UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Matevž RUPAR

LOKALIZACIJA IN SLEDENJE KROMPIRJEVEGA VIRUSA Y V RASTLINAH KROMPIRJA (*SOLANUM TUBEROSUM* L.)

DOKTORSKA DISERTACIJA

LOCALISATION AND TRACKING OF POTATO VIRUS Y IN POTATO PLANTS (*SOLANUM TUBEROSUM* L.)

DOCTORAL DISSERTATION

Ljubljana, 2014

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa 31. seje Komisije za doktorski študij UL z dne 19. 9. 2012 (po pooblastilu Senata Univerze z dne 20. 1. 2009) je bilo potrjeno, da kandidat izpolnjuje pogoje za opravljanje doktorata znanosti na Interdisciplinarnem doktorskem študijskem programu Bioznanosti, znanstveno področje biologije. Za mentorico je bila imenovana prof. dr. Maja Ravnikar, za somentorja dr. Ion Gutiérrez-Aguirre.

Doktorsko delo je bilo večinoma opravljeno na Oddelku za biotehnologijo in sistemsko biologijo Nacionalnega inštituta za biologijo (NIB) v Ljubljani. Analiza z metodo SNaPshot in priprava fluorescentno označenega virusa Y krompirja je bila opravljena na Institut National de la Recherche Agronomique (INRA), Rennes, Francija.

Komisija za oceno in zagovor:

Predsednik: prof. dr. Darja ŽGUR BERTOK
Ljubljana, Biotehniška fakulteta, Oddelek za biologijo

Član: prof. dr. Maja RAVNIKAR
Ljubljana, Nacionalni inštitut za biologijo, Oddelek za biotehnologijo in sistemsko biologijo

Član: dr. Ion GUTIÉRREZ AGUIRRE
Ljubljana, Nacionalni inštitut za biologijo, Oddelek za biotehnologijo in sistemsko biologijo

Član: prof. dr. Tatjana AVŠIČ ZUPANC
Ljubljana, Medicinska fakulteta, Inštitut za mikrobiologijo in imunologijo

Datum zagovora: 19. 5. 2014

Podpisani izjavljam, da je predložena doktorska disertacija rezultat lastnega raziskovalnega dela. Strinjam se z objavo naloge v polnem tekstu na spletni strani Digitalne knjižnice Biotehniške fakultete.

