion by protamine sulfate (Optional RuBisCO depletion step, variant B), was applied and the LC-MS/MS analysis was carried out as before. Figure 3b shows the mean and standard error of the mean for MaxQuant LFQ normalized intensities for RuBisCO small chain, the protein concentration of extracted samples after the Optional RuBisCO depletion step, the protein yield per original leaf tissue, identified peptides and proteins, and sample coverage among technical replicates. For estimation of RuBisCO intensity, a sum of MaxQuant label-free quantified (LFQ) intensities [30] for all detected peptides of Ribulose-1, 5-bisphosphate carboxylase/oxygenase small chain was used. For both depletion methods, there was an approximately 40% reduction in the recorded RuBisCO small chain LFQ intensity. The RuBisCO large subunit was also detected; however, a RuBisCO small chain was selected as a more sensitive marker, being less susceptible to cutting the extremely large peaks. It must be also noted that the MaxQuant protein intensity estimation algorithm attributes all the intensities of identified shared peptides (intensities of 'razor peptides') to the protein group with the largest number of total peptides identified, and thus, the proper intensity for pure RuBisCO small chain could also differ.

For depletion by protamine sulfate, a significant increase in both the yield and the protein concentration was visible; however, a reduced number of identified proteins, peptides, and reduced sequence coverage was also present in these samples. Protamine sulfate, a peptide-based compound, probably left some traces in the solution in which the BCA assay was carried out, leading to the overestimation of protein concentration. The reduced amount of leaf tissue proteins in the 1 µg extract injected to the LC column could result in reduced protein and peptide identification. For the other depletion step (phytate and Ca^{2+} ions), the increase due to the identification of low abundant proteins was very small using medium resolution LC-MS/MS, and could improve the detection rates probably only in combination with high resolution LC-MS/MS. If we want to analyze the consistent amount of proteins near the limit of the LC column, the protein concentration estimation step should not be omitted; thus, even though protamine sulfate could effectively reduce the RuBisCO levels, the interference with protein concentration estimation would be contra productive. For depletion of RuBisCO in our protocol, the usage of phytate and Ca^{+} ions is suggested.

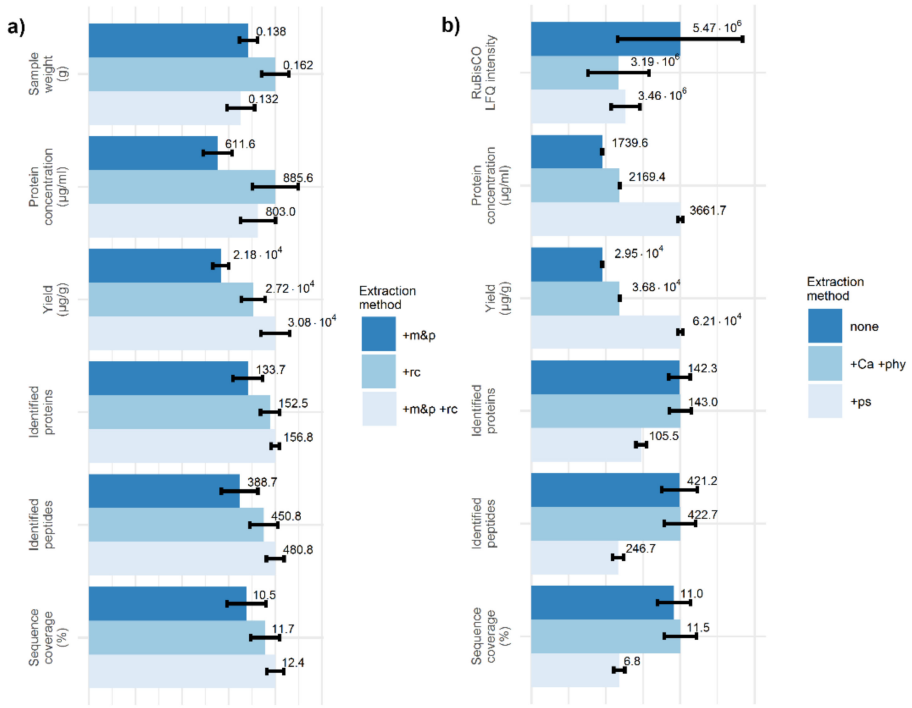

Figure 3. (**a**) Sample weights, the protein concentration of undiluted samples after the Removing solids step, protein yield per original leaf tissue weight used, identified peptides and proteins, and sample coverage for frozen winter wheat leaf tissue disrupted with a pestle in a mortar with liquid nitrogen (+m&p), or with a roller-ball crusher in urea solution (+rc), or by both (+m&p +rc). (**b**) MaxQuant LFQ normalized intensities for RuBisCO small chain, the protein concentration of undiluted samples after the Optional RuBisCO depletion step, protein yield per original leaf tissue weight used, identified peptides and proteins, and sample coverage for extraction protocol applied to frozen winter barley leaf tissue without RuBisCO depletion step (none), with a depletion step using phytate and Ca^{2+} ions (+Ca +phy), or with a depletion step using protamine sulfate (+ps). Error bars represent one standard error of the mean. All the groups contain exactly six samples ($n = 6$). For Identifed proteins, the total counts of MaxQuant proteinGroups, without contaminants, without proteins from reverse database, and without proteins only identified by site (by their peptide mass only, without supporting MS/MS spectrum) are listed.

2.2. Plant Virus Pathogen Capsid Proteins Could Be Efficiently Confirmed in Samples of Plants with Strong Infection Using the Double Crushing Extraction Protocol Followed by nanoLC-ESI-Q-TOF

For each cultivated host inoculated by a virus, the leaves were sampled and extracted (see Table 1 for a list of hosts and Supplementary File S1 for cultivation and inoculation conditions). The identified peptides in the samples were searched for pathogen-specific peptides. A list of potentially available virus pathogens for a given plant taxonomic genus was assembled using two available online databases of plant virus pathogens: Plant Viruses Online and Descriptions of Plant Viruses. Table 1 shows that the protocol is able to identify viral capsid proteins and other viral proteins in at least ten different plant hosts. In Supplementary File S2, all the identified viral proteins with the sequence isoform with the highest amino acid coverage, identified peptide fragments, and their protein alignments are listed. All identified peptides of viral proteins were then searched for occurrence in plant host protein sequences, and no such occurrence was found. Thus, the peptides identified in viral proteins were unique to identified virus sequences and could not be accidentally interpreted as identified virus sequences due to their incident colocation in the plant host proteome.

Table 1. Identified host and virus proteins from single-leaf-tissue samples of virus inoculated plants with strong symptoms.

	Sample	01a/barley *1	01b/barley	01c/barley	02/wheat *1	03a/Chinese cabbage	03b/Chinese cabbage	04a/tobacco	04b/tobacco	04c/tobacco	04d/tobacco	04e/tobacco	05/apricot	06a/plum	06b/plum	07/bean	08/goosefoot	09/sorghum	10/maize
PREPARATION	**Sample weight (g)**	0.14	0.34	0.46	0.34	0.52	0.47	0.47	0.46	0.45	0.55	0.63	0.60	0.60	0.97	0.48	0.63	0.32	0.13
	Host taxonomic classification	*Hordeum vulgare*	*Hordeum vulgare*	*Hordeum vulgare*	*Triticum aestivum*	*Brassica rapa subsp. pekinensis*	*Brassica rapa subsp. pekinensis*	*Nicotiana benthamiana*	*Nicotiana benthamiana*	*Nicotiana benthamiana*	*Nicotiana clevelandia x N. glutinosa*	*Nicotiana tabacum*	*Prunus armeniaca*	*Prunus domestica*	*Prunus Pdomestica*	*Phaseolus vulgaris*	*Chenopodium amaranticolor*	*Sorghum bicolor*	*Zea mays*
	UniProt reference proteome used		UP000011116		UP000019116	UP000011750				UP000084051 *2				*3		UP000000226	*4	UP000000768	UP000007305
	Reference genome proteins		189,799		130,673	40,809				73,605 + 53,411 + 74,802 *2				155,017 *3		30,501	1063 *4	41,380	99,254
	Known host viruses in online databases		66		63	29				369				17		227	378	22	69
	Tested viruses for sample (UniProt protein sequences available)		47		45	24				232				13		151	232	18	52
RESULTS	**Identified peptides in sample**	3233	2871	3963	3888	1844	1094	3176	2772	3412	3426	1270	2344	1648	1047	4364	346	1942	1956
	Identified host proteins *	886	810	1082	1076	537	339	756	668	865	865	287	593	435	362	1163	71	570	549
	Identified virus	Wheat dwarf virus (WDV)	Barley yellow dwarf virus (BYDV)	Brome mosaic virus (BMV)	Wheat dwarf virus (WDV)	Cauliflower mosaic virus (CaMV)	Turnip vein clearing virus (TVCV)	Plum pox virus (PPV)	Turnip mosaic virus (TuMV)	Tobacco ringspot virus (TRSV)	Tomato aspermy virus (TAV)	Tobacco mosaic virus (TMV)	Plum pox virus (PPV)	Plum pox virus (PPV)	Plum pox virus (PPV)	Bean common mosaic virus (BCMV)	Broad Bean Wilt Virus 2 (BBWV-2)	Sorghum mosaic virus (SrMV)	Sugarcane mosaic virus (SCMV)
	Identified virus family	Geminiviridae (DNA)	Luteoviridae (RNA)	Bromoviridae (RNA)	Geminiviridae (DNA)	Caulimoviridae (DNA)	Virgaviridae (RNA)	Potyviridae (RNA)	Potyviridae (RNA)	Secoviridae (RNA)	Bromoviridae (RNA)	Virgaviridae (RNA)	Potyviridae (RNA)	Potyviridae (RNA)	Potyviridae (RNA)	Potyviridae (RNA)	Secoviridae (RNA)	Potyviridae (RNA)	Potyviridae (RNA)
	Identified capsid protein peptides **	10	2	11	10	10	6	11	14	8	12	12	6	6	5	4	17	9	4
	Capsid protein sequence coverage (AA) **	147 of 260 (56.5%)	36 of 199 (18.1%)	147 of 189 (77.8%)	147 of 260 (56.5%)	110 of 489 (22.5%)	82 of 157 (52.2%)	119 of 330 (36.1%)	154 of 286 (53.8%)	104 of 513 (20.3%)	134 of 218 (61.5%)	92 of 159 (57.9%)	86 of 330 (26.0%)	65 of 330 (19.7%)	57 of 330 (17.2%)	71 of 287 (24.7%)	247 of 599 (41.2%)	95 of 320 (29.7%)	59 of 313 (18.8%)
	Capsid protein sequence coverage on ≥ 7 amino acid sequences (AA) **	147 of 189 (77.8%)	36 of 148 (24.3%)	147 of 152 (96.7%)	147 of 189 (77.8%)	110 of 333 (33.0%)	82 of 142 (57.7%)	119 of 278 (42.8%)	154 of 210 (73.3%)	104 of 437 (23.8%)	134 of 187 (71.7%)	92 of 148 (62.2%)	86 of 278 (30.1%)	65 of 278 (23.4%)	57 of 278 (20.5%)	71 of 232 (30.6%)	247 of 506 (48.8%)	95 of 211 (45.0%)	59 of 247 (23.9%)

Table 1. *Cont.*

	Sample	01a/barley *1	01b/barley	01c/barley	02/wheat *1	03a/Chinese cabbage	03b/Chinese cabbage	04a/tobacco	04b/tobacco	04c/tobacco	04d/tobacco	04e/tobacco	05/apricot	06a/plum	06b/plum	07/bean	08/goosefoot	09/sorghum	10/maize
RESULTS	**Other viral proteins identified** **	–	2 fragments of movement protein	–	–	more than 10 fragments of other viral proteins - movement protein, reverse transcriptase, aphid transmission protein, etc.	–	other more than 10 fragments of genome polyprotein	other more than 10 fragments of genome polyprotein	–	–	more than 10 fragments of replication protein	other more than 10 fragments of genome polyprotein	other 6 fragments of genome polyprotein	–	other more than 10 fragments of genome polyprotein	one other fragment of genome polyprotein from RNA 1 component	other more than 10 fragments of genome polyprotein	other more than 10 fragments of genome polyprotein
	Sequence covered (aa) **	147	87	147	147	794	82	936	695	104	134	405	291	126	57	380	275	486	343
	Putative sequence covered (aa) ***	147	87	147	147	807	82	1073	734	104	142	405	291	126	57	407	327	522	398
	Virus presence also confirmed by ****	DAS-ELISA qPCR	DAS-ELISA RT-PCR RFLP	DAS-ELISA	DAS-ELISA qPCR	DAS-ELISA	DAS-ELISA	DAS-ELISA qPCR	DAS-ELISA qPCR	DAS-ELISA	DAS-ELISA	DAS-ELISA	DAS-ELISA qPCR	DAS-ELISA qPCR	DAS-ELISA qPCR	DAS-ELISA	electron microscopy RT-PCR	DAS-ELISA	DAS-ELISA

* total count of identified MaxQuant proteinGroups containing only host proteins, without contaminants, proteins from reverse database, and without proteins only identified by site (by their peptide mass only, without supporting MS/MS spectrum); ** see also Supplementary File S2; *** see also Supplementary File S3 **** see Supplementary File S4 for method details; *[1] used extraction protocol without RuBisCO depletion step; *[2] tested against UP000084051 reference proteome of *Nicotiana tabacum* (73,605 proteins) together with assembled proteome for *Nicotiana benthamiana* (ref. [39], NbD_AA—53,411 proteins, NbE_AA—74,802 proteins); *[3] no reference proteome is available in UniProt database, and thus, tested against all available protein sequences of *Prunus* genus; *[4] no reference proteome is available in UniProt database, and thus, tested against all available protein sequences of *Chenopodium* genus.

For the total sum of amino acids in a sequence covered by the identified fragments of all detected viral proteins of inoculated viruses, more than 100 amino acids were covered for each sample, with only one exception (the 06b/plum sample, 57 amino acid covered sequence); however, in many cases, this total sum reached several hundreds of amino acids (936 AA at most, for the 04a/tobacco sample). For the distinct viral proteins of both tested DNA viruses, the highest (for WDV, 147 AA of 260 AA or 56.5%) or second highest (for CaMV, 110 AA of 489 AA or 22.5%) amino acid sequence coverage was reported for their capsid proteins. For CaMV, the only protein with higher amino acid coverage was its translational activator (ORF6, 379 AA of 519 AA or 73.0%). For RNA viruses, a genome polyprotein is often expressed, which is subsequently digested to distinct viral proteins. If only final viral proteins instead of genome polyprotein are accounted for, then the highest amino acid sequence coverage was also reached for the capsid proteins. Similarly, if we compare the relative sequence coverage of viral proteins, the relative sequence coverage for capsid proteins was higher than for other detected viral proteins; it was at least 17.2% (for PPV capsid protein in the 06b/plum sample or 57 AA of 330 AA).

For mass spectrometry peptide fragment identification, only fragments equal or longer than the selected threshold are searched, and thus, in Table 1, a sequence coverage on ≥7 amino acid sequences is also reported, i.e., the relative coverage for detected fragments on protein sequence, from which all fragments shorter than a given threshold (seven amino acids) were removed. However, even on these reduced sequences, the relative coverage does not essentially differ (20.5% to 96.7%, median 43.9%), in comparison to values on protein sequences of the original length (17.2% to 77.8%, median 32.9%).

If the detected peptide fragments for a given virus protein cannot be aligned to only one protein sequence from the database, but can be aligned to other known sequence isoform, then these alignments to other protein isoforms are reported in MaxQuant as different protein groups for the studied protein. The frequent occurrence of different protein groups for the same viral protein lead as to the construction of putative protein sequence maximizing the sequence coverage by the detected peptides. Briefly, for each viral protein, an existing protein sequence with the highest detected sequence coverage was selected as an original sequence. All peptide fragments detected in the sample were extracted and locally aligned to the original protein sequence, keeping only the alignments with few modifications to the original sequence. Then, a putative sequence was constructed based on the original protein sequence and modifications included in the aligned peptides. The resulting amino acid coverage for these putative sequences is listed in Table 1. All the constructed putative viral protein sequences with detected peptide fragments and their alignment are reported in Supplementary File S3. The highest increase in sequence coverage can be seen for PPV in tobacco (from 936 AA to 1073 AA), SCMV in maize (from 343 AA to 398 AA), and BBWV-2 in goosefoot (from 275 AA to 327 AA). However, for some other viruses, even with high amino acid coverage in the capsid protein or polyprotein, no increase is obtained this way (WDV and BMV in cereal samples, TMV in tobacco).

Regarding potential misidentification, there is high sequence similarity of SrMV and SCMV, and thus, many peptides of SCMV were also detected in the sorghum sample. However, only one of them was an unique peptide for the SrMV sequence. Similarly, in the maize sample, several peptides of SrMV and SCMV shared sequence were also detected in the sample, but no unique peptide was detected, and thus, there is no entry in the related MaxQuant proteinGroups.txt search results file. For other samples, occasionally, a few peptides of other viruses can be also detected (e.g., see tobacco or goosefoot samples), but with, at most, one unique peptide. The other detected peptides are only those which are also shared with other viral sequences. These occasional occurrences are supposed to be a computer algorithm artifact (i.e., a fragment identified by chance due to a too large search protein database and not captured by FDR or target-decoy database search strategy), validating the requirement for at least two unique peptide fragments to be identified for sequence confirmation.

2.3. The nanoLC-ESI-Q-TOF Based Detection Method with Optimized Extraction Protocol Allows Discrimination of Virus Strains or Distinct Disease Species Based on Detected Fragments

For three different virus-caused diseases (WDV, PPV, and BYDV), several different virus strains (for WDV and PPV) or distinct diseases species (for BYDV, where sources of BYDV disease are classified as distinct virus species) are present in the Czech Republic. We focused on the classification of the detected disease source in the samples even to specific virus strains or, for BYDV, to the identification of BYDV distinct disease species.

For WDV, where both WDV-Wheat (WDV-W) and WDV-Barley (WDV-B) strains occur in the Czech Republic, differences in many regions of the capsid protein amino acid sequence exist among these strains. Due to the high variability between WDV isolates of both strains, no single strain-specific sequence exists for many regions, and thus, several strain-specific sequences are often reported according to sequence subgroups. The high amino acid sequence coverage for tested samples and frequent strain-specific sequence differences allowed proper classification of the samples to be based here on many single amino acid differences (see Figure 4). The WDV strain of the barley sample was determined as WDV-B, and the WDV strain of the wheat sample was successfully determined to be WDV-W, which is in agreement with the used inoculation strain.

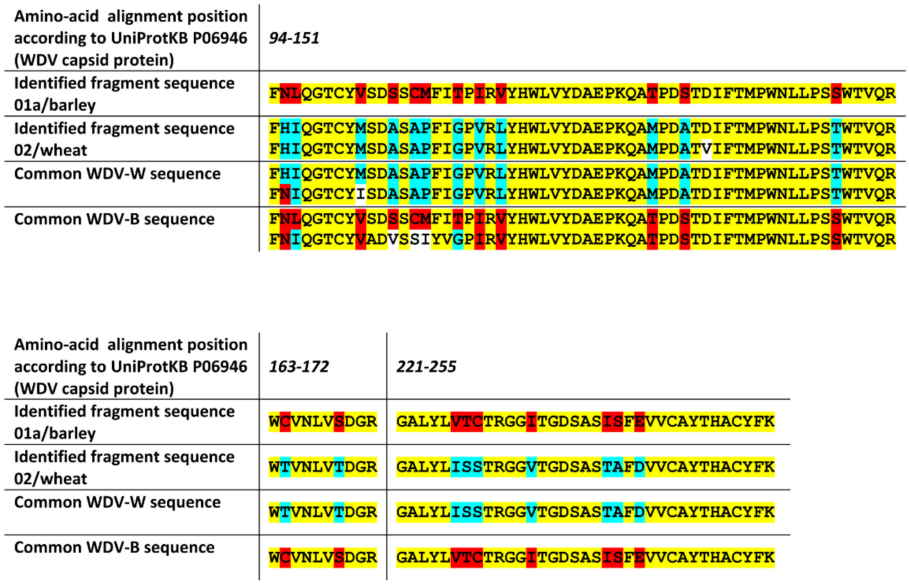

Figure 4. Alignment of detected discriminating peptide fragments of WDV capsid protein and strain-specific sequences for a given region in WDV-W and WDV-B strains. The positions with the same amino acid (yellow), with the detected amino acid in fragments similar to WDV-B (red), and with the detected amino acid in fragments similar to WDV-W (blue) are highlighted in different colors.

In the case of PPV, PPV-D, and PPV-M strains, these occur in the Czech Republic and Slovakia, together with their recombined strain PPV-Rec (sharing the C-terminus of NIb and entire capsid protein amino acid sequence with PPV-M). Most of the identified fragments for all four PPV samples were detected in regions where strain-specific sequences are equal, and only one or two peptide fragments were detected and identified in regions where strain-specific sequence differs for tested strains (see Figure 5). Based on their identification, the 04a/tobacco sample can be determined to be infected with PPV-M strain or PPV-Rec, the 05/apricot and the 06a/plum as infected with PPV-D strain, and the 06b/plum as infected with PPV-M or PPV-Rec strain, which is in agreement with the strain identity of isolates used for inoculation of the tested plants.