Matevž Rupar

KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD Dd
DK 633.491:578(043.3)
KG virus Y krompirja (PVY)/določanje in klasifikacija /sekvenciranje /evolucija gena CP / monolitna kromatografija/izolacija virusnih delcev/fluorescentno označen PVY/lokalizacija/hitrost širjenja PVY med celicami
AV RUPAR, Matevž, uni. dipl. bioteh.
SA RAVNIKAR, Maja (mentor)/GUTIÉRREZ-AGUIRRE, Ion (somentor)
KZ SI-1000 Ljubljana, Jamnikarjeva 101
ZA Univerza v Ljubljani, Biotehniška fakulteta, Interdisciplinarni doktorski študij bioznanosti, znanstveno področje biologija
LI 2014
IN LOKALIZACA IN SLEDENJE KROMPIRJEVEGA VIRUSA Y V RASTLINAH KROMPIRJA (*SOLANUM TUBEROSUM* L.)
TD Doktorska disertacija
OP IX, 98 str., 2 sl., 5 pril., 121 vir.
IJ sl
JI sl/en
AI Krompirjev virus Y (PVY) je najpomembnejši virusni povzročitelj bolezni na krompirju. Na listih povzroča znamenja mozaika, nekroze, rumenenje, odpadanje listov ter nekroze na površini gomoljev. Bolezen imenujemo obročkasta nekroza gomoljev krompirja (PTNRD) zaradi katere so gomolji neuporabni za prodajo. Različke PVY razvrščamo v skupine glede na genetske, serološke in molekulske lastnosti. Primerjali smo dve najnovejši molekulski metodi za razlikovanje med različki, s poudarkom na detekciji PVY^{NTN} različkov, ki povzročajo PTNRD. SNaPshot metoda (Rolland in sod. 2008) je razlikovala med več različnimi skupinami različkov kot metoda verižnega pomnoževanja z reverzno transkripcijo v realnem času (RT-qPCR) (Kogovšek in sod., 2008), ki je bolj zanesljivo določala PVY^{NTN} različke. Z določanjem nukleotidnih zaporedij in molekulskimi metodami smo analizirali 24 slovenskih izolatov in ocenili, da v Sloveniji tako kot drugod po Evropi prevladujejo PVY^{NTN} različki. Z bayesiansko analizo velikega nabora nukleotidnih zaporedij plaščnega proteina PVY smo pokazali, da geografski izvor in prilagoditev na gostitelja pomembno vplivata na evolucijo virusnega proteina CP, vendar pa ne pojasnjujeta celotne pestrosti. V genu CP nismo našli determinant za klasifikacijo različkov glede na gostitelja ali geografsko poreklo. Razvili smo novo metodo čiščenja filamentoznih virusnih delcev PVY z monolitno kromatografijo. Metoda dosega primerljiv izkoristek in čistost kot klasična metoda izolacije, očiščeni delci so nepoškodovani in infektivni. Čas izolacije smo skrajšali s 4 dni na 2. Metoda je tudi enostavnejša in bolj dostopna, saj ne zajema korakov ultracentrifugiranja. S tehniko infektivnih klonov smo pripravili z zelenim fluorescentnim proteinom (GFP), označen PVY. Potrdili smo, da označen virus povzroča enako močna bolezenska znamenja in v rastlini dosega primerljive koncentracije kot neoznačen PVY, zato je primeren za opazovanje širjenja PVY. Prvič smo opazovali širjenje PVY v rastlini *in vivo* ter prvič določili hitrost širjenja PVY med celicami.

KEY WORDS DOCUMENTATION

DN Dd
DC 633.491:578(043.3)
CX potato virus Y (PVY)/detection and classification/ sequencing/evolution of the CP gene/monolith chromatography /isolation of pure PVY particles /fluorescently labelled PVY/localisation/rate of cell-to-cell spread of PVY
AU RUPAR, Matevž, uni. dipl. bioteh
AA RAVNIKAR, Maja (mentor)/GUTIÉRREZ-AGUIRRE, Ion (co-mentor)
PP SI-1000 Ljubljana, Jamnikarjeva 101
PB University of Ljubljana, Biotechnical faculty, Interdisciplinary Doctoral Programme in Biosciences, Biology
PY 2014
TI LOCALISATION AND TRACKING OF POTATO VIRUS Y IN POTATO PLANTS (*SOLANUM TUBEROSUM* L.)
DT Doctoral Dissertation
NO IX, 98 p., 2 fig., 5 ann., 121 ref.
LA sl
AL sl/en
AB Potato virus Y (PVY) is the most important viral pathogen in potato, causing mosaic, yellowing and leaf drop symptoms on the leaves and necrosis on tubers. The disease was named Potato Tuber Necrotic Ringspot Disease (PTNRD). PVY strains are classified into several groups according to their genetic, serological and molecular properties. We have assessed two state of the art molecular methods for PVY sub-group classification with the emphasis on detection of PVY^{NTN} strains, which cause PTNRD. SNaPshot method (Rolland et al., 2008) was able to distinguish more isolates than real-time reverse transcription polymerase chain reaction method (RT-qPCR) (Kogovšek et al., 2008) however the latter detected PVY^{NTN} more accurately. With sequencing and molecular characterisation of Slovenian isolates we estimated that PVY^{NTN} is the prevalent strain in Slovenia, as reported for other European countries. Bayesian based analysis was applied to a large set of PVY coat protein sequences. We demonstrated that the evolution of CP protein is greatly influenced by the geographical origin and host driven adaptation, but the two factors do not explain the overall diversity. Reliable molecular determinants for classification of isolates according to the host or geographic origin were not found. We have developed a novel method for purification of filamentous PVY particles with monolithic chromatography. The method achieves comparable yield and purity as the conventional isolation method; the purified particles are intact and infectious. Isolation time was greatly reduced. The method is simple and available to wider research laboratories, since no ultracentrifugation steps are required. We prepared a green fluorescent protein (GFP) labelled PVY infectious clone. The labelled virus caused symptoms of comparable severity in reached comparable concentrations of PVY RNA in the plants as the unlabelled PVY. Thus it is suitable for monitoring the spread of PVY in the host plants *in vivo*. With the GFP labelled PVY we determined the rate of the cell-to-cell spread of PVY for the first time.