Even though only two fragments of the capsid protein of BYDV were detected, these peptides are part of the region where the BYDV disease species occurring in the Czech Republic (see [40]) differ in their amino acid sequence. Both detected capsid-protein peptide fragments for BYDV confirmed the

virus sample to be BYDV-PAV (see Figure 6), which is in agreement with the virus used for inoculation. Only one of the two detected movement-protein peptide fragments also allowed discrimination of the BYDV disease species for the detected virus. Even though the fragment is from a region where several species-specific sequences exist, this fragment also confirmed the virus sample to be BYDV-PAV.

Amino-acid alignment position according to UniProtKB E5RTT7 (PPV polyprotein)	*2775-2789*	*2904-2919 (94-109 in PPV capsid protein)*
Identified fragment sequence 04a/tobacco	KLYTDSEASETEIER	DRDVDAGSIGTFAVPR
Identified fragment sequence 05/apricot	----------	DRDVDAGSIGTFTVPR
Identified fragment sequence 06a/plum	----------	DRDVDAGSIGTFTVPR
Identified fragment sequence 06b/plum	----------	DRDVDAGSIGTFAVPR
Common PPV-D sequence	KLYTDTEASETEIER	DRDVDAGSIGTFTVPR
Common PPV-M sequence Common PPV-Rec sequence	KLYTDSEASETEIER	DRDVDAGSIGTFAVPR

Figure 5. Alignment of detected discriminating peptide fragments of PPV polyprotein and strain-specific sequences for given regions in PPV-D and PPV-M strains. The positions with the same amino acid (yellow), with the detected amino acid in fragments similar to PPV-D (red), and with the detected amino acid in fragments similar to PPV-M (blue) are highlighted in different colors. PPV-Rec strain shares its amino acid sequence at the end of the genome polyprotein (including the capsid protein sequence) with the PPV-M strain.

Amino-acid alignment position according to UniProtKB P09510 (BYDV capsid protein)	*24-35*	*49-72*
Identified fragment sequence 01b/barley	TVRPVVVVQPNR	GGANPVFRPTGGAEVFVFSVDNLK
Common BYDV-PAV sequence	TVRPVVVVQPNR	GGANPVFRPTGGTEVFVFSVDNLK
Common BYDV-PAS sequence	AIRPVVVVQPNR	RGPDSIPGSAGRTEVFIFSVDNLK
Common BYDV-MAV sequence Common BYDV-GAV sequence	AVRRMVVVQPNR	GGANLISGPAGRTEVFVFSVNDLK

Amino-acid alignment position according to UniProtKB Q45ZZ3 (BYDV movement protein)	*90-116*
Identified fragment sequence 01b/barley	SQVSVLSLSHTRPQLRPALSLLNSTPR
Common BYDV-PAV sequence	SQVSVLSLSHTRPQLRPALSLLNSTPR SQVSELSLSHTRPPIRQALSLLSSTPR SQVSELSLSHTRPPLRPALSLLNSTPR SQVSTLSLEHTRPPLRRALCLLNSTPR SQVSTLSLGHTRPPLRRALCLLNSTPR
Common BYDV-PAS sequence	SQVSVLSLSHTRPPLRRALSLLNSTPR
Common BYDV-MAV sequence	SQTSRLSLSHTRPPLQSAQCLLNSTLG

Figure 6. Alignment of detected discriminating peptide fragments of BYDV capsid and movement protein and strain-specific sequences for a given region in BYDV-PAS, BYDV-PAV, BYDV-MAV (and BYDV-GAV if the sequences are equal) strains. The positions with the same amino acid among all sequences (yellow) and positions with the same amino acids as in an identified fragment (red) highlighted by different colors.

3. Discussion

Although most methods described in this paper are generally used for proteomic analysis, the adaptation of the methods to identify viruses that may be present in low abundance is not a routine process. The presented protocol for protein extraction from leaf tissues employs techniques that are well known from nucleic acid protocol extractions—i.e., disruption of leaf samples in liquid nitrogen, the use of a chaotropic agent for protein denaturation, and techniques optimized for DAS-ELISA plant virus protein targeted methods—applying crushing by a roller-ball crusher in a preserving buffer. The high concentrations of the chaotropic agent, urea, are lowered by dilution to noninhibiting concentrations for trypsin digestion to avoid any incompatibility. The final concentration and desalting step are made using a micropipette-tip solid phase extraction, a technique which is similar to subsequent LC column protein separation. Such a protocol easily fulfills protein sample limits of LC columns, and subsequent nanoLC-ESI-Q-TOF identification makes it possible to confirm viral capsid proteins on a wide range of host plants, including grasses and flowering plants or trees. The number of identified peptides and high protein sequence coverage of viral capsid proteins even allowed us to discriminate specific virus strains or disease species based on the viral protein sequence analysis.

Even though the relative coverage of detected peptide fragments on ≥7 amino acid sequences in the samples reached 96.7% for BMV capsid protein on barley, for WDV capsid proteins, for example, this ratio stopped at 77.8%, on both highly infected barley and wheat samples. The identification of peptide fragments in recorded spectra is still based on the provided protein sequences and their modifications. Any missed modification in the setup of the database search could lead to missed identification of peptides, despite the fact that the peaks of these peptides are still present in the recorded spectra. Even if the proper modifications are included, for some of them, like phosphorylation, their detection by mass spectrometry techniques is generally complicated due to their different charge states and subsequent problems with ionization [41].

While the identification of peptides from MS/MS spectra is continuously improving [42,43], the significant reduction of the assembled protein database to be searched is an important factor for the elimination of false-negative results, where all these sequences are also used through control target-decoy database search strategy. For the detection of virus pathogens, a reduction to only a host and potentially available virus species is suggested. General online sources for potential plant host–virus interactions are currently not easily available. Frequently used sources, such as Plant Viruses Online or Descriptions of Plant Viruses, are reportedly not updated, even though they are still important sources of information. Selective information aggregated by plant hosts can be found for important agricultural crops and viruses [44,45]; however, a manual search in literature through protein database sources is required to obtain an up-to-date list of potential virus pathogens for a specific host, based on existing virus taxonomy [46].

Even though the absolute amino acid coverage and relative coverage of potentially misidentified sequences in the samples remain zero or very low (for unique detected viral protein fragments) or at least low (for all detected fragments including the shared ones) in comparison to detected coverages of proteins supposed for confirmation, the potential random identification of peptides of other viral sequences requires a relatively high amount of the protein to be present in the sample, resulting in high sequence coverage with several unique peptides identified. The advantage of the peptide-based identification method is its ability to reconstruct the protein sequence, provided that the sequence information is available in proteome databases. Even for the new viruses or mutated virus strains, if the amino acid sequences of their viral proteins share at least some parts with other related and already identified viruses, there is a high probability of identification of the part of the protein and confirmation of its presence. A putative sequence can be then reconstructed if there is an assumption for mutated virus strain or new virus present. However, a special caution must be made in case of co-infections. For distinct viruses with viral proteins sharing the large regions of the same protein sequence, many peptides could be identified from these regions. The decision of whether coinfection or infection by only a new species occurs will be then dependent on the identification of a few unique

peptides from regions where the sequences should differ. A high sequence coverage could alleviate the issue, but generally, confirmation by another detection method would be then required, although the supporting method could be highly target-specific, like PCR.

The presented double crushing step extraction protocol combined with at least medium resolution LC-MS/MS allows confirmation of viral proteins even from single-leaf-tissue samples and can serve as an efficient viral protein confirmation method for highly infected samples.

4. Materials and Methods

4.1. Protein Sample Extraction and Preparation Protocol

Crushing step 1: Leaf tissue was cut and homogenized in liquid nitrogen, ground into a fine powder using mortar and pestle, and collected in an Eppendorf tube and weighted. Crushing step 2: The tissue from the previous step was transferred (or the remains were eluted by pipette) to a thick-walled plastic bag, and 5 mL of preservation solution (0.4 M ammonium hydrogen carbonate, 8 M urea, pH 7.8–8.0) was added. The content of the plastic bags was homogenized with a hand roller ball crusher homogenizer (Bioreba, Reinach, Switzerland, see Supplementary Figure S1) without removing the content from the bags. Removing solids: 1 mL of the liquid part was pipetted to an Eppendorf tube and centrifuged for 10 min at 5000× g to remove any remaining solids. The supernatant was pipetted to a new tube. Optional RuBisCO depletion step: Optionally, RuBisCO depletion procedure variant A (using phytate and Ca^{2+}) or variant B (using protamine sulfate) can be subsequently included, or the extraction can continue directly to the next step (protein concentration estimation). Variant A: 200 µL of supernatant was pipetted to a new tube, and 20 µL of 100 mM $CaCl_2$ (dissolved in 8 M urea) was added. Subsequently, 20 µL of 100 mM phytate (dissolved in 8M urea) was added. The sample was left incubated for 10 min in a shaker (42 °C, 500 rpm) and centrifuged for 15 min (16,100× g, room temperature). Variant B: 200 µL of supernatant was pipetted to a new tube, and 20 µL of 5% protamine sulfate (dissolved in 8M urea) was added. The sample was incubated for 30 min at 4 °C on ice and centrifuged for 15 min (12,000× g, room temperature). Protein concentration estimation: For each sample, an aliquot was diluted eight times by distilled deionized water and the concentration was estimated using the BCA assay (Thermo Fisher Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). Pipetting the same protein amount: For subsequent steps, the same protein amount (here 100 µg) from each extracted sample of its original concentration (not diluted as in the previous step) was pipetted to a new tube to proceed to subsequent steps in a convenient volume. Reduction step: Dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA) was added to the final concentration of 5 mM, and the samples were left in a thermoblock for 30 min (60 °C, 300 rpm). Alkylation step: After cooling to room temperature, iodoacetamide (Sigma-Aldrich) was added to give a final concentration of 10 mM. The samples were then left for 30 min at room temperature in the dark. Neutralization of remains of iodoacetamide: Dithiothreitol was added to give a final concentration of 5 mM, and the samples were left for 30 min at room temperature. Trypsin digestion step: An aliquot of 2 µg of protein was diluted eight times by distilled deionized water, and 1 µL of trypsin (0.1 µg/µL fresh solution in distilled deionized water) was added. If required, the aliquot used for digestion can be lowered down to 1 µg of protein, and the dilution ratio can be also optionally lowered to four times for samples of low protein concentration, to keep the maximum volume of 20 µL for the reaction mixture in order to maintain the efficiency of the digestion and subsequent concentration. The reaction mixture was then incubated for 16 h (overnight) at 37 °C. Concentration step: The digestion was stopped by the addition of 10% trifluoroacetic acid (Sigma-Aldrich) to a final concentration of 0.5%, and the sample was concentrated using ZipTip (Millipore, Burlington, MA, USA) containing reverse phase C18 according to the supplied protocol with the elution solution of 5 µL of 50% acetonitrile and 0.1% TFA in deionized water. The tubes were then left open in air to evaporate the supernatant, leaving the solid extract.

4.2. LC-MS/MS

Mass spectrometry measurements were carried out using liquid chromatography column with nanoliter per min flow ranges (nanoLC), electrospray ionization (ESI), and quadrupole time-of-flight (Q-TOF) technology; i.e., here a UHPLC Dionex Ultimate 3000 RSLCnano (Dionex, Sunnyvale, CA, USA) was connected to a mass spectrometer ESI-Q-TOF Maxis Impact (Bruker, Billerica, MA, USA). The extracted sample was dissolved in the mixture of water:acetonitrile:formic acid (97:3:0.1%), and then 1 µg of peptides in the injection volume of 1 µL was loaded on a trap column, Acclaim PepMap 100 C18 (100 µm × 2 cm, particle size 5 µm, Dionex, Germany), with a mobile phase A (0.1% formic acid in water) flow rate of 5 µL/min for 5 min. The peptides were eluted from the trap column to the analytical column, Acclaim PepMap RSLC C18 (75 µm × 150 mm, particle size 2 µm), by mobile phase B (0.1% formic acid in acetonitrile) using the following gradient: 0–5 min 3% B, 5–95 min 3–35% B, 97 min 90% B, 97–110 90% B, 112 min 3% B, 112–120 min 3% B. The flow rate during gradient separation was set to 0.3 µL/min. The peptides were eluted directly to the ESI source—Captive spray (Bruker Daltonics, Germany). Measurements were performed in data-dependent analysis mode with precursor-ion selection in the range of 400–1400 *m/z*; an MS spectrum was recorded every 3 s and MS/MS spectra were collected at the speed of 4–16 Hz, depending on the intensity of the precursors. Dynamic exclusion was set to 1 min, preferred charge state to 2–5, and singly charged precursors were excluded. Collision induced dissociation MS/MS spectra were recorded in the range of 50–2200 *m/z* and profile spectra were saved. The mass spectrometry proteomics data and search results were deposited to the ProteomeXchange Consortium via the PRIDE partner repository [47] with the dataset identifier PXD022456.

4.3. Protein Identification and Protein Databases Searched

The peptides in raw spectra were identified and quantified by MaxQuant [11] 1.6.7.0 for Windows using reverse sequences for target-decoy database search strategy [48] and applying a 1% false discovery rate (FDR) for both the peptide spectrum match and protein group levels. The processed files were set as one experiment per each file to report the detected intensities in each of the processed files separately. Trypsin was set as the proteolytic enzyme and two missed cleavages were allowed. Cysteine carbamidomethylation was selected as a fixed modification. Oxidation of methionine and protein N-terminal acetylation were searched as variable protein modifications. Match between runs was switched off for samples of the same plant genus analyzed together. A Bruker Q-TOF instrument was selected and default tolerances were used (0.07 Da for the first search and 0.006 Da for the main peptide search at the MS level). Protein identification was performed using default 40 ppm as the mass tolerance at the MS/MS level for the TOF analyzer. The minimal required peptide length was set to seven amino acids. For each tested plant host, either reference proteome with all isoforms was downloaded from the UniProt protein knowledgebase [49] (accessed 3 January 2020) or, if not present, all available protein sequences for the given taxonomic plant genus from UniProt were used instead. For *Nicotiana benthamiana*, a homology-guided proteome [39] derived from *Nicotiana tabacum* was also included (see UniProt identifiers in Table 1). For each tested plant host, a host range property was searched in Plant Viruses Online [34] and Descriptions of Plant Viruses [35,36] online databases, and a list of potential virus pathogens for the tested plant genus was assembled. All available protein sequences for the obtained viruses were downloaded from the UniProt protein knowledgebase (accessed 3 January 2020, see also the PREPARATION section of Table 1) and their UniProt protein identifiers were supplied with the "UNIPROT_VIRUS_" prefix to easily identify detected virus proteins among the MaxQuant results. Thus, a protein sequence database for each tested host consisted of specific host proteins with original UniProt identifiers and virus proteins of potential virus pathogens for a selected host (with "UNIPROT_VIRUS_" prefix followed by their original UniProt identifier). The MaxQuant proteinGroups.txt and peptides.txt files were used for subsequent analyses of detected proteins and peptides in the samples and are included as Supplementary File S5.

4.4. Virus Protein Sequence Coverage, Virus Protein Putative Sequence

For each identified virus protein, all amino acid sequence isoforms, listed as separate entries in MaxQuant proteinGroup file, were examined. As a representative sequence for the isoform, the leading detected protein in each MaxQuant proteinGroup record was selected; if it was reported as a fragment in its fasta header, then other proteins of the protein group with the same or longer sequence and with the same number of detected fragments were considered. All the identified peptide fragments for the isoforms were aligned to the sequence using R Software 4.0 [50] and in-house scripts (for download, see http://uprt.vscht.cz/aaseq). The viral protein isoform sequence with the highest sequence coverage was then selected as a representative sequence for the identified virus protein at all. For these sequences with highest detected coverages, detected peptide fragments, their alignment, and supposed trypsin sites, see Supplementary File S2. For viruses expressing the polyprotein, the protein sequence, detected peptide fragments and their alignment are reported only if peptide fragments were also detected in other regions than in the capsid protein sequence; otherwise, only their capsid protein sequence is listed.

In MaxQuant software, minimal length for peptide detection was set to seven amino acids. Thus, for each reported protein sequence, a relative coverage was also computed for the sequence with all the peptide fragments shorter than seven amino acids omitted (see Table 1 and Supplementary File S2). The task was done using the trypsin digest sites detected according to the rules of ExPASy PeptideCutter online tool [50] and R Software 4.0 in-house scripts (for download, see http://uprt.vscht.cz/aaseq).

To construct a putative sequence maximizing the sequence coverage by detected peptide fragments, all the identified peptide fragments in the sample were extracted from MaxQuant search result files. For each examined viral protein, a protein sequence isoform with the highest sequence coverage was selected as its original sequence. All the local alignments of detected peptides to the protein sequence were then determined using R Bioconductor package 'Biostrings' (version 2.58). The alignments overlapping the sequence borders were removed. For each remaining peptide alignment, the number of modifications (substitutions, insertions, deletions, or improper alignments to trypsin digest sites) to the original sequence was determined and only peptide alignments with at most ⌊fragment length/7⌋ modifications were used. For the conflicting alignments of the peptides, those with the higher ratio of modifications per length were removed (but are also separately reported). The putative sequence was then constructed based on the original protein sequence and modifications detected in the aligned peptides. For constructed putative sequences, detected peptide fragments, their alignment, the peptides with conflicting alignments, and the putative sequence amino acid coverage, see Supplementary File S3. The used in-house scripts can be downloaded at http://uprt.vscht.cz/aaseq.

4.5. Identification of Strain-Specific Sequences

For the determination of strain-specific protein sequences (WDV, PPV) or distinct BYDV disease species protein sequences, all available complete genome nucleotide sequences were downloaded from the NCBI database [51] for virus strains and BYDV disease species occurring in the Czech Republic, and the sequences were aligned using the ClustalX 2.1 tool [52]. The sequences of open reading frames were then translated into an amino acid sequence and the amino acid sequences were aligned again in ClustalX 2.1. For each tested region and virus strain/pathogen species, the consensus amino acid sequence was determined and reported as a strain-specific sequence. If no consensus sequence was detected, then the consensus amino acid sequences for sequence subgroups were determined and used instead.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-0817/9/11/966/s1. Table S1: Taxonomic classification and brief description of tested plant virus pathogens; Figure S1: Mechanical roller steel-ball crusher homogenizer (Homogenizer Hand Model, Art. No. 400010, Bioreba, Switzerland) for proper tissue homogenization; File S1: Plant cultivation conditions and virus inoculation for tested hosts; File S2: Viral protein sequences, detected peptide fragments, their alignment, and amino acid sequence coverage—isolate sequences with most detected peptides); File S3: Putative viral protein sequences maximizing the sequence

coverage, together with detected peptide fragments. The sequences are based on identified protein database sequence with the highest coverage, followed by the inclusion of other detected peptides with only a few modifications to the original sequence (see the Materials and Methods Section 4.4); File S4: Other Biochemical methods used for confirmation of detected viral pathogens; File S5: The MaxQuant proteinGroups.txt and peptides.txt files from virus inoculated samples used for subsequent analyses of detected proteins and peptides. References [53–86] are cited in the supplementary materials.

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Conflicts of Interest: The authors declare no conflict of interest.

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RESEARCH ARTICLE

Recombinant expression of osmotin in barley improves stress resistance and food safety during adverse growing conditions

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Abstract

Background

Although many genetic manipulations of crops providing biofortified or safer food have been done, the acceptance of biotechnology crops still remains limited. We report on a transgenic barley expressing the multi-functional protein osmotin that improves plant defense under stress conditions.

Methods

An *Agrobacterium*–mediated technique was used to transform immature embryos of the spring barley cultivar Golden Promise. Transgenic barley plants of the T0 and T1 generation were evaluated by molecular methods. Transgenic barley tolerance to stress was determined by chlorophyll, total protein, malondialdehyde and ascorbate peroxidase content. Methanol extracts of i) *Fusarium oxysporum* infected or ii) salt-stressed plants, were characterized by their acute toxicity effect on human dermal fibroblasts (HDF), genotoxicity and affection of biodiversity interactions, which was tested through monitoring barley natural virus pathogen–host interactions–the BYDV and WDV viruses transmitted to the plants by aphids and leafhoppers.

Results

Transgenic plants maintained the same level of chlorophyll and protein, which significantly declined in wild-type barley under the same stressful conditions. Salt stress evoked higher ascorbate peroxidase level and correspondingly less malondialdehyde. Osmotin expressing barley extracts exhibited a lower cytotoxicity effect of statistical significance than that of wild-type plants under both types of stress tested on human dermal fibroblasts. Extract of *Fusarium oxysporum* infected transgenic barley was not able to damage DNA in the Comet

assay, which is in opposite to control plants. Moreover, this particular barley did not affect the local biodiversity.

Conclusion

Our findings provide a new perspective that could help to evaluate the safety of products from genetically modified crops.

Introduction

Osmotin is a 26-kDa protein belonging to the 5th class of pathogenesis-related proteins (PR 5) together with thaumatin, zeamatin and others. Osmotin was first characterized in *Nicotiana tabacum* plants adapted to saline conditions [1, 2]. Later, its homologs were found in both monocots and dicots across the whole plant kingdom [3]. Osmotin is a multifunctional protein that plays an important role in the plant immune system during stress. Abiotic originators of stress such as drought, salt and cold induce osmotin expression. As the name indicates, osmotin plays a role as an osmoprotectant, providing enzyme protection and protein chaperone functions. Biotic stress resistance is related to the ability of osmotin to activate the fungal receptor that induces the programmed cell death of fungi. Being a promising protein, it has served as a tool for biotechnology engineering for many decades [1].

The first transgenic plants containing the heterologous osmotin gene in their genomes were potato and tobacco [4] and many others followed, as summarized in reviews [3, 5]. More recently, transgenic monocotyledonous plants bearing the osmotin gene were developed as well. Osmotin was first expressed in a monocot wheat in 2008 [6] followed by rice another three years later [7]. The genetic manipulation of monocots is a promising and still developing field, because their agriculture is significantly endangered by climatic changes. Monocots play an important role in agriculture for animal feed and for human nutrition, as they are among the most often planted crops (*e.g.* wheat, barley, rice, maize and others), however, the application of their GM variants still remains limited.

Even though transgenic crops are one of the most characterized and toxicologically tested plants, many of them have never been applied (*e.g.* Golden Rice [8]). The economic aspects, environmental benefits and higher yield of GM crops [9] usually do not affect public opinion significantly. Even though biofortified crops (reviewed *e.g.* in [10]) with improved properties and safer food [11] were reported years ago, the usage of GM crops is still restricted. No conventionally bred crop has been so thoroughly tested for toxicity, allergenicity or effect on the environment, intestinal microbiome and nutrition. However, what is often neglected is the increased toxicity of crops protecting themselves against harsh environmental conditions.

As a protection against stress, plants have adapted their own immune system [12] in order to survive. Nonetheless, many of the secondary metabolites formed this way can be toxic compounds, *e.g.* glycoalkaloids [13], steroidal alkaloids [14], flavonoids [15, 16] and glycosides [17, 18]. Furthermore, the toxicity of plant immune system compounds is often supplemented by the toxicity of the pathogen's secondary metabolites, *e.g.* mycotoxins [19].

The aim of this work is the development of a transgenic barley with improved resistance to stress originators, documentation of this higher resistance by biochemical methods, and a proof of better toxicological properties for consumers compared to commercially used crops. As an often-mentioned disadvantage of GM plants, its influence on biodiversity is described. Here, we also focused on studying the effect of this GM barley on the spreading of viruses by

aphids and leafhoppers. Two economically most important virus pathogens for barley cereals in Europe were selected–Barley yellow dwarf virus, transmitted by aphids, and Wheat dwarf virus, transmitted by leafhoppers.

Materials and methods

Barley transformation

The osmotin gene (*OSM*, GenBank: M29279.1, NCBI) was synthetized artificially (GeneArt Gene Synthesis, Thermo Fisher Scientific), therefore codon optimization for barley was performed. The osmotin gene was further cloned into the vector pDONR207 (Invitrogen) by BP clonase reaction (Gateway, Thermo Fisher Scientific). The construct was verified by digestion. The osmotin gene was inserted by LR clonase reaction (Gateway LR Clonase, Thermo Fisher Scientific) into the destination vector pBract214 (http://www.bract.org). The pBract214 vector was designed for the transformation of barley. The gene of interest is under the control of the maize Ubi promoter. The vector contains the *hpt* gene conferring hygromycin resistance under the CaMV 35S promoter. Correct orientation of the transgene was verified by restriction analysis and sequencing. The Vector pBract214::*osm* (S1 Fig) and the helper plasmid pSoup were transformed into the *Agrobacterium tumefaciens* strain AGL1 by electroporation. The *Agrobacterium*-mediated transformation of the immature zygotic barley embryo genotype 'Golden Promise' was performed according to the transformation protocol by Harwood et al [20]. Explants were cultivated *in vitro* on selection—callus induction, and regeneration medium and transferred into soil. Putative transgenic T0 plants were screened by PCR analysis. The analysis was performed with genomic DNA that was isolated from leaf tissue of the regenerated plants. For PCR reaction, premix REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich, USA,) was used. The presence of the osmotin gene was determined by amplifying a 222-bp fragment using the primers F: 5´-GCCCTGCCTTCATACGCTAT-3´ and R: 5´-TACGGGCAGTTGTTCCTCAC-3´. The presence of the *hpt* selection gene by amplifying a 275-bp amplicon using the primers F: 5´-GATTGCTGATCCCCATGTGT-3´and R: 5´-GCTGCTCCATACAAGCCAAC-3´.

Transgene expression was verified at the mRNA level. Where not stated otherwise, all procedures were done according to the manufacturer's instructions. RNA was extracted from young leaf tissue of the transgenic plant with an RNAqueous Total RNA Isolation Kit (Thermo Fisher Scientific). The sample was treated with a Turbo DNA-free Kit (Thermo Fisher Scientific) and RNA concentration was assessed spectrophotometrically (DeNovix, DS-11 Spectrophotometer). 1 ug of total RNA was reverse transcribed using RevertAid H minus Reverse transcriptase (Thermo Fisher Scientific). To analyze the reaction efficiency, dilution series of the selected cDNA samples were prepared. The endogenous gene for elongation factor [21] was selected as an internal control. For mRNA expression verification, a SensiFAST SYBR No-ROX Kit (Bioline) was used. Three-step PCR was conducted using a MyGo Mini real-time cycler (IT-IS Life Science Ltd.). Primer sequences for osmotin transcript detection were F: 5´-TCAGGTCCAGCTTCGTGTTC-3´and R: 5´-TACGGGCAGTTGTTCCTCAC-3´and produced an amplicon of 85 bp. Initial denaturation at 95˚C for 180 s was followed by forty cycles of denaturation at 95˚C, annealing at 60˚C and elongation at 72˚C. Reaction was terminated by a final 5 min extension at 72˚C. Melting analysis and electrophoretic separation of PCR products were done to verify primer specificity. Transgenic barley plants with verified expression of osmotin gene were grown in greenhouse to maturity. Immature embryos (T1 generation) were dissected from young caryopses of T0 plants and were selected on half-MS medium containing hygromycin 75 mg/L. Subsequently, the germinating plants were transferred to pots. All the germinated plants were analyzed for the presence of the osmotin and *hpt* transgenes by PCR.

Exposure of transgenic and control plants to the stress

Isolated embryos of the T1 generation of obtained GM plants and of the non-GM barley were sown in individual pots (10 x 10 cm) filled with universal gardening substrate and placed in growing chambers under controlled conditions (temperature 24/16˚C, air humidity 70%, 16/8 day/night). Until 50 days after sowing, all plants had four developed leaves and were divided into groups according to the planned stress treatment. Each group consisted of 4 transgenic and 4 non-transgenic plants. The control group was planted under the same conditions, another group was irrigated with 200 mM NaCl every second day, the last group was treated with spores of *Fusarium oxysporum* DBM 4199 (OD = 0.7) once per week. One fully developed leaf was cut off every 3 days and kept at -80˚C until the biochemical analysis. 16 days after the beginning of the stress conditions, all biomass was harvested and lyophilized for toxicity testing.

The BYDV virus infection was implemented using the aphids *Rhopalosiphum padi* carrying the BYDV-PAV species [22]. At the stage of two unfolded leaves, approximately five viruliferous aphids were transmitted onto each of 6 transgenic and 6 control group plants. The aphids were maintained there for four days, after that, the plants were treated with insecticide (Mospilan, Nippon Soda Co.) and kept under controlled conditions (temperature 19/15˚C, 16/8 day/night).

The WDV virus infection was administered using leafhoppers *Psammotettix alienus* carrying the WDV-B barley strain [22]. At the stage of the main shoot and one tiller being detectable, approximately five viruliferous leafhoppers were transmitted onto each of 6 transgenic and 6 control group plants. The leafhoppers were maintained there for fourteen days, after that the leafhoppers were removed by hand and the plants were closely checked for any missing leafhoppers. The plants were then kept under controlled conditions as before (temperature 19/15˚C, 16/8 day/night).

Both WDV and BYDV isolates, as well as infected aphids and leafhoppers, were obtained from the Virus Collection of the Crop Research Institute, Prague (Virus Collection, 2017). The aphid and the leafhopper count of insect vectors required for successful infection were estimated based on the long-term experience with testing of cereal cultivars in the Crop Reseach Institute, Prague [23, 24].

Biochemical assays

For chlorophyll content determination, 0.02 g of barley leaf tissue was used. Samples were incubated in 99% alcohol (1:100 (w/V)) in the dark for 24 h. The subsequent measurement was the same as described in the method by [25]. The preparation of samples for the determination of protein content and enzyme activities was universal in order to decrease sample weight and consumption of plant material. 0.1 g of leaf tissue was homogenized in liquid nitrogen into powder. Then, 2 ml of extraction buffer (50 mM phosphate buffer, 1 mM EDTA and 2% (w/v) polyvinylpyrrolidone of pH 7.8) was added. The homogenized sample was centrifuged at 14,000 × g at 4˚C for 30 min. Protein content was measured according to the method by Bradford [26]. The reaction mixture contained plant extract, extraction buffer and reagent in the ratio 1:1:8. The reagent and subsequent measurement was the same as in the original paper. The enzyme activity of ascorbate peroxidase was determined according to [27] without any modifications. Malondialdehyde content was determined according to [28] with slight modifications. Extract was prepared by the homogenization of 0.1 g of leaf tissue in liquid nitrogen and the addition of 2 ml of 80% ethanol followed by centrifugation at 14,000 × g at 4˚C for 20 min. In contrast to the original paper, a 0.4 ml aliquot was used for the preparation of the reaction mixture. After heating followed by cooling, the mixture was centrifuged at 1,000 g at 4˚C for 20 min.

Hemolytic and cytotoxicity studies

Hemolytic activity was determined according to a previous report [29]. 1 μl of plant methanol extract (100 $mg.ml^{-1}$) was used per spot. Triton X-100 (1%, 1 μl per spot, Sigma Aldrich) was used as a known hemolytic agent. The toxicity in mammalian tissue culture was studied on HDF–human dermal fibroblasts, Sigma-Aldrich, 106-05N).

HDF cells were cultivated in DMEM (Sigma-Aldrich) enriched with 10% fetal bovine serum (Sigma-Aldrich). The cells were maintained in media without antibiotics; however, for experiments media supplemented with Antibiotic Antimycotic Solution were used (commercial mixture of penicillin, amphotericin and streptomycin, Sigma-Aldrich).

Cells were harvested from exponential-phase cultures by a standardized detachment procedure using 0.25% Trypsin-EDTA, and the cell number was counted automatically using a Roche's CASY Cell Counter and Analyzer. 100 ml of 10^5 $cells.ml^{-1}$ was seeded into the wells for cytotoxicity experiments. Each concentration was tested in quadruplicate within the same experiment in the concentration range 62.5–1,000 $\mu g.ml^{-1}$. Viability was evaluated after 72 h by standard resazurin assay [30] using fluorescent measurement (560/590 nm). Viability was calculated as (sample fluorescence–fluorescence of resazurin) / (fluorescence of cells without treatment—fluorescence of resazurin).

Genotoxicity assay was determined using Hek 293T cells (Human embryonal kidney cells, Sigma-Aldrich, USA) according to the Comet assay as previously described [31]. The cells were cultivated as described for HDF cells. For the experiment, 10^5 $cells.ml^{-1}$ were seeded into the 12-well plates. After 24 hours of cultivation in CO_2 incubator, the medium was changed and the plant extracts were added to the final concentration of 3 $mg.ml^{-1}$. After 24 hours of incubation, the positive control of genotoxicity was realized by addition of H_2O_2 (4.2 $mg.l^{-1}$) for 10 min to the cells. As a negative control served cells without any treatment. After the cells harvesting and electrophoresis, fluorescent microscopy (Olympus IX81 equipped with Texas Red filtr) was used for comets visualization and ImageJ software was used for evaluation TailDNA (%).

Detection of BYDV infection

RNA was isolated by the traditional method using TRIzol reagent (Thermo Fisher Scientific, USA). RT-qPCR assay was performed in a Roche LightCycler 480 Instrument II using LightCycler 480 SYBR Green I Master (Roche Applied Science, Germany), RT Enzyme Mix (ArrayScript UP Reverse Transcriptase and RNase Inhibitor, Thermo Fisher Scientific) and PVinterF and YanRA primers [32]. The RT-qPCR was performed at 48˚C for 30 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min and a melting-curve analysis (95˚C 15 s, 60˚C 1 min, 95˚C 15 s, 60˚C 15 s). The qPCR efficiency was determined as E = 105.51%, R2 = 0.9964 and the linear standard curve interval as 6.89×10^1–6.89×10^7 copies using triplicates for the standard and tested samples. For the standard curve, a specific BYDV nucleotide sequence (294 bp, primers `PVinterF+YanRA`) amplified by RT-PCR was inserted into the vector pGem-T Easy (Promega) and cloned into *E. coli* JM-109. The selected colony with confirmed insertion sequence was cultivated and DNA extracted (Plasmid Plus Midi Kit, Qiagen, Netherlands) and then excised from the gel (Sigma-Aldrich x-tracta Gel Extraction Tool, Sigma-Aldrich, USA) and purified (GenElute Gel Extraction kit, Sigma-Aldrich). Thereafter, tenfold serial dilution of the transcripts were prepared.

For all samples, the mean detected BYDV concentration was calculated based on the tested triplicates and subsequently normalized using the RNA sample concentration detected spectrophotometrically. For these normalized BYDV titers, their log10 values were calculated and for each week, the log10 mean BYDV titers of the tested group and control group are depicted, together with the interval of plus and minus one standard error of the mean.

Detection of WDV infection

DNA was isolated by adding 0.5 ml of extraction buffer (1 M guanidine thiocyanate, 20 mM Na2H2EDTA, 0.1 M MOPS, pH 4.6, mercaptoethanol added to 0.2% just prior to use) to 50–100 mg of sampled tissue that had been disrupted and homogenized in liquid nitrogen. The solution was incubated for 30 min in a 60˚C water bath with occasional vortexing followed by phenol-chloroform-isoamyl alcohol (25:24:1, Affymetrix, USA) extraction, chloroform extraction, isopropanol and sodium acetate precipitation and two steps of 70% ethanol purification. The qPCR assay was performed on a Roche LightCycler 480 Instrument II using LightCycler 480 Probes Master (Roche Applied Science, Germany), UniWDVfw and UniWDVrv primers [33] and a TaqMan probe (`6-FAM-AGGCGAAGAATGATTCACCCT-BHQ-1`). The qPCR was performed at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min. The qPCR efficiency was determined as E = 99.49%, R^2 = 0.9989 and the linear standard curve interval as 2.9×10^3–2.9×10^8 copies using triplicates for the standard and tested samples. For the standard curve, a specific WDV nucleotide sequence (140 bp, primers `UniWDVfw+UniWDVrv`) amplified by PCR was inserted into the vector pGem-T Easy (Promega) and cloned into *E. coli* JM-109. The selected colony with confirmed insertion sequence was cultivated and DNA extracted (Plasmid Plus Midi Kit, Qiagen, Netherlands) and then excised from the gel (Sigma-Aldrich x-tracta Gel Extraction Tool, Sigma-Aldrich, USA) and purified (GenElute Gel Extraction kit, Sigma-Aldrich). Thereafter, tenfold serial dilution of the transcripts were prepared.

For all samples, the mean detected WDV concentration was calculated based on the tested triplicates and subsequently normalized using the DNA sample concentration detected spectrophotometrically. For these normalized WDV titers, their log10 values were calculated and for each week, the log10 mean WDV titers of the tested group and control group are depicted, together with the interval of plus and minus one standard error of the mean.

Statistical analysis of experimental data

The statistical significance of results was tested by analysis of variance followed by Duncan's test in *STATISTICA 12* (data analysis software system, StatSoft. Inc. 2013). For all statistical tests, the significance level was established at $p < 0.05$.

Results and discussion

Transgenic barley expressing tobacco osmotin gene

In order to avoid potential mutations originating from the cloning process, the osmotin gene was commercially synthetized *de novo*. The codon optimized osmotin gene was cloned into the expression vector pBract214. The possibility of tobacco osmotin gene expression under the constitutive promoter of the cauliflower mosaic virus has been already by our group demonstrated in barley previously [29]. Therefore, the same promoter was used in this case as well. Transgenic barley plants expressing the osmotin gene were prepared via *Agrobacterium*-mediated transformation. In total, 210 immature embryos were transformed, providing 26 regenerating plants (S2 Fig) of the T0 generation that were transferred into pots and grown to maturity in a greenhouse. The presence of the osmotin transgene was confirmed by PCR in 25 regenerated plants (S3 Fig). The ratio corresponds to a transformation efficiency of 12%, which is typically lower than for the transformation of dicotyledonous plants [34], but comparable with other barley transformation experiments [35]. Notably, transformation efficiency for *Agrobacterium*-mediated barley immature embryos utilizing hygromycin selection is 25% in average [20]. Incomplete T-DNA integration into host genomic DNA might occur during *Agrobacterium*-mediated transformation leading to an unintended loss of the selection

marker. Previously, it was reported that incomplete integration of T-DNA can reach up to 44% in monocot wheat [36]. In our experiment, *hpt* selection marker was detected in all transgenic plants thus confirming complete integration of T-DNA cassette (S4 Fig). Heterologous peptide expression can be driven by tissue specific or constitutive promoters. [37] demonstrated usage of root tip specific promoter to induce resistance to biotic stress induced by nematodes. Alternatively, constitutive promoter CaMV35S [38, 39] can be used for heterologous protein production in plants. In our study, expression of osmotin protein was driven by the strong constitutive maize *ubi* promoter that provides strong stable expression in all plant tissues. Broad expression of osmotin should enhance plant response to various biotic and abiotic stresses such as salt stress or fungal infection whose symptoms are not restricted into specific plant tissues but more likely tend to affect the whole plant. The expression of the osmotin gene was confirmed by transgene-specific RT-PCR, while transgene-specific amplicons were not detected in the WT. The specificity of PCR product was additionally verified by melting analysis after performed PCR reaction. The osmotin gene expression was demonstrated in all transgenic plants. There were not observed any visible abnormal phenotypic manifestations of transgenic plants comparing to WT plants suggesting that accumulation of heterologous osmotin protein in plant cells of transgenic barley do not affect substantially plant growth performance. Transgenic plants were prepared for the analysis of the effect of biotic and abiotic stress. First, immature embryos were selected on a medium containing hygromycin. Then, germinating plants were transferred to the pots and the presence of the osmotin transgene was verified by PCR. The segregation ratio of the osmotin transgene in the T1 generation showed Mendelian inheritance. Verified transgenic plants were used in the subsequent experiments.

Higher stress resistance of transgenic barley

Both transgenic and non-transgenic tobacco plants were exposed to stress caused by salinity (200 mM NaCl) and pathogen infection (*Fusarium oxysporum*). As can be seen e.g. in the S5 Fig, the transgenic plants showed a significant reduction in disease symptoms. Both types of stress were able to induce a decrease in protein content in wild-type barley. Many researchers have reported that the level of total soluble protein in crop plants decreases under abiotic stress. Stress usually leads to protein damage caused by *e.g.* reactive oxygen species [40] or by increased activity of proteases [41]. A downregulation of photosynthesis under several stresses was reported at the proteome level [42]. Salt and drought induced a decrease in the main proteins of photosystem II and in both chlorophyll *a* and *b* binding proteins as well as producing a downregulation of RuBisCO and key Calvin cycle enzymes in barley and wheat [43, 44]. However, under stress the transgenic barley expressing the osmotin gene maintained the same protein level as the non-stressed transgenic control plants (Fig 1). Similarly, a higher protein level was detected in strawberries recombinantly expressing osmotin [45] in comparison to non-transgenic plants during salt conditions.

As was mentioned above, photosynthesis is significantly affected during stress conditions; therefore, chlorophyll content in barley was measured in the presence of both types of stressing factors. Ongoing stress was detected in wild-type barley, where both *Fusarium* infection and salinity exhibited an influence on chlorophyll content (Fig 2). However, similarly to protein content, the transgenic barley maintained the same chlorophyll level as the control non-stressed plants. In agreement with our results, it has been [46] already reported that transgenic tomato plants expressing the osmotin gene had higher chlorophyll content during the drought and salt stress than the non-transgenic plants. Similarly, osmotin-expressing transgenic soybean, chilli pepper and strawberry exhibited higher chlorophyll content than the non-transgenic variants during salinity [45, 47, 48]. The connection between osmotin and

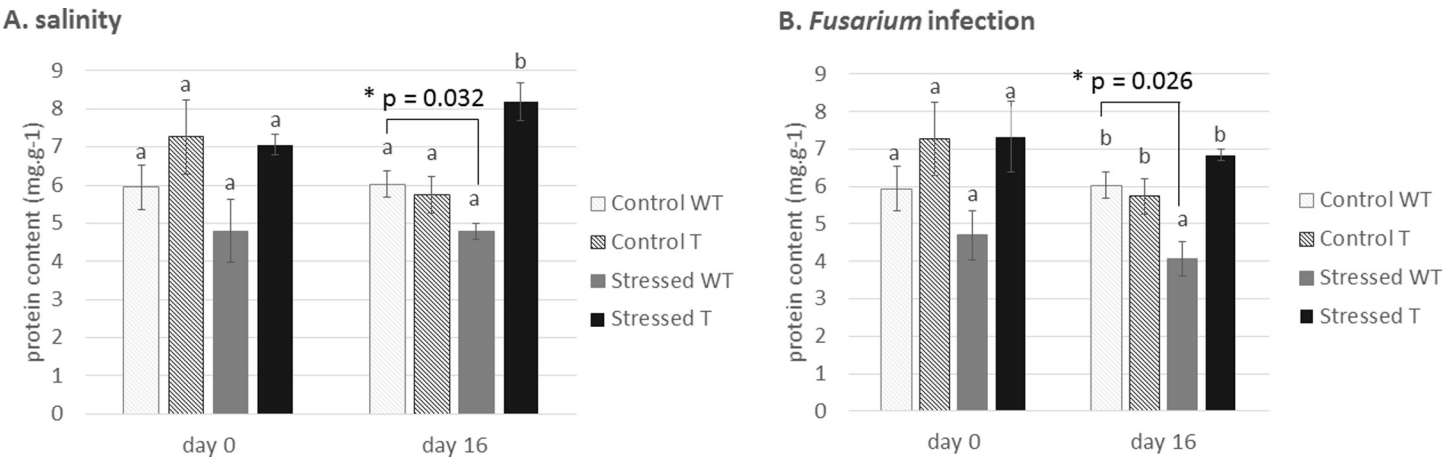

Fig 1. Protein content determined under both types of stress: (a) plants under abiotic stress and (b) plants under biotic stress. WT: non-transgenic (wild type) barley *Hordeum vulgare* L. var. Golden Promise, T: transgenic barley expressing tobacco osmotin gene. The results are shown as the average value of four plants measured in four replicates. Data are presented with the standard error of the mean (SEM). The statistical significance of results was tested by analysis of variance followed by Duncan's test ($p < 0.05$). * the groups were compared by two-choice t-test, the p values are presented.