KAZALO VSEBINE

KAZALO SLIK

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

PVY	(Potato virus Y), virus Y krompirja
PTNRD	(Potato Tuber Necrotic Ringspot Disease), bolezen obročkaste nekroze gomoljev krompirja
PIPO	(Pretty interesting potyviral protein), protein potivirusov, ki se nahaja v 2+ odprtem bralnem okviru znotraj proteina P3, in sodeluje pri premikanji virusa med celicami
CP	(Coat protein), protein plašča
DPI	(days post inoculation), dni po inokulaciji
ELISA	(Enzyme-linked immunosorbent assay), encimskoimunski test
RT-PCR	(reverse transcription polymerase chain reaction), verižna polimerizacija z reverzno transkripcijo
RT-qPCR	(real-time reverse transcription polymerase chain reaction), verižna polimerizacija z reverzno transkripcijo v realnem času
EDTA	(Ethylenediaminetetraacetic acid), Etilendiamintetraocetna kislina
CIM	(Convective Interactive Media), vrsta metakrilatnih monolitnih kolon proizvajalca BiaSeparations
QA	(Quaternary amine), kvartarni amin, ligand na površini močne anionsko izmenjevalne monolitne kolone
DEAE	(Diethylamine) dietilamin, ligand na površini šibke anionsko izmenjevalne monolitne kolone
SDS-PAGE	(Sodium dodecyl sulfate polyacrylamide gel electrophoresis), poliakrilamidna gelska elektroforeza z natrijevim dodecil sulfatom, metoda za ločevanje proteinov na gelu
GFP	(Green fluorescent protein), zeleni fluorescentni protein
dsRed	(Discosoma red fluorescent protein) rdeči fluorescentni protein
TEM	(Transmission electron microscopy), presevna elektronska mikroskopija
SNP	(Single nucleotide polymorphism), polimorfizem posameznega nukleotida
NGS	(Next generation sequencing), sekvenciranje nove generacije
RuBisCO	(Ribulose-1,5-bisphosphate carboxylase/oxygenase) RuBisCO ribulozo-1,5-bifosfat-karboksilaza/oksigenaza, encim, ki se nahaja v stromi kloroplasta in sodeluje v temotnih reakcijah fotosinteze pri nastanku sladkorjev v Calvinovem ciklu.
FEL	Fixed-effects likelihood
IFEL	Internal branches fixed-effects likelihood
MEME	Mixed Effects Model of Evolution

VLP	(Virus-like particle), virusu podoben delec

Okrajšave virusov in viroidov:

TMV	virus mozaika tobaka
ToMV	virus mozaika paradižnika
BDMV	virus pritlikavosti in mozaika navadnega fižola
TRV	virus šelestenja tobaka
PEBV	virus zgodnjega rjavenja navadnega graha
PVA	virus krompirja A
TVBMV	virus združevanja žil in mozaika tobaka
TVMV	virus lisavosti žil tobaka
TEV	virus razjed tobaka
PepMoV	virus lisavosti paprike
PepRSV	virus obročkaste pegavosti paprike
PSTVd	viroid vretenatosti krompirjevih gomoljev