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photosynthesis has been already reported, demonstrating an osmotin affinity to brassinosteroids, plant hormones affecting photosynthesis activity [49, 50].

As a major part of their defense system, plants have evolved an antioxidant strategy for overcoming stress conditions. Antioxidants (both enzymatic and non-enzymatic) prevent osmotic stress, oxidative stress, molecular damage, and even cell death [51]. Salt stress induces the production of reactive oxygen species (ROS), which causes oxidative stress. Therefore, the amount of antioxidant plays an important role during stressful conditions. Here, attention was focused on APX (ascorbate peroxidase). When influenced by stress, the transgenic barley plants exhibited a higher level of this antioxidant (Fig 3), indicating a lower susceptibility to salinity than the non-transgenic control plants. The connection between a higher level of APX and salt stress tolerance was demonstrated in genetically modified sweet potato [52]. Similarly

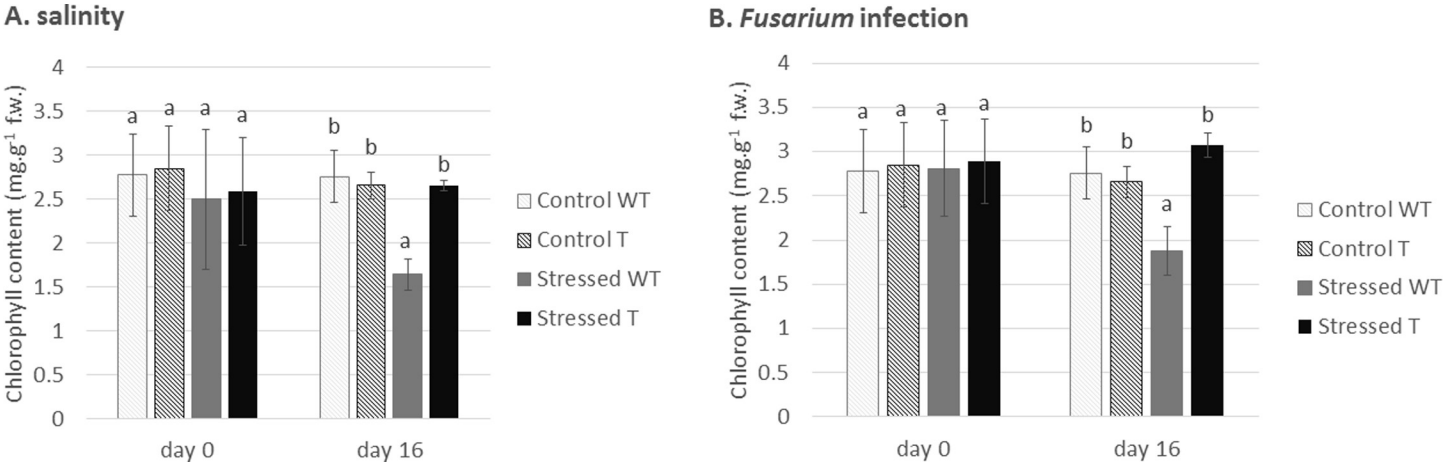

Fig 2. Chlorophyll content determined in both types of stress: (a) plants under abiotic stress and (b) plants under biotic stress. WT: non-transgenic (wild type) barley *Hordeum vulgare* L. var. Golden Promise, T: transgenic barley expressing tobacco osmotin gene. The results are shown as the average value of four plants measured in four replicates. Data are presented with the standard error of the mean (SEM). The statistical significance of results was tested by analysis of variance followed by Duncan's test ($p < 0.05$).

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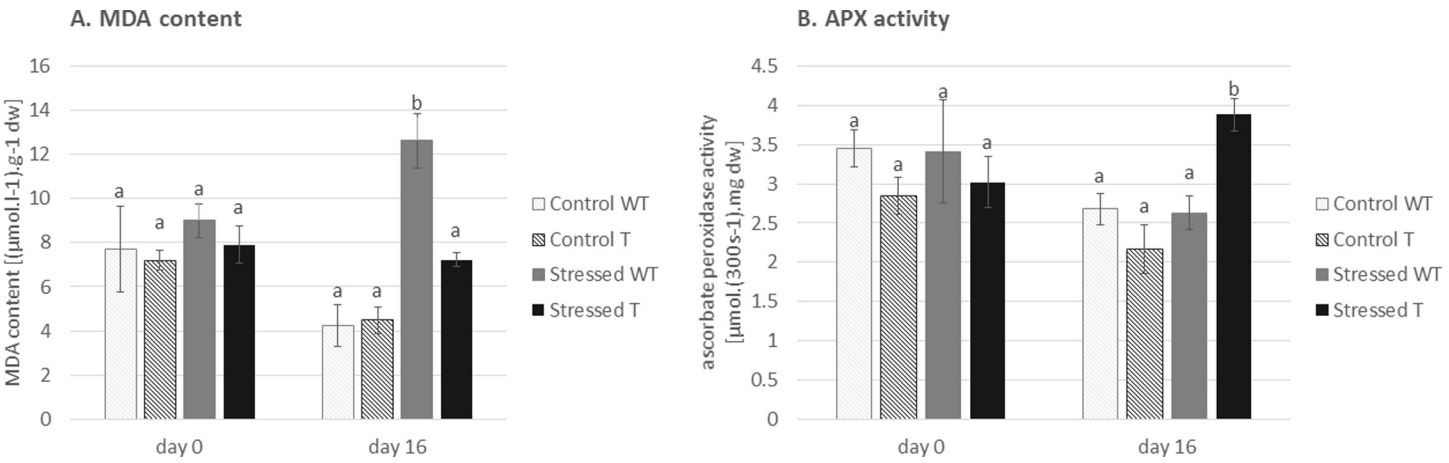

Fig 3. Effect of salinity on lipid peroxidation demonstrated as malondialdehyde (MDA) content (a) and antioxidant activity presented as ascorbate peroxidase (APX) activity (b). WT: non-transgenic (wild type) barley *Hordeum vulgare* L. var. Golden Promise, T: transgenic barley expressing tobacco osmotin gene. The results are shown as the average value of four plants measured in four replicates. Data are presented with the standard error of the mean (SEM). The statistical significance of results was tested by analysis of variance followed by Duncan's test ($p < 0.05$).

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to our results, transgenic chilli pepper and soybean expressing the tobacco osmotin gene exhibited a higher level of APX and improved salt stress tolerance [48, 53].

Lipid peroxidation is a process caused by free radicals (*e.g.* ROS) attacking unsaturated lipids in membranes. Malondialdehyde (MDA) is one of the end products of lipid peroxidation [54]. As we demonstrated that an increased level of APX prevents radical formation in transgenic plants, logically, we also found a lower amount of MDA in transgenic plants during salinity (Fig 4). Less MDA, indicating the effect of osmotin on cell membrane protection from damage by lipid peroxidation, has been already reported in transgenic olive plants exposed to drought [55].

Both types of stress led to the induction of stress markers such as a decrease in chlorophyll and protein content in wild-type barley plants, however, osmotin-expressing barley plants did not show evidence of ongoing stress, indicating their better preparedness for coping with the

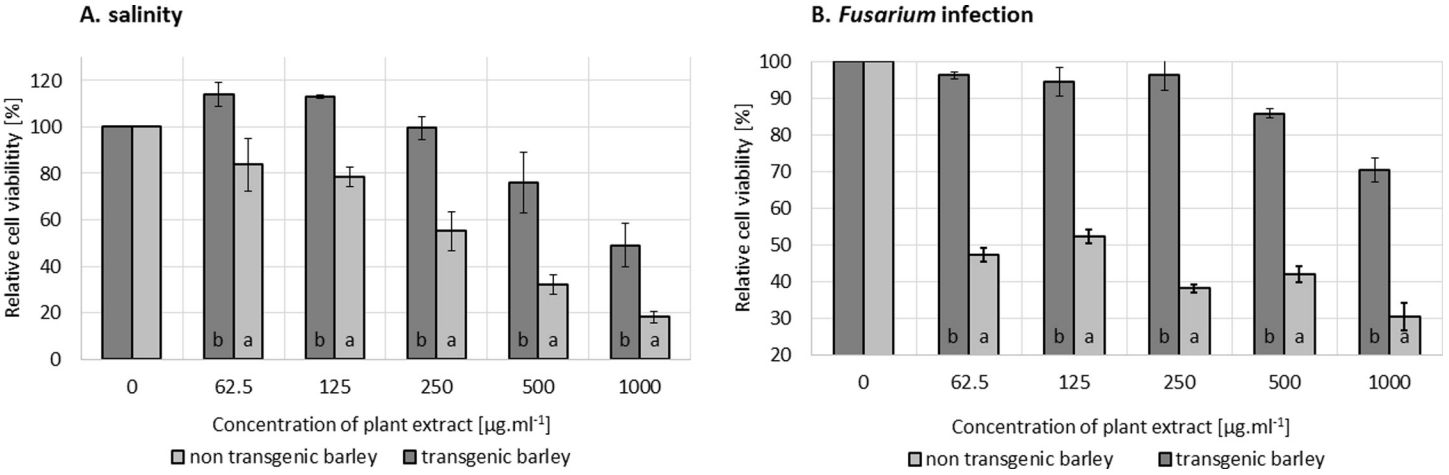

Fig 4. Cytotoxicity of methanol barley extracts on human dermal fibroblasts (HDF). The toxicity was evaluated after 72 hours of cells cultivation over a range of concentrations of extracts by standardized resazurin-based viability assay. The results are shown as the average value of four plants measured in four replicates. Data are presented with the standard error of the mean (SEM). The statistical significance of results was tested by analysis of variance followed by Duncan's test ($p < 0.05$).

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stressful conditions. Moreover, during conditions of salt stress, transgenic barley has a higher level of antioxidant and corresponding lower amount of MDA.

Lower toxicity of stressed transgenic barley in comparison to WT

At the end of the exposure to stress, the aboveground biomass was extracted by methanol. The extracts were then added to the growth medium of human fibroblasts in the concentration range from 0 to 1,000 $\mu g.ml^{-1}$. In both types of stress, there is evidence that transgenic plant extracts are less toxic than these of the non-transgenic (wild-type) ones. The cytotoxicity experiment was done in four technical repetitions for each plant sample, and the viability of fibroblasts (Fig 5) was evaluated as the average of four biological repetitions (meaning four independent plants, both transgenic and wild-type variant). The viability of cells decreased with a higher concentration of plant extracts. However, the toxicity of wild-type barley extracts of plants exposed to both types of stress was detected at the lowest tested concentration (62.5 $\mu g.ml^{-1}$). On the other hand, a toxicity of osmotin-expressing barley extracts was detected at a significantly higher concentration (500 $\mu g.ml^{-1}$). This finding could confirm our hypothesis that transgenic plants are better prepared for stressful conditions by osmotin expression and therefore do not produce so many secondary metabolites, which are mostly responsible for their toxicity. This finding was confirmed by genotoxicity comparison of transgenic and non-transgenic extracts as well where the plants expose to *Fusarium*

GM plants, which were modified to cope with environmental stress, have their internal metabolism significantly changed, preventing plant defense system over-response and the accumulation of toxicants, anti-nutrients and secondary metabolites during the ongoing stress [56]. The changes in toxic secondary metabolite content have been already demonstrated [57] in transgenic potatoes exposed to a pathogen. The genetic manipulation of carbohydrate

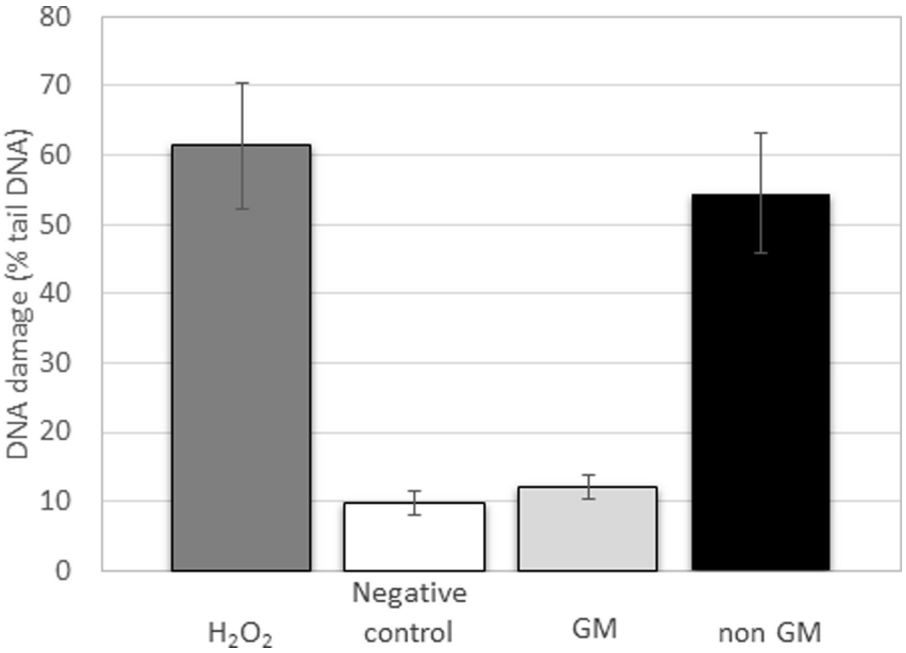

Fig 5. Genotoxicity was evaluated after 24 h of co-cultivation of human embryonic kidney cells with methanol extracts of both transgenic and non-transgenic barley exposed to *Fusarium oxysporum* infection. Cells incubated with H_2O_2 served as a positive control of DNA damage. Cells without any treatment served as a negative control. Data are presented as the average of 50 individual determinations with the standard error of the mean (SEM).

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metabolism and pathogen resistance in these potatoes led to changes in the profile of plant defense compounds, which were mainly characterized by a reduction in the level of the main glycoalkaloids R-solanine and R-chaconine.

As well as the expression of plant secondary metabolites, the secondary metabolites formed by the pathogen could have a significant effect on the acute toxicity of crop extracts. In particular, the toxicity of mycotoxins has been reported many times [58]. A lower amount of mycotoxins as a secondary effect of genetic manipulation was detected *e.g.* in a comprehensive study focused on transgenic maize [59].

The mycotoxins, as a secondary metabolite of fungi, could be responsible for the genotoxicity, which we detected in case of methanol extracts from the non-transgenic barleys infected by *Fusarium oxysporum*. However, the extracts from transgenic barley expressing the antifungal protein osmotin showed no toxicity in the same test as shown in Fig 5. The genotoxicity was evaluated after co-cultivation of plant extracts with human embryonal kidney cells (Hek 293T) by standardized Comet assay with appropriate controls.

Weak or no impact of transgenic barley on viral infection spread by aphids and leafhoppers

The influence of GM crops on biodiversity has been discussed and tested many times (reviewed *e.g.* by [60] and [61]), mostly demonstrating that GM crops have reduced the impacts of agriculture on biodiversity. Nevertheless, confirmation of this hypothesis is still needed. In this paper, we focused on the effect of barley expressing a multi-functional osmotin

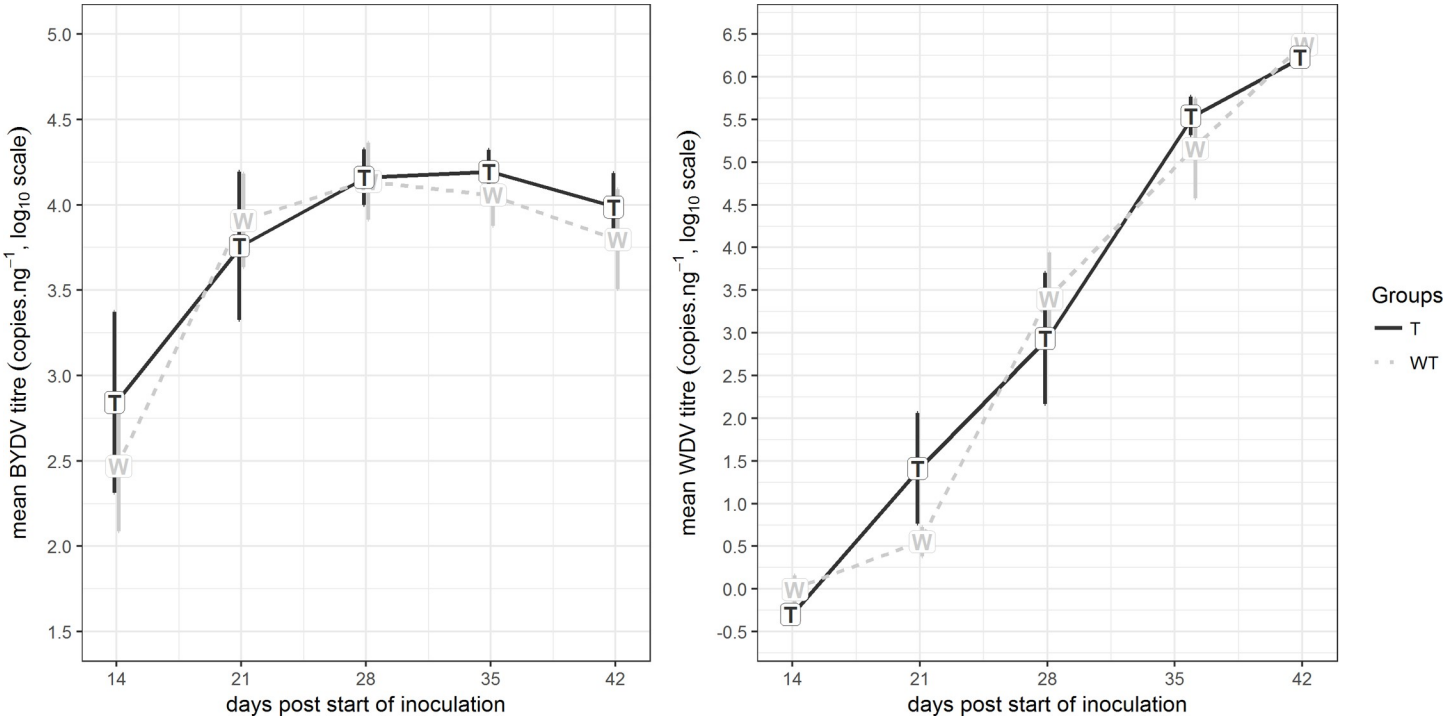

Fig 6. BYDV and WDV titres measured in plant leaves in 42-day period following inoculation (DPI–days post inoculation). BYDV and WDV titres were measured as the mean qPCR identified in titres from triplicates, normalized to (divided by) the RNA sample concentration (for BYDV) or DNA sample concentration (for WDV) and then a base-10 logarithm transformation was applied. From each tested period, the mean from all six tested plants of each group is depicted (WT: non-transgenic (wild type) barley *Hordeum vulgare* L. var. Golden Promise, T: transgenic barley expressing tobacco osmotin gene). Data are presented with the standard error of the mean (SEM). The statistical significance of results was tested by analysis of variance followed by Duncan's test ($p < 0.05$).

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protein on virus pathogen–host interactions. For barley, the effect of aphids spreading BYDV and the effect of leafhoppers spreading WDV was studied. Both viruses cause worldwide diseases of the most important crops including barley, wheat, rice and maize [62]. As is shown in Fig 6, the genetic manipulation of barley by the osmotin gene insertion had no effect on obtained virus titres through the whole tested period of first 6 weeks after inoculation. Both aphids and leafhoppers were able to attack GM barley and insert the virus into the phloem and to infect the tested plants. For phenotype observations, the difference in plant height and tiller count between wild-type infected and transgenic infected plants was tested 6 weeks after inoculation. For both the WDV and BYDV virus, neither the plant height nor the tiller count after 6 weeks from inoculation was proved to differ. The smallest t-test value for two-sample two-tailed t-test was measured for tiller count for WDV infection ($t = 0.086$), where the infected transgenic barley plants showed smaller, but not significantly different, tiller count (2.29) than the infected wild-type plants (3.38), i.e. exhibiting slightly less severe symptoms of WDV infection, where strongly infected plants often exhibit a high tiller count at the tillering stage. Similarly no impact of potato genetic manipulation on aphids was determined neither by [63] or by another research group, which found Bt corn to have no effect on aphids [64].

Conclusions

Many investigations have been carried out to elucidate the mechanisms of the response of the transgenic plant to abiotic and biotic stress, however, the acceptance of transgenic crops in agriculture and industry is still limited, particularly in the EU. We compared osmotin-expressing GM and non-GM barley exposed to biotic and abiotic stress in order to investigate whether their toxicity level under adverse conditions is comparable. The results clearly show that our transgenic barley has a decreased toxicity to human cells under conditions of a/biotic stress for which it is better prepared and exhibits higher stress resistance. These findings provide a new perspective that could help to evaluate the safety of products from genetically modified crops.

Supporting information

S1 Fig. Scheme of the expression vector pBRACT214 containing the osmotin gene.
(TIF)

S2 Fig. Regeneration of transgenic barley var. Golden Promise after transformation with osmotin gene. a) Regenerating plantlets from calluses after 6 wk on selection medium. b) Putative transgenic plants on regenerating medium. c) Transgenic plants T0 generation in greenhouse.
(TIF)

S3 Fig. Detection of osmotin transgene in the genomic DNA of T0 regenerants. Lane 1–11: samples; lane 12: negative control (DNA/RNA free water); lane 13: positive control (plasmid pBRACT214::osm); lane 14: negative control (genomic DNA of WT plants); lane 15: DNA standard (50 bp DNA ladder, Bioline). Size of PCR product is 222 bp.
(TIF)

S4 Fig. Detection of transgene encoding the marker for selection (hpt gene) in the genomic DNA of T0 regenerants. Lane 1–3: samples; lane 4: negative control (DNA/RNA free water); lane 5: negative control (genomic DNA of WT plants); lane 6: positive control (hpt positive plant), lane 7: DNA standard (2-kb DNA ladder, Bioline). Size of PCR product is 275 bp.
(TIF)

S5 Fig. Exposition of transgenic and control barley to fungi infection. left: Non-transgenic barley (left) versus transgenic barley bearing tobacco osmotin gene (right) after biotic stress (15 days after first spraying of Fusarium oxysporum spores). Right: symptoms recognized on non-transgenic barley leaves after stress (chlorosis, necrosis, premature leaf drops and wilt of whole plant).
(JPG)

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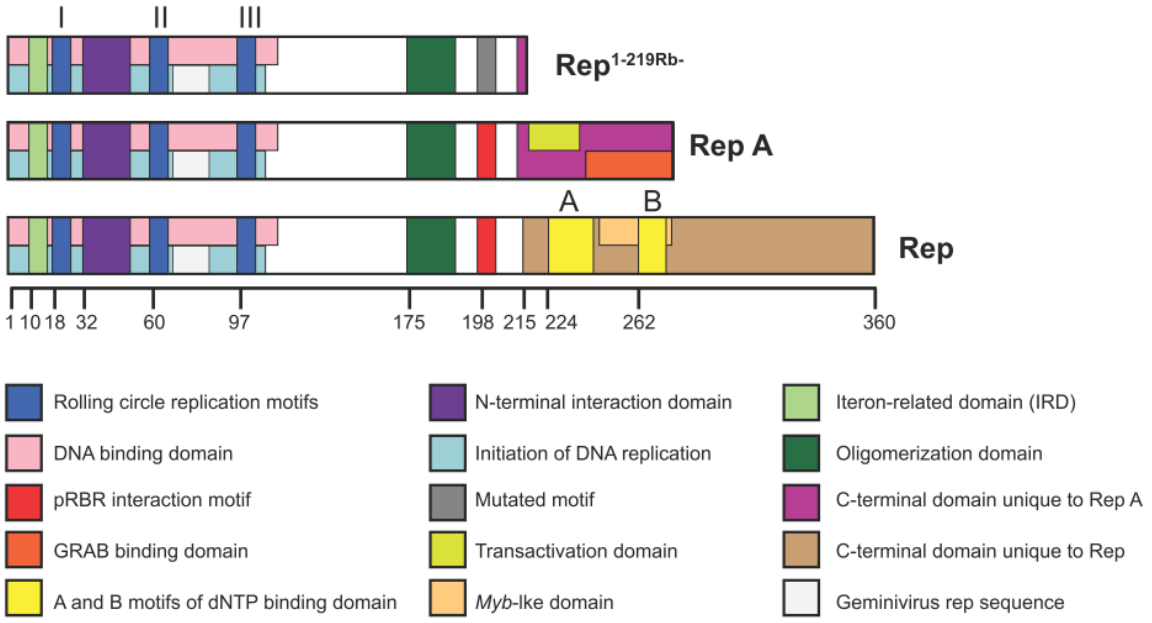
4 Sumární diskuze

4.1 O možnosti navození rezistence obilnin pomocí transkripce či exprese některých úseků virové DNA

Shepherd a kol. (2007b) studovali mechanismus replikace u MSV, tzn. prakticky nejbližšího příbuzného viru WDV, z hlediska podobnosti sekvence aminokyselin jednotlivých proteinů, organizace genomu či mechanismů šíření viru. I když v mnoha případech rezistence na principu RNA-interference u geminivirů vede k úspěšnému navození rezistence (Shepherd a kol. 2009), transkripce zkrácených DNA sekvencí pro Rep v antisense orientaci neměly žádný efekt na replikaci viru (Shepherd a kol. 2007b) v buněčné kultuře kukuřičných buněk. Pouze transkripce nezkrácené DNA sekvence pro Rep v antisense orientaci vykazovala snížení virové akumulace (Mangwende 2000). Nicméně několik různých konstruktů viru s mutacemi uvnitř původní sekvence proteinu Rep bylo schopno prakticky zastavit replikaci viru MSV v buněčné kultuře kukuřičných buněk. Příslušná mutovaná sekvence Rep proteinu ovšem v transformovaných rostlinách způsobovala řadu růstových a vývojových defektů, což velmi pravděpodobně souvisí se známými funkcemi samotného Rep proteinu a jeho interakcí s hostitelskými buňkami. Fenotypově normální zdravé rostliny rezistentní vůči viru proužkovitosti kukuřice se podařilo

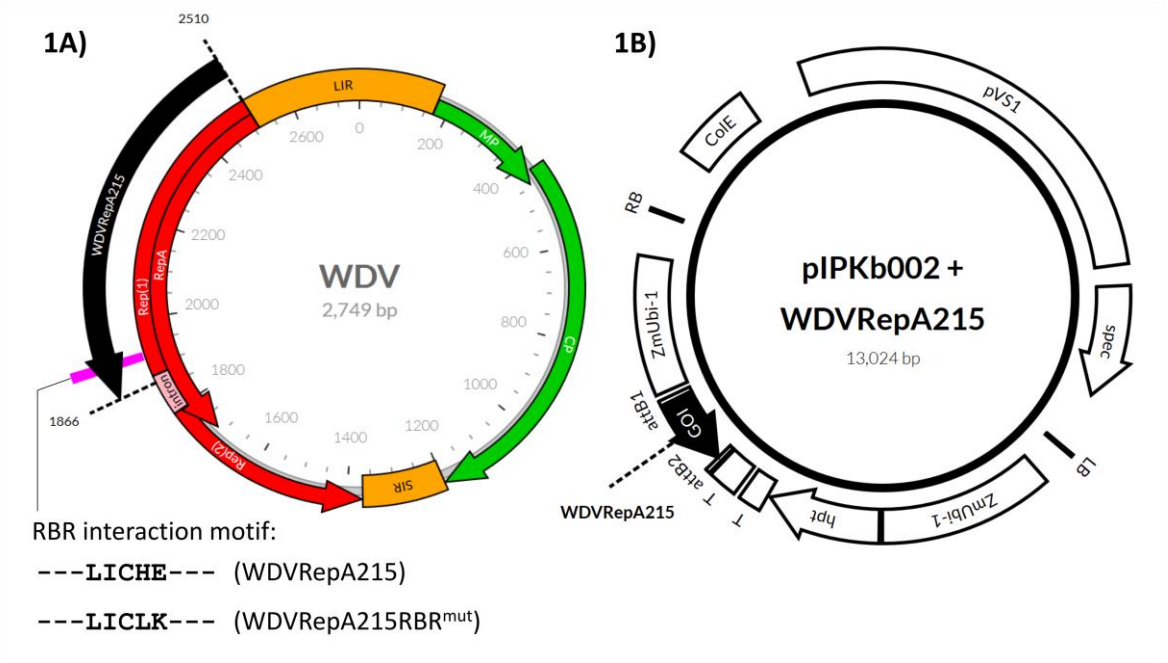
Obrázek 4. Známé sekvenční motivy a funkční domény MSV Rep proteinu, MSV Rep A proteinu a zkrácené verze Rep proteinu exprimované v transgenních rostlinách (Shepherd a kol. 2007b). Číslování aminokyselin je relativní vůči počátečnímu (N-koncovému) methioninu. Potenciální DNA vazné domény a GRAB-vazná doména jsou zobrazené na pozicích analogických jejich lokacím v Rep proteinu begomoviru, respektive v WDV Rep A proteinu.



Obrázek 5. Srovnání netransgenních (vlevo) a transgenních rostlin kukuřice (vpravo) exprimujících příslušnou zkrácenou Rep sekvenci cca 30 dní po inokulaci MSV (Shepherd a kol. 2007b).

připravit až při použití zkrácené sekvence proteinu Rep s mutacemi. Příslušná zkrácená verze Rep sekvence stále zahrnovala motivy pro iniciaci replikace pomocí RCR, motivy pro vazbu na DNA, doménu pro interakci s RBR (s přidanými mutacemi) i motivy pro oligomerizaci, nicméně již tam chyběla jak příslušná homologická doména pro interakci s GRAB proteiny, tak doména s možnou vazností na další transkripční faktory (*myb*-podobné transkripční faktory), které mohly způsobovat pozorované vývojové defekty u transformovaných rostlin (viz také Obrázek 4). Rostliny s příslušnou zkrácenou verzí replikačního proteinu vykazovaly fenotypově znatelně nižší míru infekce (viz také Obrázek 5) i vyšší počty přeživších infikovaných rostlin než kontrolní rostliny. Navíc 90 % transgenních hybridních kříženců vzešlých křížením transgenní linie a hospodářsky využívaných, avšak k MSV náchylných, linií kukuřice vykazovalo zvýšenou rezistenci (Shepherd a kol. 2007a). Příslušný transgen sdílí cca 94% podobnost sekvence aminokyselin i s dalšími, agresivnějšími variantami viru MSV vyskytujícími se v dané oblasti, a tedy byl předpoklad pro dosažení obecné rezistence vůči MSV, která byla následně potvrzena (Shepherd a kol. 2014).

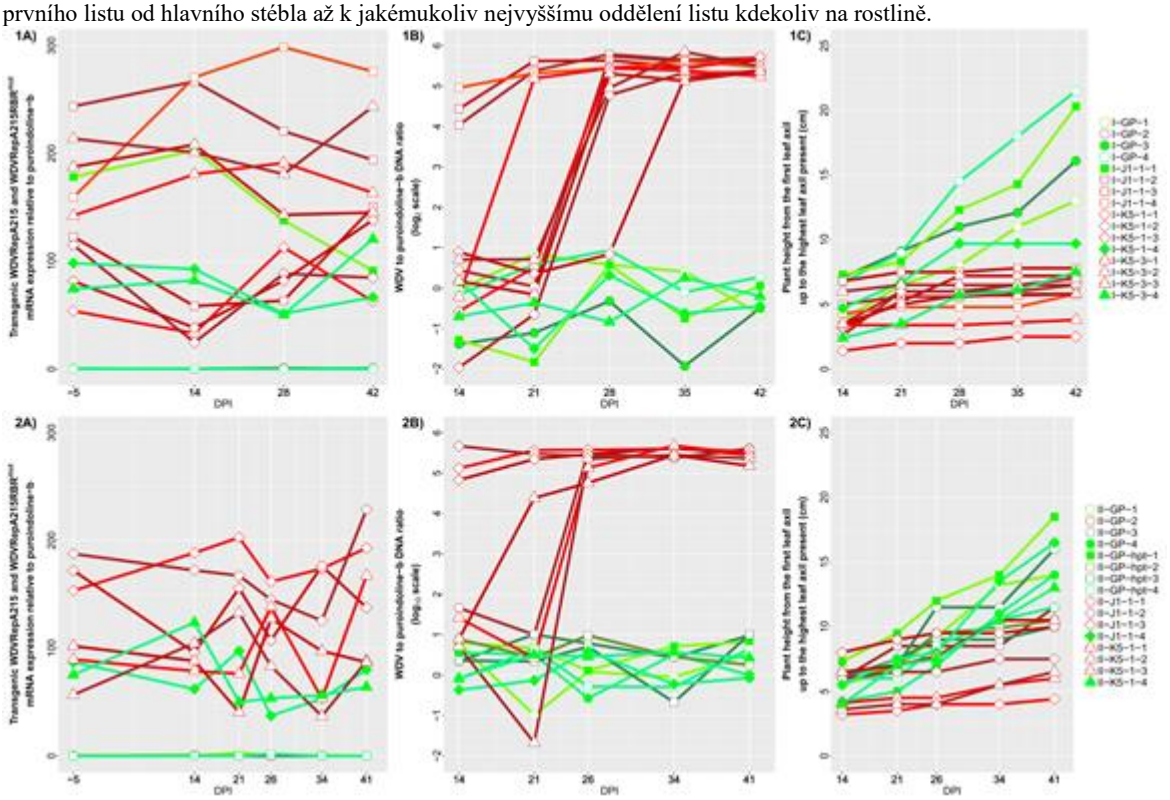
Obrázek 6. Struktura genomu WDV (izolát WDV FJ546189) s vyznačenou částí použitou pro transgenní konstrukt (1A) a struktura plasmidu pIPKb002+WDVRep215 (1B) použitého pro vnesení cílové sekvence do trostlin: (a) Struktura a pozice otevřených čtecích rámců v sekvenci viru zakrslosti pšenice v cirkulární DNA: MP – pohybový protein, CP – obalový protein, Rep – replikační protein, RepA – replikační asociační protein, LIR – dlouhý mezigenový úsek obsahující replikační počátek, SIR – krátký mezigenový úsek, RBR – interakční doména RBR, ColE – ColE1 replikační počátek, pVS1 – pVS1 replikační počátek, spec – streptomycin/spectinomycin adenyltransferáza, LB, RB – levy a pravý okraj T-DNA, ZmUbi-1 – ubikvitinový promotor následovaný ubikvitinovým intronem, hpt – hygromycin fosfotransferáza, T - terminátor, GOI – vnášený gen, attB1, attB2 – reakční místa pro LR klonázu.



V publikaci (Cejnar a kol. 2018b), jež je součástí této disertační práce, byla popsána příprava transformačních plasmidů (viz také Obrázek 6) vhodných pro přenos DNA obdobné části zkráceného replikačního proteinu WDV Rep do cílových rostlin. Byl připraven jednak konstrukt pIPKb002 + WDVRepA215 obsahující sekvenci homologickou k příslušnému zkrácenému úseku MSV Rep schopnému navodit rezistentní chování u kukuřice a rovněž i expresní plasmid pIPKb002 + WDVRepA215RBR^{mut} obsahující obdobnou dvoubodovou mutaci pro zesílení případného rezistentního projevu. Oba plasmidy byly konstruovány z vektoru pIPKb002 (Himmelbach a kol. 2007) pro vnesení DNA do cílových rostlin za pomoci agroinokulační metody.

V T0 generaci rostlin ječmene s vnesenými úseky pro WDVRepA215 a WDVRepA215RBR^{mut} byly úspěšně regenerovány rostliny ze 4 různých transgenních událostí WDVRepA215 (J1-1 až 4) a ze 6 transgenních událostí WDVRepA215RBR^{mut} (K5-1 až 6). Přibližně polovina odnoží z každého květináče úspěšně regenerovaných rostlin byla přesunuta do oddělených květináčů na konci fáze odnožování (DC 28-30 podle Zadokse). Pro zamezení projevů získané rezistence z důvodu inokulace v příliš pozdní fázi růstu (viz také (Lindblad a kol. 2004)) byla tato skupina otestována na odolnost proti WDV. Křísci polní byli ponecháni

Obrázek 7. Podrobné sledování rostlin J1 (WDVRepA215), K5 (WDVRepA215RBR^{mut}), GP (kontrola – původní rostliny ječmene odrůda Golden Promise) a GP-hpt (kontrolní rostliny hpt+) v prvních 6 týdnech po inokulaci WDV ve *screening experiment* 1 a 2: inokulované rostliny (bílá výplň) a neinokulované kontrolní rostliny (zelená výplň); rostliny, kde byl detekován virus WDV (červené čáry) nebo nebyl detekován (zelené čáry) 6 týdnů po inokulaci. (A) úroveň mRNA transgenních transkriptů WDVRepA215 a WDVRepA215RBR^{mut} stanovených pIPKbRepA215 AB SYBR RT-qPCR RNA a PINB AB SYBR RT-qPCR RNA assay. (B) Titry WDV v DNA stanovené WDV Roche TaqMan DNA qPCR normalizované na množství DNA stanovené PINB Roche SYBR DNA qPCR assay. (C) Výška rostlin měřená od oddělení prvního listu od hlavního stébla až k jakémukoliv nejvyššímu oddělení listu kdekoliv na rostlině.



na silném zdroji infekce po dobu 5 dnů před inokulací a následně po 4 dny na inokulovaných rostlinách, vždy přibližně 15 křísků na testovanou skupinu odnoží v každém květináči. Rostliny vykazovaly pouze mírné příznaky infekce (snížený růst, menší či chřadnoucí listy než u neinokulované kontrolní skupiny) a vzorky z jejich listového pletiva byly odebrány 40 dnů od zahájení inokulace (days following the start-of-inoculation – d.p.i.). Odnože jedné z testovaných rostlin uvadly již přibližně 30 d.p.i. DNA byla extrahována a následně kvantifikována UV spektrofotometricky a stanoven počet kopií DNA (zde relativně vůči počtu kopií referenčního genu puroindolin-b (PINB)). Titry viru WDV vykazovaly vysokou variabilitu ve výsledcích – byly přítomné jak rostliny s extrémně vysokým tak s extrémně nízkým titrem viru. Pro screening T1 generace byly vybrány pouze transgenní události s detekovaným extrémně nízkým titrem viru.

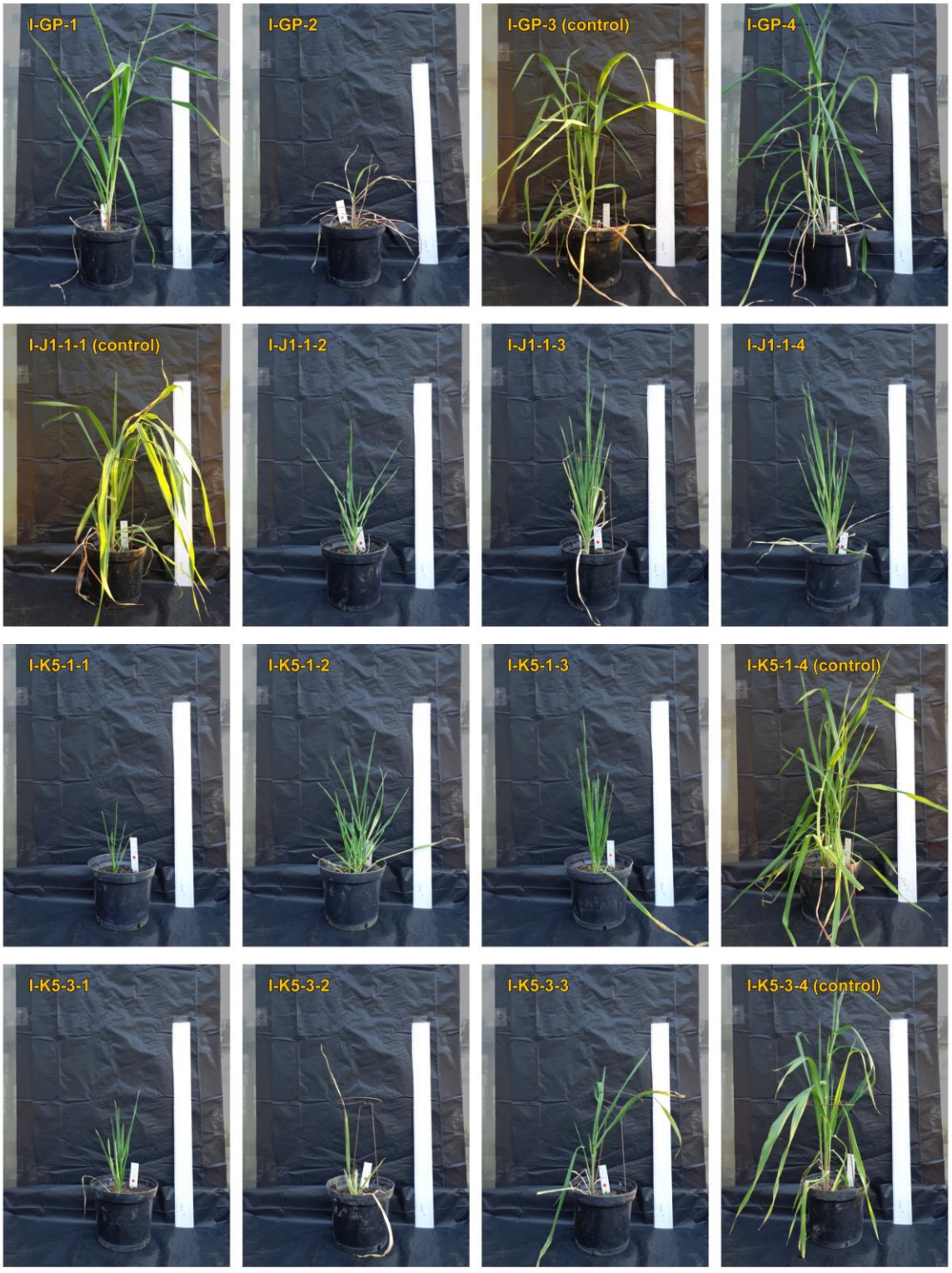
Pro detailní studium průběhu infekce WDV v rostlinách byl vybrán středně silný zdroj infekce. *Screening experiment 1* zahrnoval 3 inokulované rostliny z každé slibné transgenní události (J1-1, K5-1, K5-3), 3 inokulované kontrolní rostliny (odrůda ječmene Golden Promise) a 4 neinokulované kontrolní rostliny (jedna pro každou testovanou skupinu, tzn. celkem 16

testovaných rostlin). Ve fázi třetího listu (DC13 podle Zadokse) byli křísci polní z infekčního zdroje přeneseni na testované rostliny a ponechány po 6 dnů. Po dalších pět týdnů byly rostliny sledovány fenotypově, každý týden byl jeden list odstřihnut pro sledování průběhu infekce WDV i na molekulární úrovni a extrahována z něj DNA. Jednou za dva týdny byla rovněž extrahována mRNA a sledována úroveň transkripce WDVRepA215 či WDVRepA215RBR^{mut}. Zjištěná úroveň titru viru WDV i fenotypové pozorování (viz Obrázek **7** (1B, 1C)) potvrdily infekci u všech transgenních rostlin. U inokulovaných kontrolních rostlin byla infikována pouze 1 ze 3 kontrolních rostlin. Výrazný nárůst titru viru byl viditelný (pro rostliny z transgenních událostí K5-1, K5-3 a kontrolní rostlinu) přibližně 4 týdny po začátku inokulace. Pro rostliny Jl-1 byl tento nárůst patrný již zhruba o 2 týdny dříve – cca po 2 týdnech od inokulace. Rostliny nevykazovaly žádné další významné rozdíly po ukončení pokusu, kdy byly dále udržovány v řízených podmínkách (viz také Obrázek 8).