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

Izziv predloženega doktorskega dela je omejevanje in razumevanje širjenja najpomembnejšega virusnega povzročitelja bolezni na krompirju, krompirjevega virusa Y (PVY). Ker edino celostni pristop lahko zagotovi uspešno obvladovanje tovrstne problematike, smo raziskovali tako diagnostične pristope za določanje in klasifikacijo virusa kot tudi zaporedje genoma virusa, ki omogoča vpogled v virusno evolucijo. Razvili smo novo metodo izolacije čiste raztopine virusa ter raziskovali lokalizacijo in širjenjem virusa v gostiteljskih rastlinah.

1.1 SOLANUM TUBEROSUM

Krompir (*Solanum tuberosum* L.) je v prehrani ljudi tretja najpomembnejša poljščina, takoj za rižem in pšenico (Centro Internacional de la Papa, 2010). Bruto vrednost proizvodnje krompirja je bila samo v državah Evropske unije leta 2011 višja od 15,5 milijard ameriških dolarjev (Food and Agriculture Organisation, 2014). Odlikuje ga široko podnebno območje pridelave. Uspeva namreč od najjužnejših področij Južne Amerike do Grenlandije, od regij tik nad morsko gladino pa vse do 4700 m nadmorske višine v izvornem področju gorovja Andov (Centro Internacional de la Papa, 2010).

Pridelava krompirja potrebuje do kar sedemkrat manj vode v primerjavi z žiti, krompir pa ima tudi bogato hranilno vrednost. Kuhan krompir je odličen vir ogljikovih hidratov z nizko vsebnostjo maščob ter relativno visoko vsebnostjo proteinov (2,1 odstotek sveže mase, kar je več kot koruza), vitamina C, kalcija, železa in drugih esencialnih mikroelementov ter vlaknin. Pridelava krompirja postaja globalno vse pomembnejša, saj je krompir temeljni element prehranske varnosti v razvijajočih se državah Južne Amerike, Afrike in Azije (Centro Internacional de la Papa, 2010).

Pridelavo krompirja ogroža širok spekter bolezni in škodljivcev, od koloradskega hrošča (*Leptinotarsa decemlineata*), ogorčic (*Globodera pallida* in *G. rostochiensis*), gliv (*Phytophthora infestans*), bakterijskih gnilob (*Ralstonia solanacearum, Clavibacter michiganensis* pv. *sepedonicus, Dickea spp*.) in virusnih okužb (Centro Internacional de la Papa, 2010).

V tej doktorski nalogi smo preučevali kompleksno problematiko enega izmed najpomembnejših virusnih patogenov krompirja, krompirjevega virusa Y (Potato virus Y, v nadaljevanju PVY). PVY na krompirju povzroča številna bolezenska znamenja, katerih videz in jakost sta močno odvisna od kultivarja krompirja, različka virusa, ki ga okužuje, tipa okužbe e.g. primarna (z žuželčjim vektorjem v rastni sezoni) ali sekundarna okužba (preko okuženih semenskih gomoljev), pojavnost znamenj pa je odvisna tudi od klimatskih pogojev. Najpogostejša bolezenska znamenja so: mozaik, pegavost, nagubanost in nepravilna rast listov, lokalne in sistemske nekroze na listih, ki jih pogosto spremlja rumenenje in odpadanje listov. Rast rastlin je lahko zmanjšana in