Biologické opakování (*screening experiment 2*) předchozího experimentu bylo provedeno s použitím skupiny 3 inokulovaných rostlin T1 generace z transgenní události J1-1, 3 inokulovaných rostlin z transgenní události K5-1, 3 inokulovaných kontrolních rostlin (odrůda ječmene Golden Promise) a 3 rostlin ječmene odrůdy Golden Promise s vloženým genem hpt pro selekci na antibiotiku ovšem bez transgenu WDVRepA215 či WDVRepA215RBR^{mut} (hpt+ kontrolní rostliny, artefakty screeningu pozitivních transformantů z předchozích transformací pomocí *A. tumefaciens*). Ke každé skupině byla přidána jedna rostlina jako neinokulovaná kontrola. Síla zdroj infekce byla stanovena na přibližně stejné úrovni jako pro předchozí experiment, podmínky a rozsah inokulace zůstaly také zachovány. Obrázek 7 (2B, 2C) následně shrnuje naměřené titry viru WDV a výšku rostlin ve sledovaném období. Všechny inokulované rostliny Jl-1 a K5-1, žádná z inokulovaných kontrolních rostlin hpt+ a jedna z kontrolních rostlin původní odrůdy Golden Promise byly infikovány. Na molekulární úrovni vykazovaly všechny inokulované rostliny J1 dřívější nástup výrazného nárůstu titru viru WDV v rostlině (2 týdny po inokulaci) ve srovnání s rostlinami K5-1 a infikovanou kontrolní rostlinou odrůdy Golden Promise (přibližně 4 týdny po inokulaci). S přihlédnutím k úrovni transkripce mRNA v pokusu *screening experiment 1* úroveň sledovaných transkriptů WDVRepA215 či WDVRepA215RBR^{mut} v mRNA v pokusu *screening experiment 2* potvrdila stabilní hladinu transkripce transgenních konstruktů nezávisle na přítomné WDV infekci ve sledovaných rostlinách (viz také Obrázek 7 (1A, 2A)).

Aby se potvrdilo, zda transgenní rostliny jsou náchylnější na WDV prostřednictvím snížené potřebné inokulační dávky, byl proveden *screening experiment 3* s extrémně slabým zdrojem infekce. Konečná testovaná skupina rostlin zde zahrnovala 3 inokulované rostliny

Obrázek 8. WDV inokulované rostliny a neinokulované kontroly (*screening experiment 1*) 77 dnů po inokulaci: I-GP-1 až 4 (ječmen odrůda Golden Promise), I-J1-1 až 4 (transgenní rostliny s WDVRepA215), I-K5-1-1 až 4 a I-K5-3-1 až 4 (oboje transgenní rostliny s WDVRepA215RBR^{mut}).



z transgenní události J1-1, 4 inokulované rostliny z transgenní události K5-1, 4 inokulované netransgenní kontroly a 4 inokulované hpt+ kontrolní rostliny. S extrémně slabým zdrojem

infekce byla infikována jedna rostlina ze skupiny J1-1 a jedna rostlina ze skupiny K5-1 a nebyly infikovány žádné kontrolní rostliny (ani netransgenní kontroly ani hpt+ kontrolní rostliny). Dokonce i v tomto experimentu byl mezi rostlinami infikovanými J1-1 a K5-1 pozorován přibližně dvoutýdenní posun na začátku detekovatelného zvýšení titru WDV.

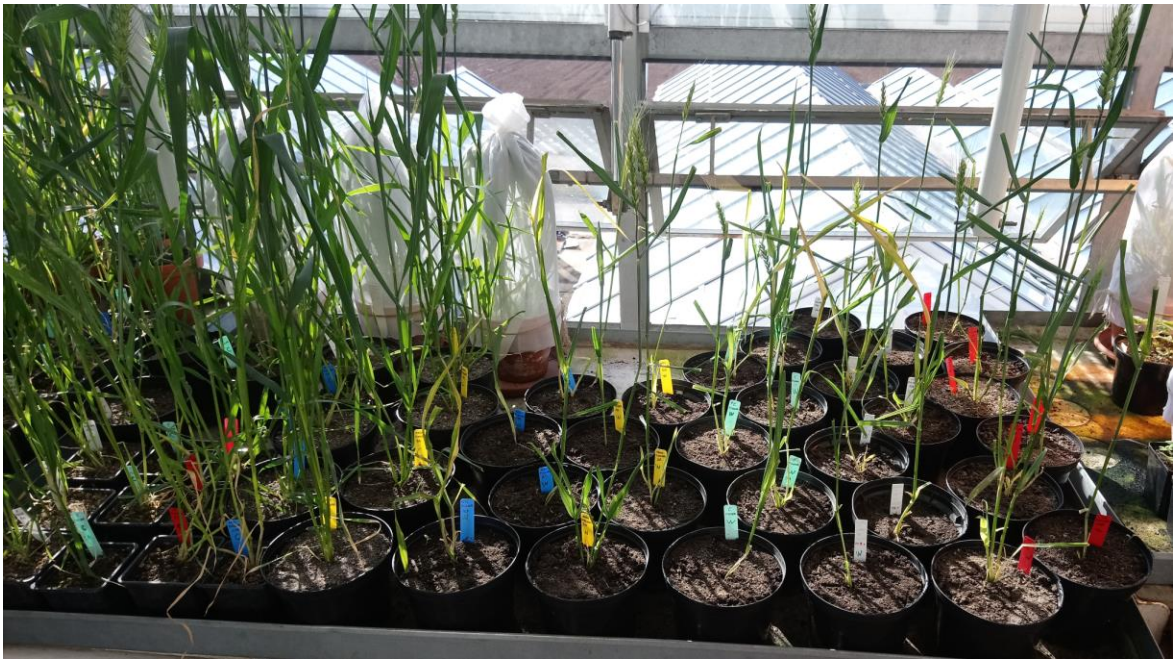
Různé načasování výrazného nástupu titru viru mezi transgenními rostlinami s transkripty – rostlinami s transkripty WDVRepA215 oproti rostlinám s transkripty WDVRepA215RBR^{mut}, tzn. navíc s dvoubodovou mutrací v interakční sekvenci pro doménu pRBR, bylo potvrzeno ve všech třech pokusech *screening experiment 1-3*. Přítomnost dvoubodové mutace nebyla letální pro infekci WDV v ječmeni jak je známo např. u projevů infekce viru MSV na kukuřici, nicméně z pokusů je patrně, že daná dvoubodová mutace má vliv na šíření viru WDV v ječmeni patrně prostřednictvím ovlivnění příslušných hostitelských proteinů. I když příslušná mutace přímo nezastaví šíření viru v ječmeni, narušení mechanismu infekce je stále patrně detekovatelné jako zdroj zpoždění pozorovaného vzestupu titru viru u rostlin WDV K5 ve srovnání s rostlinami WDV J1.

Všechny tři pokusy dále podporují hypotézu, že vnesený konstrukt prvních 215 kodonů aminokyselin pro replikační protein viru WDV pro zvolený izolát zvyšuje náchylnost rostlin ječmene k infekci WDV při srovnání s infekcí probíhajících na kontrolních rostlinách, na rozdíl od průběhu infekce blízce příbuzného viru MSV na kukuřici (viz také Shepherd a kol., 2007a, 2014). Avšak i u rostlin kukuřice seté se zvýšenou odolností proti viru MSVbyla zjištěna naopak zvýšená náchylnost vůči MSV u rostlin s ještě kratšími vloženými konstrukty sekvence MSV Rep. Tato zvýšená náchylnost rostlin kukuřice s vloženými velmi krátkými verzemi konstruktů sekvence MSV Rep je způsobena přítomností sekvencí pro vazebné domény na DNA v daném vytvářeném proteinu, které jsou dále zodpovědné za urychlení mechanismu replikace viru MSV, což může být také příčinou u WDV na ječmeni. V příslušné sekvenci WDV Rep jsou vazebné domény na DNA v odpovídajích úsecích sekvence rovněž přítomné (Koonin a Ilyina 1992). Pro MSV odpovídající strategie rezistence významně snižovala titry viru MSV i u izolátů s max. 60,6% shodou sekvence aminokyselin (Shepherd et al., 2014), čímž byla prokázána i robustnost takovéto strategie. Zde byly všechny experimenty prováděny s izoláty WDV běžně se vyskytujícími v přírodě, které společně vykazovaly nejméně 88% shodu sekvence aminokyselin a žádný z nich nevykazoval odlišné příznaky nebo změny v progresi onemocnění. Pokusy *screening experiment 1-3* rovněž ukázaly, že titr virů u inokulovaných transgenních i netransgenních rostlin ječmene odrůdy Golden Promise v 5. nebo 6. týdnu po inokulaci dosáhl velmi podobných hodnot, tzn. akumulace viru v testovaných listových pletivech pravděpodobně nebyla ovlivněna přítomností WDVRepA215 ani

WDVRepA215RBR^{mut}. Průběh pozdějšího pokusu (Cejnar a kol. 2018b) s výrazně slabším zdrojem infekce ukázal zvýšenou náchylnost transgenních rostlin k infekci patrně prostřednictvím snížené počáteční dávky viru nutné pro infekci ve srovnání s rostlinami bez transgenních konstruktů WDVRepA215 a WDVRepA215RBR^{mut}, což vedlo k ovlivnění celkové náchylnosti rostlin k infekci i přes obdobné cílové hladiny viru v infikovaných rostlinách.

V experimentech prováděných u transgenních rostlin pšenice (T1 generace rostlin) se stejnými vnesenými úseky pro WDVRepA215 a WDVRepA215RBR^{mut} (konstrukty vloženy do pšenice odrůdy Fielder) nicméně nedochází k projevům zvýšené náchylnosti vůči viru WDV a tyto mají chování více podobné transgenním rostlinám kukuřice a jejich reakci vůči MSV. Transgenní rostliny pšenice s vnesenými úseky vykazují po inokulaci virem WDV fenotypové charakteristiky velmi obdobné neinokulovaným kontrolám (viz Obrázek 9). Potenciální mechanismus takového rozdílného chování by se patrně mohl nalézat v odlišnostech vzájemných interkací patogen-host, jež se vyvinuly po specializaci mastreviru na jejich cílové hostitele (WDV u ječmene, WDV u pšenice, MSV u kukuřice), nebo jako vedlejší efekt jakékoliv jiné události reagující na vzájemnou koevoluci vztahů pathogen-host (viz také Shepherd et al., 2005), Guiu-Aragones a další (2015), Pinel-Galzi a kol. (2016)).

Obrázek 9. Neinokulované kontroly (vlevo) a rostliny inokulované WDV (vpravo) 2,5 měsíce od inokulace křískem polním (prováděno na T1 generaci rostlin, 3 křísi na každé rostlině po dobu 12 dnů): modrá – transgenní konstrukt NO2, žlutá – transgenní konstrukt NAC4, světle zelená – netransgenní kontroly, bílá – transgenní konstrukt J1 (WDVRepA215), červená – transgenní konstrukt K5 (WDVRepA215RBR^{mut}).



4.2 O zhodnocení možnosti přenosu viru na rostlinu molekulárně genetickými technikami

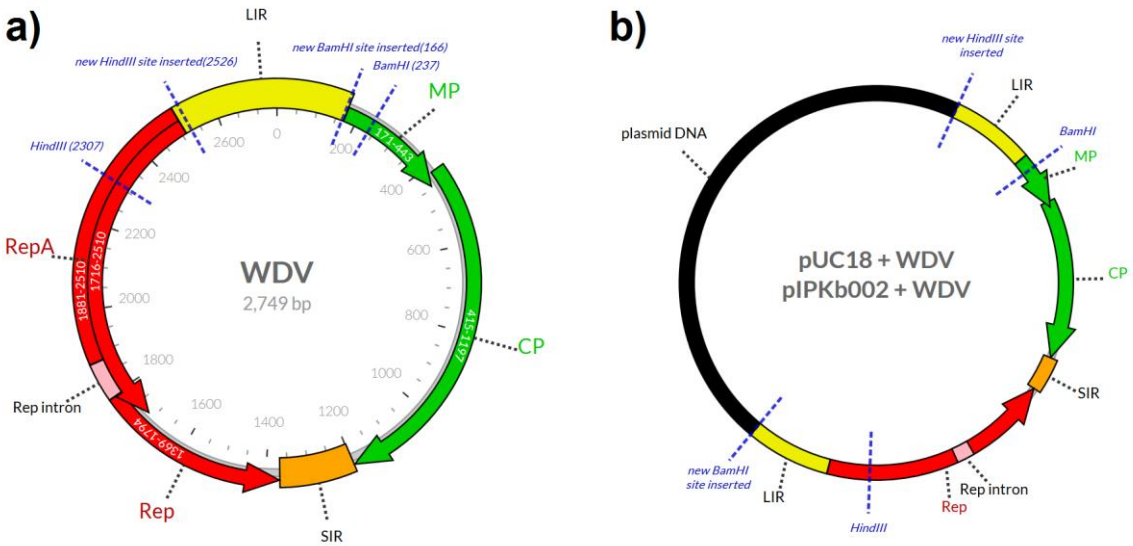
Metody pro inokulaci rostlinného materiálu viry jsou založené na zjednodušení inokulace přírodní cestou nebo na analogii vnášení sekvencí DNA do rostlinných buněk za účelem jejich transkripce či exprese (transformace rostlin). Umělé metody pro inokulaci rostlin slouží především ke zjednodušení testování chování viru nebo pro odhad stupně rezistence příslušného rostlinného materiálu, pokud přírodní inokulace je složitá nebo neekonomická pro daný účel. V literatuře je zmiňováno mnoho různých metod pro inokulaci rostlinného materiálu. Pokud ovšem je třeba vyvozovat informace z výsledků inokulace umělými inokulačními metodami pro celé rostliny či odrůdy, zůstáváme u metod, které nevyžadují speciální rostlinný materiál, jednu konkrétní odrůdu, či dlouhou fázi regenerace. Proto se další práce zaměřuje především na uzpůsobení či optimalizaci metod známých z úspěšného přenosu DNA do rostlin používaných zejména u přípravy transgenních rostlin, jako např. biolistická inokulace či agroinokulace. V literatuře jsou zmiňovány i jiné inokulační metody (např. inokulace elektroporací protoplastů (Lörz a kol. 1985, Woolston a kol. 1989)), ovšem, z výše zmíněných důvodů, metody vyžadující před nebo po inokulaci mnoho komplexních kroků se samotným testovaným materiálem nejsou nadále uvažovány. Podobně některé nové a velmi inovativní metody pro vnášení cizí DNA do genomické DNA hostitele mohou být patrně uzpůsobeny i pro vnesení virové DNA, nicméně nadále nejsou uvažovány z důvodu jejich překotného vývoje v současnosti, jež často teprve předchází jejich úplnému pochopení a rutinnímu použití (prime-editing (Anzalone a kol. 2019), CRISPR/Cas9 (Cong a kol. 2013)), některé jsou navíc doprovázené vysokou cenou při jejich aplikaci, jako např. zinc fingers, transcription activator–like effector nucleases (TALENs) nebo homing meganucleases.

Většina inokulačních metod pro viry nepracuje přímo s virovou DNA, jež se nesnadno získává ve větších množstvích v laboratorních podmínkách, ale vychází z infekčního klonu příslušného viru. V literatuře bylo prezentováno několik postupů jak získat přímo infekční klon viru zakrslosti pšenice (Hayes a kol. 1988a, Woolston a kol. 1988, Bendahmane a kol. 1995, Boulton 2008, Ramsell a kol. 2009). Důsledně je většinou doporučováno, aby DNA infekčního klonu obsahovala dvakrát úsek s vlásenkou potenciálního replikačního počátku, příslušná virová DNA se pak preferenčně vyštěpuje (viz také práce Redinbaugh (2003) a Ramsell a kol. (2009)). Infekční klon dále musí obsahovat replikační počátek pro replikaci v bakteriích (např. *E. coli*) a geny pro selekci plasmidu. Při agroinokulaci je třeba mít infekční klon i s replikačním počátkem pro zajištění stability a množení plasmidu i přímo v agrobakteriích. Nezávisí-li

ovšem replikační metoda na agrobakteriích, bylo dosaženo úspěšné inokulace u blízce příbuzných virů či při biolistické transformaci pšenice i při použití infekčních klonů v jednoduchých bakteriálních plasmidech (Redinbaugh 2003, Jones a kol. 2009).

Agroinokulační metoda přímo pro WDV je popsána např. v pracích Hayes a kol. (1988a), Woolston a kol. (1988), Boulton (2008), obecný protokol pro transformace pšenice či ječmene pomocí agrobakterií je možné nalézt např. v pracích Jones a kol. (2009), Ohnoutkova a kol. (2012). Z agrobakterií používaných pro transformaci obilnin jsou nejvíce používány kmeny C58C1, AGL1 nebo LBA4404. Jones a kol. (2005) pak doporučují hypervirulentní AGL1 či virulentní C58C1 oproti slabě virulentnímu LBA4404. Boulton (2008) uvádí podrobně samotný agroinokulační protokol. Obecně při přípravě média je doporučeno kultivovat agrobakterie na miskách místo v roztoku, kdy residuální antibiotikum může způsobovat u inokulovaných rostlin chlorotické příznaky. Podle Boulton (2008) technika kultivace nemá na efektivitu inokulace vliv, a to ani při až 100násobných rozdílech v koncentraci agrobakterií v inokulačním médiu, rovněž tak indukce agrobakterií v acetosyringonu před samotnou agroinokulací (studováno na *A. tumefaciens* kmenu C58C1, u ostatních kmenů může být jinak). Podle Boulton (2008) je také výrazně těžší agroinokulovat ječmen, naopak snadné je agroinokulovat kukuřici. Ramsell a kol. (2009) se zabývali agroinokulací ječmene včetně několika alternativních metod vpravení agrobakterií do rostliny. Nejvyšší účinnosti bylo dosaženo při pokapání rostlinných embryí agroinokulačním roztokem a případným následným propichem (viz také práce Redinbaugh a kol. (2001)). Někteří autoři

Obrázek 10. Struktura genomu WDV a odpovídající struktura zkonstruovaného infekčního klonu WDV: (a) Struktura a pozice otevřených čtecích rámců v sekvenci viru zakrslosti pšenice v cirkulární DNA: MP – pohybový protein, CP – obalový protein, Rep – replikační protein, RepA – replikační asociační protein, LIR – dlouhý mezigenový úsek obsahující replikační počátek, SIR – krátký mezigenový úsek. Nukleotidy číslované podle izolátu viru zakrslosti pšenice pšeničného kmene vyskytujícího se na území ČR (NCBI accession number FJ546188). (b) Struktura infekčních klonů pUC18 + WDV a pIPKb002 + WDV.



zmiňují konkrétní případy virů, kdy při inokulaci hrálo také významnou roli i případné namočení semen před samotnou inokulací (Chen a kol. 1992, Louie 1995). Je-li WDV zaměřen potenciálně na buňky floému, alternativně se nabízí i vpravovat inokulační roztok přímo do floému (viz také (Oparka a kol. 1994)).

Biolistické metody používané pro transformaci obilnin jsou popsány např. v práci Jones a kol. (2009). Při biolistické inokulaci jsou používány zlaté či wolframové mikročástice zvolené velikosti a stejných aerodynamických vlastností (typicky kulovitého tvaru), na kterých je vysrážena DNA (různými protokoly zahrnujícími spermidin, Ca^{+2} ionty a případně adhezivum polyvinylpyrrolidon pro přichycení mikročástic s DNA k povrchu, viz také (BioRad M1652411 Rev C). Samotná biolistická transformace je prováděna buď ve vakuové komoře (např. Biorad PDS-1000/He), kdy rychlá expanze plynu (hélia či jiného plynu s nízkou atomovou vahou) nejprve akceleruje tzv. disk makronosiče (macrocarrier disk) s nanesenými mikročásticemi (mikronosič) s DNA, a ty jsou následně nárazem vymrštěny vůči transformované části rostliny. Alternativním postupem je pak inokulace či biolistická transformace pomocí přenosného biolistického zařízení, jako např. Bio-Rad Helios Gene Gun, kde expanze plynu (opět He) přímo akceleruje částice v patroně. Výhodou mobilního zařízení je možnost inokulovat i pouze zvolenou část přímo na rostlině, bez nutnosti vkládat rostlinu do vakuové komory, nevýhodou může být naopak prudký výstřel proudu plynu vůči inokulované části či jejímu nosiči, což výrazně komplikuje inokulaci kultur kultivovaných na agaru. U biolistických transformací se parametry optimalizují nejprve pomocí sledování transientní exprese plasmidu s reportérovým genem, např. beta-glukuronidázou (GUS).

Pokud bychom uvažovali i o mechanické inokulaci jako použitelné cestě, taková metoda by musela překlenout nutnost cíleného navádění virové kapsle s virovou DNA do jádra, ať už pomocí výrazného nadbytku inokulované DNA nebo např. alternativně jako případná mechanická inokulace přímo extrahovanými virovými částicemi s již obsaženými proteinovými směrovacími sekvencemi. U geminivirů či přímo mastrevirů, v rámci jejich odlišností, ovšem protokoly pro extrakci virových částic konkrétního viru obecně nejsou aplikovatelné úspěšně na všechny ostatní příbuzné viry (viz např. Dollet a kol. (1986)). Speciálně pro WDV zde může hrát nezanedbatelnou roli i nízká koncentrace WDV virionů v rostlinné šťávě oproti např. příbuznému MSV (Markham a kol. 1989, Lindsten 1991). Kromě protokolů pro obecnou extrakci virů (Vajda 1978, Salazar a kol. 1999) je možné najít i protokoly přímo pro extrakci WDV (Lindsten a kol. 1980, Bisztray a kol. 1989), nicméně i když v tomto směru byly autorem provedeny experimentální pokusy, oproti inokulaci pomocí biolistické metody či agroinokulaci nebylo dosaženo významných výsledků.

V publikaci (Cejnar a kol. 2019), jež je součástí této disertační práce, byla popsána příprava infekčního klonu (viz také Obrázek 10) viru WDV. Byly zkonstruovány dva infekční klony ječného kmene WDV (pUC18+WDV a pIPKb002+WDV) pro navození infekce WDV v rostlinách. Oba infekční klony obsahují 1,1-násobek délky sekvence WDV, začínající v dlouhém mezigenovém úseku (LIR) obsahujícím potenciální replikační počátek (vlásenku DNA), procházející všemi virovými otevřenými čtecími rámci až po další přidanou kopii sekvence dlouhého mezigenového úseku včetně sekvence potenciálního replikačního počátku na konci. Infekční klon pUC18 + WDV obsahuje také počátek replikace pMBl z bakterie *Escherichia coli* (ColEl a pBR322) a gen pro rezistenci na ampicilin (sekvence beta-laktamázy z *E. coli*) z původního plasmidu pUC18 pro úspěšnou řízenou kultivaci v *E. coli*. Infekční klon pIPKb002 + WDV rovněž obsahuje replikační počátek ColEl pro replikaci v *E. coli*, replikační počátek pVS1 (z bakterií rodu *Pseudomonas*) pro replikaci v dalších rodech bakterií, jako např. *Agrobacterium tumefaciens*, dále gen pro hygromycin fosfotransferázu (řízený promotorem ubikvitinu z kukuřice) pro získání rezistence na hygromycin v rostlinách a také gen pro streptomycin/spektinomycin adenyltransferázu pro rezistenci na streptomycin/spektinomycin v bakteriích. Všechny přidané sekvence genů pocházejí z původního plasmidu pIPKb002.

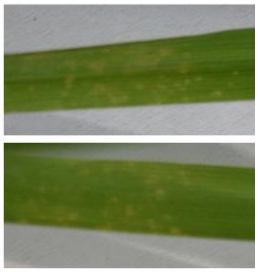
Při experimentech s agroinokulační metodou bylo otestováno přes 500 rostlin – pro optimalizaci parametrů agroinokulace a pro samotné testování pomocí agroinokulace. Nebyly zjištěny žádné významné rozdíly, pokud byly bakterie *Agrobacterium* pro inokulum kultivovány na misce nebo v roztoku se všemi nezbytnými antibiotiky, pouze s antibiotikem streptomycin pro agroinokulovaný plasmid nebo zcela bez antibiotik. U použitých protokolů bylo dosaženo PCR systémově pozitivní a ELISA pozitivní inokulace v 1-3 případech z 25-35 testovaných vzorků a 1-4 PCR pozitivních vzorků, ovšem bez ELISA pozitivního testu.

Pro ozimou pšenici odrůdy Svitava a pro pšenici jednozrnku (*Triticum monococcum*) se podařilo dosáhnout úspěšnosti až třech ELISA pozitivních a qPCR pozitivních případů na 25 inokulovaných rostlin a pro pšenici odrůdy Alana se podařilo dosáhnout jednoho ELISA pozitivního a qPCR pozitivního vzorku z 25 inokulovaných rostlin a jednoho ELISA pozitivního a qPCR pozitivního vzorku z 35 inokulovaných rostlin. Tento poměr se nezlepšil ani v případě indukce agrobakterií v indukčním pufru (acetosyringon 150 µM, 10 mM $MgCl_2$ ve sterilní destilované vodě) 4 hodiny ve tmě při pokojové teplotě těsně před inokulací. Inokulace rostlin starších než ve fázi DC12 podle Zadokse (cca 2 týdny staré) nevedla k pozitivním výsledkům, rovněž tak agroinokulace při níž místo vpichů do stonku bylo prováděno nasávání inokulačního roztoku do seříznutých listů (obě 0 z 16 testovaných rostlin). Samotná vizuální kontrola agroinokulovaných rostlin neumožňovala jasné rozlišení úspěšně

infikovaných rostlin. Žloutnutí listů, vadnutí nebo zakrnělost rostlin byla často způsobena pouze jako vedlejší efekt agroinokulačního protokolu. Samotná zakrslost byla pozorovatelná, nicméně infikované rostliny byly často vyšší než některé kontrolní rostliny silně trpící pouze vedlejším efekty agroinokulačního protokolu.

Postupně přibližně 250 rostlin pšenice bylo inokulováno biolisticky s infekčními klony umístěnými v plasmidech pUC18 nebo pIPKb002. Během optimalizace protokolu biolistické inokulace za použití barvení GUS se jako optimální tlak pro výstřel ukázal tlak 150-200 psi (1034 – 1379 kPa) s co možná nejmenšími zlatými částicemi (0,6 μm) a co možná nejmladšími rostlinami (fáze DC10-11 podle Zadokse). Vystřelované částice o větším průměru (1,0 μm) způsobovaly viditelně větší poškození cílových pletiv. U velmi mladých rostlin bylo nutné fixovat inokulační zařízení Bio-Rad Helios Gene Gun v dodatečném stojanu, aby byla s jistotou zasažena cílová oblast rostliny. Při inokulaci rostlin starších než 10 dní se nepodařilo dosáhnout ani jednoho pozitivního záchytu. Při pokusech o biolistickou inokulaci rostlin starších než 7 dnů (DC12-14 podle Zadokse) nebyly získány žádné pozitivní výsledky (0/16, pUC18 + WDV u rostlin 10, 14 a 21 dnů). Při použití biolistické inokulace se podařilo získat infikované rostliny ELISA pozitivní i qPCR pozitivní pro oba testované infekční klony (pUC18 + WDV 1 pozitivní případ z 12 inokulovaných rostlin a 2 pozitivní případy z 35 inokulovaných rostlin, pro pIPKb002 1 pozitivní případ z 35 inokulovaných rostlin). U všech infikovaných rostlin bylo množství zbývající DNA kvantifikováno jako nejvýše 275 kopií / ng extrahované DNA, tj. o několik řádů menší než detekované množství WDV v DNA. Rostliny úspěšně inokulované za použití částic o průměru 0,6 μm vykazovaly za několik dní po inokulaci žluté skvrny v oblasti cílové plochy výstřelu ve srovnání s ošetřenými kontrolami (viz také Obrázek 11). Nicméně při použití částic o průměru 1,0 μm nebo větších se objevily také velmi malé skvrny i u ošetřených kontrol.

Obrázek 11. Žluté skvrny objevující se cca 2 dny po úspěšné inokulaci (pšenice setá, odrůda Alana), použitý tlak hélia 180 psi, 0,6 μm zlaté částice, 0,5 μg v kartridži pro výstřel, 0,5 μg DNA WDV-infekčního klonu v kartridži.



Jako kontrolní pokus bylo deset rostlin z každé testované odrůdy inokulováno křískem polním. Úspěšně infikované rostliny byly získány se 100% účinností přenosu (viz Tabulka 4). Všechny rostliny s detekovanou infekcí WDV pomocí qPCR byly také potvrzeny jako infikované pomocí ELISA. Obrázek 12 udává naměřené titry viru WDV zjištěné pro každou inokulační metodu a testované odrůdy. Vysoká účinnost přirozené inokulační metody nám umožnila odhadnout průměrný titr WDV pro pšenici odrůd Svitava a Alana a pšenici

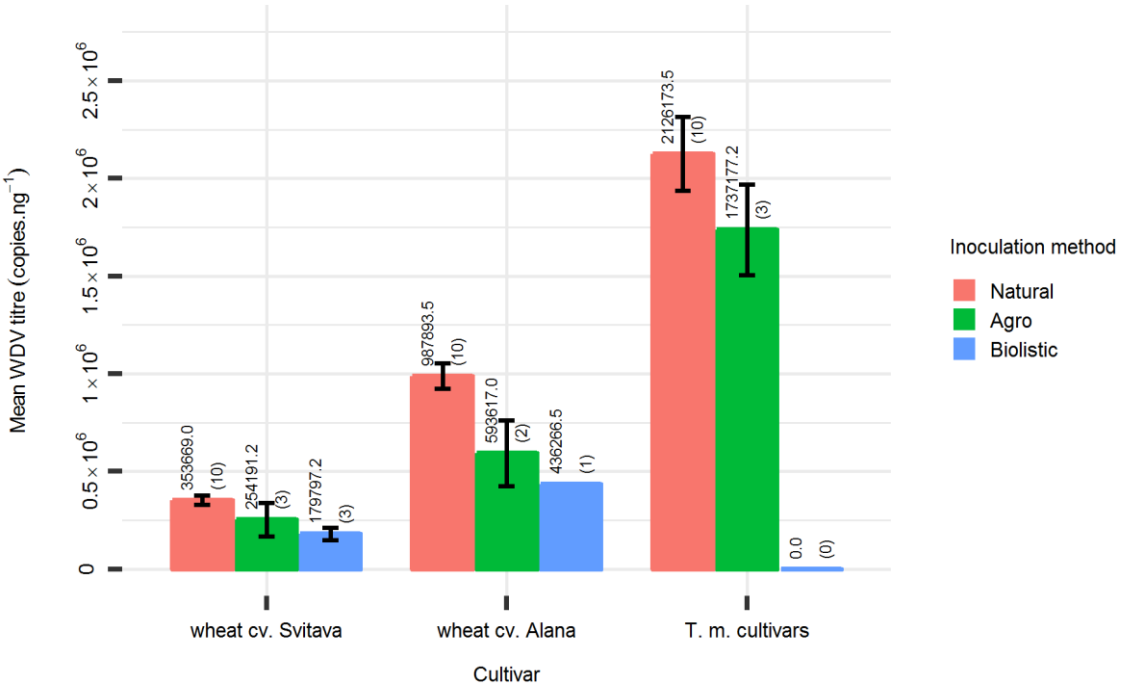
Tabulka 4. Nejlepší výsledky dosažené různými inokulačními metodami. Je uveden počet infikovaných rostlin ze všech inokulovaných rostlin v pokusu a příslušná úspěšnost. Výsledky jsou uvedeny vždy pro pokus s nejvyšší dosaženou úspěšností.

Metoda	Ozimá pšenice (Svitava)	Ozimá pšenice (Alana)	Pšenice jednozrnka
Inokulace kříškem polním (*Psammotettix alienus*)	10/10 (100 %)	10/10 (100 %)	10/10 (100 %)
Agroinokulační metoda	3/25 (12 %)	1/25 (4 %)	3/25 (12 %)
Biolistická inokulační metoda	1/12 (8,33 %)	1/12 (8,33 %)	0/35 (0 %)

jednozrnku a porovnat je s výsledky získanými agroinokulací a biologickou inokulací za použití infekčních klonů WDV.

Úspěch agroinokulace s infekčním viremu příbuzných virů, jako např. agroinokulace s infekčním klonem blízce příbuzného viru MSV na rostlinách kukuřice, byla popsána již dávno (Grimsley a kol. 1987, Boulton a kol. 1989a). Agroinokulace rostlin s pIPKb002 + WDV za použití kmene *A. tumefaciens* AGL1 potvrdila vhodnost zkonstruovaného infekčního klonu k zahájení infekce WDV u rostlin. Bylo dosaženo slibných výsledků při agroinokulaci *A. tumefaciens* AGL1 ať už s, nebo bez předchozí indukce acetosyringonem před agroinokulací

Obrázek 12. Průměrný titr viru WDV dosažený pro zvolenou inokulační metodou a testované odrůdy spolu s intervalem ±jedna standardní chyba průměru (SEM). Čísla v závorkách ukazují celkový počet infikovaných rostlin potvrzených qPCR i DAS-ELISA pro danou metodu a testovanou odrůdu.



nebo s různými metodami kultivace agrobakterií. Inokulace pomocí *A. tumefaciens* AGL1 získaných kultivací na miskách vedla k obdobným výsledkům jako při inokulaci z kultivace agrobakterií v kapalném médiu. Dosažená účinnost agroinokulace u testovaných odrůd (4 až 12 %) je podobná jako u jiných autorů (Bendahmane a kol. 1995, Ramsell a kol. 2009), avšak jsou také uváděny i výsledky s vyšší dosaženou účinností při použití jiných kmenů agrobakterií (Marks a kol. 1989, Benkovics a kol. 2010). Pro obdobný virus MSV ukazují srovnávací studie, že přítomnost dodatečné promotorové sekvence (podobně jako u použitého plasmidu pIPKb002) může zvýšit účinnost agroinokulace podobně jako výběr orientace vnášené virové DNA u použitého plasmidu (Martin a kol. 2000). Nicméně podobnost WDV a MSV v daných případech nemusí být směrodatná, protože u infekčního klonu MSV je potrzena i mechanická přenositelnost na jeho preferenčního hostitele, kukuřici (Redinbaugh 2003) a rovněž je v některých pracech uváděna i extrémně vysoká dosahovaná účinnost agroinokulace na kukuřici (Grimsley a kol. 1987, Grimsley a kol. 1988), málo běžná pro jednoděložné rostliny.

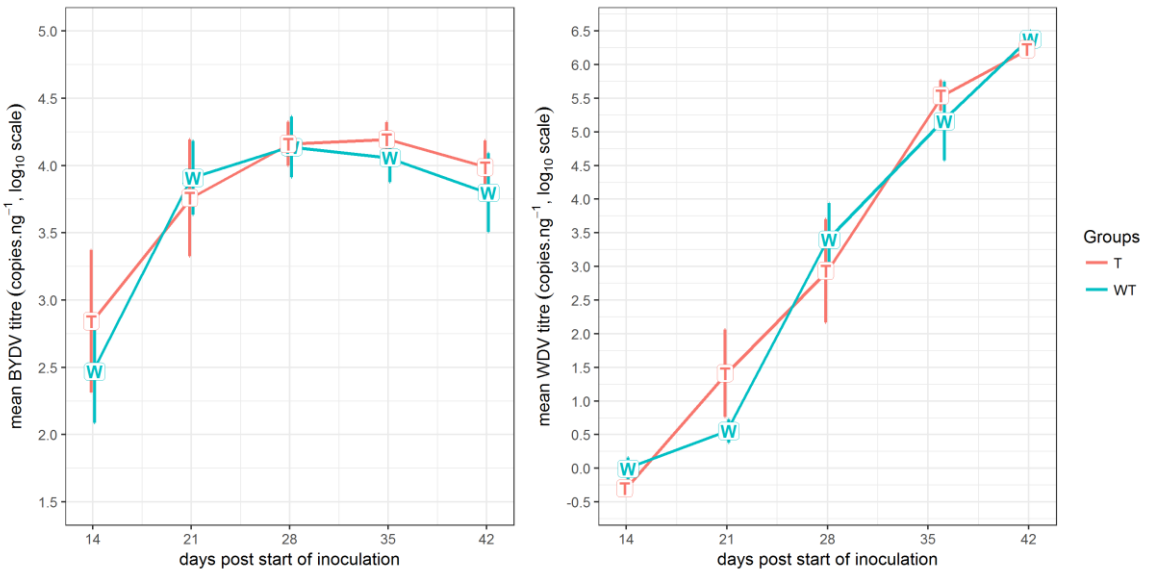
Úspěšná biolistická transformace pšenice nebo ječmene je založena na použití nezralých embryí, indukovaných kalusů kultivovaných na agaru (Jones a kol. 2009), nebo velmi mladých rostlinných pletiv, pro něž je často Bio-Rad Helios Gene Gun méně vhodný než například Bio-Rad Helios PDS1000/He s vakuovou komorou. Přesto, pokud jsou nastaveny optimální parametry, byly získány rostliny infikované WDV za použití každého ze zkonstruovaných infekčních klonů. Účinnost transformace pomocí biolistické inokulace však byla nižší než účinnost agroinokulace. Zde uváděné výsledky ukazují, že je možné úspěšně inokulovat virus cílený na floém, jako např. WDV (Dinant a kol. 2004, Tholt a kol. 2018), i bez hmyzího přenačeče, a to za použití infekčního klonu viru a agroinokulační nebo biolistické inokulační metody. Mnohé dřívější poznatky ukázaly, že agroinokulace je velmi účinná metoda pro přenos infekční virové DNA do hostitelských buněk (Grimsley a kol. 1986), jež následně je schopná navodit rozvoj onemocnění u testovaných rostlin včetně pozorovatelných symptomů (Czosnek a kol. 1993, Kheyrpour a kol. 1994). Podobně, i u biolistické inokulace bylo dosahováno dostatečné účinnosti pro úspěšný přenos virové DNA na rostlinu a navození infekce (Lapidot a kol. 2007). V jednoděložných rostlinách, jako jsou obilniny, může být biolistická inokulace méně účinná (Helloco-Kervarrec a kol. 2002), což se také projevuje i zde. Kvantitativní analýza zde pak ukazuje, že v agroinokulovaných rostlinách je v cílové sledované době titr WDV mnohem vyšší než při použití biolistické inokulace a je podobný titru viru v rostlinách inokulovaných přirozeným způsobem pomocí křísky polního.

4.3 O možnosti detektce viru WDV dalšími metodami

Podrobné zkoumání vývoje choroby na cílových rostlinách je v současnosti limitováno potřebou poměrně velkého množství infikovaného materiálu pro úspěšnou kvantifikaci titru viru, např. pomocí metody ELISA. V případě kvantifikace pomocí DNA kvantifikačních metod, jako např. qPCR (Gadiou a kol. 2012), může adekvátní potřebné množství být menší, nicméně extrakce pomocí různých extrakčních kitů stále vyžaduje použití většího množství rostlinného materiálu, i s ohledem na variabilitu extrahovaného množství DNA. Pro extrakci DNA či RNA existuje více různých metod (Tan a kol. 2009), jež jsou následně optimalizovány pro odlišnosti v cílovém pletivu nebo pro požadavky na kvalitu získané DNA či RNA (Triant a kol. 2009) a dané tedy nabízí i možnost optimalizovat příslušný extrakční protokol tak, aby bylo možné sledovat vývoj titru zvoleného viru i na samostatných rostlinách v delším časovém úseku.

V publikaci Viktorova a kol. (2019), jež je součástí této disertační práce, byla popsána optimalizovaná metoda pro detekci titru viru WDV a BYDV na bázi detekce nukleových kyselin, vyžadující méně než jeden list z testované rostliny pro následnou kvantifikaci titru viru. Extrakční protokol byl vyvinut optimalizací jednotlivých kroků používaných při extraci nukleových kyselin (Triant a kol. 2009) a studiem vlivu jednotlivých kroků na výtěžek. Pomocí této uvedené techniky bylo možné následně zkoumat vývoj titru viru i v jednotlivých rostlinách v delším časovém úseku či podrobně analyzovat vznik případných odlehlých hodnot. Uvedený

Obrázek 13. Průměrný titr viru BYDV a WDV měřený v kontrolních netransgenních rostlinách (W) a transgenních rostlinách (T) ječmene odrůdy Golden Promise s vloženým genem pro expresi osmotinu – genu pro větší odolnost vůči nepříznivým abiotickým podmínkám jako salinita, či sucho (Viktorova a kol. 2019). Titry BYDV a WDV měřené v listech rostlin ve 42-denním období po inokulaci. Titr viru BYDV a WDV byl měřen jako průměr stanovený pomocí metody qPCR (triplikáty) a normalizovaný na koncentraci vzorku RNA (pro BYDV) nebo koncentraci vzorku DNA (pro WDV). Zobrazené hodnoty jsou po transformaci $\log_{10}$. Z každého testovaného období je zobrazen průměr ze všech šesti testovaných rostlin v každé skupině (W, T) a standardní chyba průměru (SEM). Analýza rozptylu následovaná Duncanovým testem ($p < 0,05$) nepotvrdila odlišné hodnoty titru virů pro transgenní a kontrolní skupinu.



protokol pak umožnil posoudit vliv sledované linie ječmene na případný vývoj titru viru v rostlinách vůči kontrolní skupině (viz také Obrázek 13).

V rámci možností detekce virů pomocí širokospektrých technik byla pozornost zaměřena na širokospektré proteomické techniky, zejména hmotnostní spektrometrii, jež je v současné době rutinně používanou metodou pro analýzu struktury a složení v mnoha různých oblastech. Hmotnostní spektrometrie je používána jako následná detekční technika pro plynovou (GC-MS) či kapalinovou chromatografii (LC-MS), či pro přímou identifikaci peptidů a proteinů na základě laserové desorpční ionizace z matrice (MALDI-TOF, matrix-assisted laser desorption/ionization – time of flight). Hmotnostní spektrometrie byla s úspěchem uplatněna pro detekci virových proteinů již dříve (Thomas a kol. 2000, Trauger a kol. 2003), včetně rostlinných virů (Blouin a kol. 2010). Ve starší literatuře se objevovaly postupy, kdy nejprve byla provedena extrakce virionových částic nebo čistých virových proteinů a jejich následná ionizace a identifikace byla provedena pomocí MALDI-TOF nebo ESI-TOF (ionizace vzorku elektrosprejem sdružená s hmotnostním spektrometrem rozlišujícím částice na základě času jejich letu, electrospray ionization – time of flight). Později je detekce virových proteinů zmiňována také v extraktech všech proteinů ze vzorku ve studiích změn zdravého a infikovaného rostlinného proteomu (Cooper a kol. 2003, Di Carli a kol. 2012), využívajících 2D gelovou elektroforézu doprovázenou TOF (hmotnostně spektrometrický detektor rozlišující částice na základě času jejich letu, time-of-flight) či později LC-MS/MS (kapalinová chromatografie sdružená s dvěma hmotnostními spektrometry propojenými v tandemu, liquid chromatography – tandem mass spectrometry) (Di Carli a kol. 2012, Fang a kol. 2015), nicméně jako vzorkovací materiál byla běžně odebírána celá rostlina nebo i více rostlin současně.

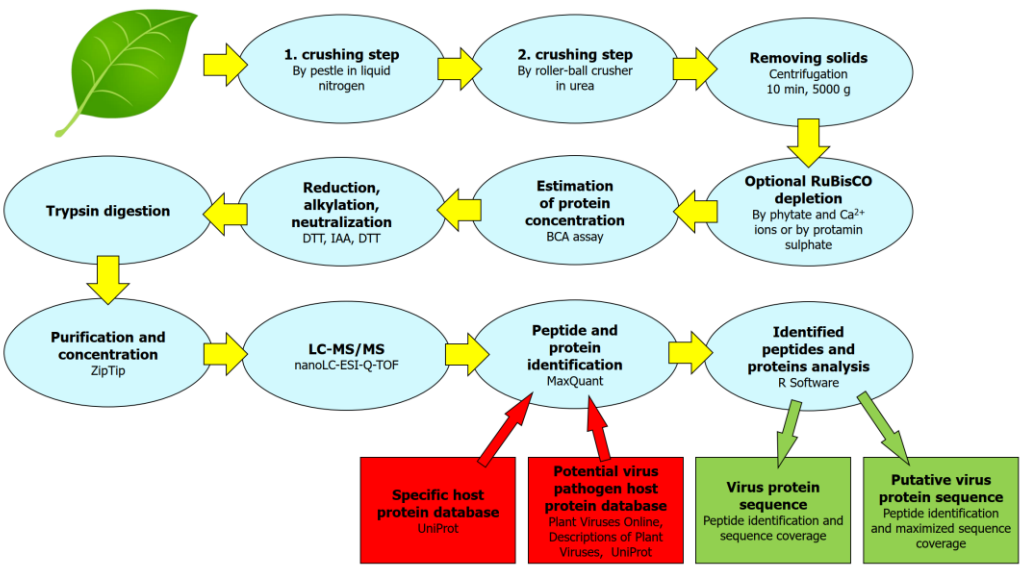
Pro techniku MALDI-TOF analýza naměřených spekter vzorků umožňuje identifikaci peptidů, proteinů i případně včetně jejich modifikací (Junkova a kol. 2013, Plaks a kol. 2015, Sugioka a kol. 2017). Přesná identifikace změn mezi skupinami vzorků je často prováděna na základě souhrnného (průměrného) spektra (Karamonova a kol. 2013, Svobodova a kol. 2017) vizuálně identifikující nejvíce rozlišující pozice s ohledem na změny v zaznamenané intenzitě, či na základě použití diskriminační analýzy na extrahovaných vrcholech intenzit (Jombart a kol. 2010). Identifikace rozdílů ve vzorcích, jež jsou ovlivňovány mnoha možnými faktory, vyžaduje ovšem sofistikovanější analýzu, kde je každý faktor identifikován odděleně a robustně. Pro snazší analýzu rozdílů v naměřených hmotnostních spektrech byly připraveny dva programy – *ms-alone* a *multiMS-toolbox* a informace o nich publikovány v samostatném článku (Cejnar a kol. 2018a). *ms-alone* je samostatný program pro rozhraní příkazového řádku,

který umožňuje import dat z běžných formátů, aplikaci základních spektrálních operací, identifikaci vrcholů intenzit nalezením lokálních maxim nad zvolenou hladinou poměru signál-šum, aproximaci vrcholů zvolenou tvarovou funkcí pomocí nelineární regrese pro přesné určení polohy maxima intenzity, export seznamu identifikovaných vrcholů a spekter pro analýzu v jiném softwaru. *multiMS-toolbox* je softwarový modul (toolbox) do statistického prostředí R (R Core Team 2020), který následně umožňuje vzájemné ztotožnění (registraci) příslušných vrcholů nebo klastrů vrcholů z různých vzorků pomocí dvoustupňového algoritmu, normalizaci dat podle zvolené metody a aplikaci pokročilých statistických metod (PCA – Principal Component Analysis, ANOVA – Analysis of Variance) na ztotožněných vrcholech intenzit či na původních spektrech a následnou vizualizaci výsledků v grafech a textových výstupech. Praktické uplatnění ilustruje i jejich úspěšné použití v dalších publikovaných pracích (Bozik a kol. 2018). Nicméně aplikace techniky MALDI-TOF na celkový proteinový extrakt z rostlin nakažených virem zakrslosti pšenice nevedla k úspěšné detekci virových proteinů ve vzorcích ani po optimalizacích extrakčního protokolu (nepublikovaná data). Samotná detekce virových proteinů z celkových extraktů proteinů z nakažených rostlin tak patrně vyžaduje použití složitějších separačních technik před aplikací hmotnostní spektrometrie, jako např. 2D gelové elektroforézy (2DE/MALDI-TOF) či kapalinové chromatografie (LC-MS/MS).

Při identifikaci virových proteinů v celkových extraktech proteinů z rostlinných vzorků pomocí LC-MS/MS je pro potřeby vzorkování běžně odebírána (homogenizována) celá rostlina nebo i souhrnný vzorek z více rostlin. Pro účely detekce virových proteinů v rámci jedné rostliny či snížení potřebného množství materiálu na minimum bylo nutné optimalizovat extrakční protokol pro extrakci proteinů ze vzorku. Velká pozornost byla věnována srážecím krokům (často jsou v protokolech používány srážecí kroky na bázi kyselina trichloroctová-aceton, kyselina mravenčí-acetonitril atd.). Srážecí kroky umožňují odfiltrovat nechtěné složky ze směsi, ale také přinášejí do protokolu potenciálně další zdroj variability výsledného výtěžku, který by mohl vést k nízké koncentraci sledovaných proteinů. K eliminaci tohoto negativního vlivu je pak nutná vyšší hmotnost vzorkovaného materiálu. I když existují techniky srážení dosahující až 100% účinnosti (Crowell a kol. 2013, Feist a kol. 2015), pro vzorky jednotlivých listů (s hmotností přibližně 50 – 200 mg), jsme vyvinuli extrakční protokol bez srážecích kroků. U rostlinných vzorků musí extrakční protokol pro proteiny zvládnout narušení buněčných stěn (např. pomocí cyklu zmrazování-rozmrazení, mechanického narušení v kapalném dusíku, přidání detergentu, denaturace zahřátím, mechanického drcení v extrakčním pufru apod.) spolu se současnou inhibicí jakéhokoliv procesu degradace proteinů (např. přidáním chaotropního činidla nebo inhibitorů proteázy) (Bodzon-Kulakowska a kol. 2007, Feist a kol. 2015). Na to

často navazuje vybraný proces odstraňování kontaminujících látek (Burgess 2009, Crowell a kol. 2013). Pro následnou LC-MS analýzu po těchto krocích ještě bývá aplikována redukce disulfidových vazeb, alkylace volných cysteinových zbytků a štěpení enzymy pro fragmentaci proteinů na peptidy pro účely jejich identifikace (Gundry a kol. 2009, Suttapitugsakul a kol. 2017). Správné použití všech technik musí zabránit nekompatibilitě přidaných činidel s jakýmkoliv následným štěpením enzymy, a proto musí být případná nekompatibilní činidla odstraněna buď sražením, extrakcí špičkou mikropipety s pevnou fází nebo alespoň zředěním na kompatibilní koncentrace. Kroky, které jako vedlejší efekt mohou vést k modifikacím peptidů (Sun a kol. 2014, Muller a kol. 2017), však také mohou významně snížit počet identifikovaných peptidů. Kapacita chromatografické kolony je rovněž omezená, a pro identifikaci proteinů o velmi nízké koncentraci by vzorky sice mohly být dále rozfrakcionovány, avšak takové řešení by značně navyšovalo počet vzorků analyzovaných na LC-MS/MS. Pokud by měl, v rámci analýzy jednoho odebraného vzorku, být zpracováván na LC-MS/MS pouze jeden vzorek či případně jen jeho další technická opakování, přistupuje se alespoň k odstranění nejhojnějších proteinů ze vzorku, pokud tyto nesouvisí se sledovanými změnami. U rostlinných vzorků je tak zejména odstraňován enzym RuBisCO (Ribulóza-1,5-bisfosfátkarboxyláza/oxygenáza). Pro jeho odstranění se běžně používají dvě účinné metody (Krishnan a kol. 2009, Kim a kol. 2013, Gupta a kol. 2015): srážení s protamin-sulfátem nebo srážení s fytátem v přítomnosti iontů Ca^{+2}. V práci Cejnar a kol. (2020), jež je součástí této disertační práce, je představen optimalizovaný protokol extrakce rostlinných proteinů (viz také Obrázek 14), který umožňuje extrahovat proteiny z rostlinných vzorků a následně potvrdit přítomnost virových proteinů, za použití i pouze jediného listu rostliny se silnými příznaky

Obrázek 14. Schéma optimalizovaného protokolu pro identifikaci virových proteinů z rostlinných vzorků.



infekce. Navržený protokol kombinuje techniky, které jsou dobře známy z extrakcí protokolů nukleových kyselin – narušení buněčné stěny a homogenizace vzorků drcením listů v kapalném dusíku, použití chaotropního činidla pro denaturaci bílkovin a technik běžně používaných pro DAS-ELISA – homogenizace ručním ložiskovým drtičem v konzervačním pufru. Vysoké koncentrace chaotropního činidla, močoviny, jsou následně sníženy zředěním na neinhibující koncentrace pro štěpení trypsinem, aby se zabránilo jakékoliv nekompatibilitě. Konečné zakoncentrování a odsolení je provedeno pomocí extrakce špičkou mikropipety s pevnou fází, což je technika velmi podobná následné separaci proteinů na chromatografické koloně. Takový protokol umožňuje dosáhnout výtěžku postačujícího pro zaplnění kapacity LC kolony a následná identifikace pomocí nanoLC-ESI-Q-TOF umožnila v testovaných vzorcích úspěšné potvrzení virových obalových proteinů na široké škále hostitelských rostlin, včetně trav, kvetoucích rostlin nebo stromů. Počet identifikovaných peptidů a vysoké pokrytí sekvencí proteinů u virových obalových proteinů dokonce umožnilo rozlišit specificky i jednotlivé virové kmeny na základě analýzy sekvencí virových proteinů. Uvedený protokol v kombinaci s přístrojem nanoLC-ESI-Q-TOF s alespoň středním rozlišením se ukázal jako účinná metoda pro potvrzení virových proteinů u rostlin diagnostikovaných jinými nízkoprahovými detekčními metodami a ponechaných pro vývoj silných příznaků choroby. Použití méně než jednoho listu umožňuje uchovat živou rostlinu pro další experimenty. Vhodnost protokolu byla otestována na více druzích rostlin, jednoděložných i dvouděložných: ječmen (*Hordeum vulgare* L.), pšenice (*Triticum aestivum* L.), brukev (*Brassica rapa subsp. Pekinensis* (Lour.) Hanelt), tabák (*Nicotiana tabacum* L., *Nicotiana benthamiana* Domin), švestka (*Prunus domestica* L.), meruňka (*Prunus armeniaca* L.), fazol (*Phaseolus vulgaris* L.), huseníček (*Chenopodium amaranticolor* H.J. Coste & A. Reyn), čirok (*Sorghum bicolor* (L.) Moench) a kukuřice (*Zea mays* L.). Na jednotlivých vzorcích se podařilo úspěšně prokázat přítomnost různých virových proteinů. Detekované viry z různých vzorků zahrnovaly širokou škálu virů (jak DNA, tak RNA virů): virus zakrslosti pšenice (WDV – *Wheat dwarf virus*) z čeledi Geminiviridae, virus žluté zakrslosti ječmene (BYDV – *Barley yellow dwarf virus*) z čeledi Luteoviridae, virus mozaiky sveřepu (BMV – *Brome mosaic virus*) a virus aspermie rajčete (TAV – *Tomato aspermy virus*) z čeledi Bromoviridae, virus mozaiky květáku (CaMV – *Cauliflower mosaic virus*) z čeledi Caulimoviridae, virus tabákové mozaiky (TMV – *Tobacco mosaic virus*) a virus prosvětlení žilek vodnice (TVCV – *Turnip vein clearing virus*) z čeledi Virgaviridae, virus šarky švestky (PPV – *Plum pox virus*), virus mozaiky vodnice (TuMV – *Turnip mosaic virus*), virus obecné mozaiky fazolu (BCMV – *Bean common mosaic virus*), virus mozaiky čiroku (SrMV – *Sorghum mosaic virus*), virus mozaiky cukrové třtiny (SCMV – *Sugarcane mosaic virus*), vše

z čeledi Potyviridae, virus kroužkovitosti tabáku (TRSV – *Tobacco ringspot virus*) a virus vadnutí bobu 2 (BBWV-2 – *Broad bean wilt virus* 2) z čeledi Secoviridae. Publikovaný protokol tak umožňuje široké využití pro studium změn v proteomu při virových chorobách rostlin či jako účinná metoda potvrzení přítomnosti virového proteinu pro vysoce infikované vzorky.

Závěr

Všechny tři uvažované hypotézy byly postupně potvrzeny. Byly připraveny transgenní rostliny T0 a T1 generace ječmene odrůdy Golden Promise s vnesenými konstrukty WDVRepA215 nebo WDVRepA215RBR^{mut}. Významné zpoždění nárůstu titru viru WDV jako odezva na přítomnou dvoubodovou mutaci v oblasti domény pRBR ve vnášené sekvenci je pravděpodobně následkem cíleného narušení mechanismu infekce v rostlinách. Nicméně ani vnesený transgen, ani zahrnutá mutace nevedly k zamezení šíření infekce viru v rostlinách ječmene. Zvýšená náchylnost transgenních rostlin k infekci WDV ve srovnání s netransgenními rostlinami byla identifikována jako zvýšená náchylnost rostlin i vůči nižšímu infekčnímu tlaku. U rostlin pšenice nicméně dochází k výraznému potlačení fenotypových projevů infekce virem.

Byly připraveny dva různé infekční klony v bakteriálním plasmidu pUC18 s replikačním počátkem pro replikaci v *Escherichia coli* a v plasmidu pIPKb002 s replikačním počátkem jak pro replikaci v *Escherichia coli*, tak také i v *Agrobacterium tumefaciens*. Pomocí těchto infekčních klonů se podařilo navodit infekci v rostlinách za použití agroinokulace či biolistické inokulace, tzn. bez přítomnosti přirozeného hmyzího přenašeče. Úspěšně infikované rostliny vykazovaly podobné titry WDV ve srovnání s rostlinami inokulovanými přirozeným přenosem.

Byly optimalizovány metody extrakce nukleových kyselin z obilnin a následné detekce a normalizace množství viru WDV tak, že bylo možné podrobně sledovat průběh infekce WDV na molekulární úrovni v jednotlivých rostlinách během prvních 6 týdnů po inokulaci.

Byl optimalizován extrakční protokol pro extrakci proteinů z listů rostlin umožňující přímou detekci virových proteinů za použití kapalinového chromatografu zapojeného v tandemu s hmotnostním spektrometrem. Optimalizovaný extrakční protokol vyžaduje méně než 0,2 g rostlinného materiálu, tzn. typicky méně než jeden list rostliny, a umožňuje tak studium změn v proteomu rostliny či potvrzení přítomnosti virových proteinů bez nutnosti nenávratného zničení sledované rostliny.

Použité zkratky

2DE	Dvoudimenzionální gelová elektroforéza (2D electrophoresis)
ATP	adenosintrifosfát (adenosine triphosphate)
BeYDV	virus žluté zakrslosti fazolu (*Bean yellow dwarf virus*)
BYDV	virus žluté zakrslosti ječmene (*Barley yellow dwarf virus*)
CP	obalový protein (coat protein)
CRISPR	segmenty nahromaděných pravidelně rozmístěných krátkých palindromických repetic (clustered regularly interspaced short palindromic repeats)
d.p.i.	dnů od zahájení inokulace (days following the start-of-inoculation)
DNA	deoxyribonukleová kyselina (deoxyribonucleic acid)
ELISA	immunochemická metoda ELISA (enzyme-linked immunosorbent assay)
ESI	technika ionizace vzorku elektrosprejem (electrospray ionization)
GFP	zelený fluorescenční protein (green fluorescent protein)
GUS	beta-glukuronidáza
LC-MS/MS	technika detekce založená na separaci pomocí kapalinové chromatografie a detekci pomocí hmotnostních spektrometrů propojených v tandemu (liquid chromatography – tandem mass spectrometry)
LIR	dlouhý mezigenový úsek (long intergenic region)
MALDI	technika ionizace vzorku laserovou desorpční ionizací z matrice (matrix-assisted laser desorption/ionization)
MP	pohybový protein (movement protein)
MSV	virus proužkovitosti kukuřice (*Maize streak virus*)
nanoLC	chromatografická kolona pro kapalinovou chromatografii s průtokem v řádu přibližně stovek nanolitrů za minutu
NTP	nukleosidtrifosfát (nucleoside triphosphate)
PCR	polymerázová řetězová reakce (polymerase chain reaction)
PINB	puroindolin-b
Q-TOF	hmotnostně spektrometrometrický detektor založený na architektuře kvadrupól-čas letu (quadrupole – time of flight)
qPCR	kvantitativní polymerázová řetězová reakce (quantitative polymerase chain reaction)
RBR	proteiny podobné retinoblastomu (retinoblastoma-related proteins)
RCR	replikace pomocí replikace kruhových plasmidů (rolling circle replication)
RDR	rekombinant-dependentní rekombinace (recombination dependent recombination)
Rep	replikační protein (replication protein)
Rep A	replikační asociační protein (replication associated protein)
RNA	ribonukleová kyselina (ribonucleic acid)

RuBisCO	Ribulóza-1, 5-bisfosfátkarboxyláza/oxygenáza (Ribulose-1, 5-bisphosphate carboxylase/oxygenase)
SIR	krátký mezigenový úsek (short intergenic region)
ssDNA	jednovláknitá DNA (single-stranded DNA)
TOF	hmotnostně spektrometrickým detektor rozlišující částice na základě času jejich letu (time-of-flight)
WDV	virus zarkslosti pšenice (*Wheat dwarf virus*)

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