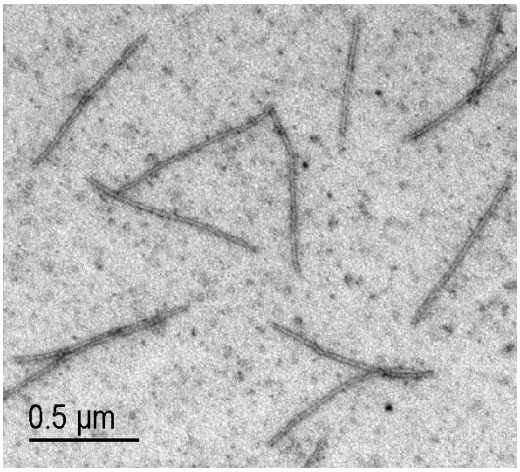
zakrnela. Nekateri različki povzročajo tudi nekroze na površini gomoljev, ki so značilno obročkaste oblike, v začetni fazi izbočene, nato pa močno vbočene. Bolezen imenujemo obročkasta nekroza gomoljev krompirja (Potato Tuber Necrotic Ringspot Disease ali PTNRD), zaradi katere so gomolji neuporabni za prodajo (Karasev in Gray, 2013). Pojav PTNRD je podobno kot bolezenska znamenja na listih močno odvisen od kultivarja in od pogojev rasti (Singh in sod. 2008), pogosto pa se pojavi šele med skladiščenjem gomoljev. PVY močno vpliva na proizvodnjo krompirja, saj zmanjša obseg pridelka za 40-70% (Nolte in sod., 2004), vrednost preostalega pridelka pa je lahko še dodatno zmanjšana zaradi bolezenskih znamenj (Beczner in sod., 1984; Le Romancer in sod., 1994).

1.2 KROMPIRJEV VIRUS Y

1.2.1 Osnovne značilnosti krompirjevega virusa Y

PVY je RNA virus predstavnik rodu Potyvirus družine Potyviridae, katerega virusni delec je sestavljen iz približno 10 kb dolge enoverižne pozitivno usmerjene RNA molekule, obdane s cca. 2000 molekulami proteina plašča. Na 5' konec RNA molekule je kovalentno vezan en protein VPg. Virusni delec ima filamentozno obliko in meri cca 740 nm v dolžino in 11 nm v preseku (Kerlan, 2006). Virusna RNA kodira 340-360 kDa velik poli-protein, ki se po translaciji razreže v 10 manjših večfunkcijskih proteinov (Urcuqui-Inchima in sod., 2001). Trije izmed njih imajo proteazno aktivnost (P1, Hc-PRO in NIa), protein plašča (CP) sestavlja virusni delec, sodeluje pa tudi v prenosu z vektorji. Protein NIb je virusna RNA polimeraza. Proteini P3, 6K1, CI, 6K2 in VPg sodelujejo pri premikanju virusa po rastlini, pomnoževanju RNA in translaciji proteinov (povzeto po Blanchard in sod., 2008; Grangeon in sod., 2013; Quenouille in sod., 2013). V območju genoma, kjer je kodiran protein P3 pa se v +2 odprtem bralnem okviru nahaja ali bolje rečeno »skriva« še enajsti protein t.i. PIPO (ali P3N-PIPO) (Chung in sod., 2008). Nastane zaradi zamika bralnega okvira pri translaciji in je nujno potreben za premikanje virusa med celicami (Vijayapalani in sod., 2012; Wei in sod., 2010).

PVY ima širok krog gostiteljskih rastlin, večinoma iz družine razhudnikovk (*Solanaceae*), med katerimi sta ekonomsko najpomembnejši vrsti krompir (*Solanum tuberosum*) in tobak (*Nicotiana tabaccum*). PVY je razširjen po celem svetu, v naravi ga prenaša vsaj 40 vrst listnih uši (primarna okužba), poleg tega se preko gomoljev prenaša še z materinske na hčerinsko rastlino (sekundarna okužba) in mehansko z rastlinskim sokom. Na lestvici desetih najpomembnejših rastlinskih virusov na svetu zaseda peto mesto predvsem zaradi ekonomskega vpliva v proizvodnji krompirja, tobaka ter paprike in paradižnika.



Slika 1. Filamentozni virusni delci PVY (presevna elektronska mikroskopija, foto: Rupar, M.)

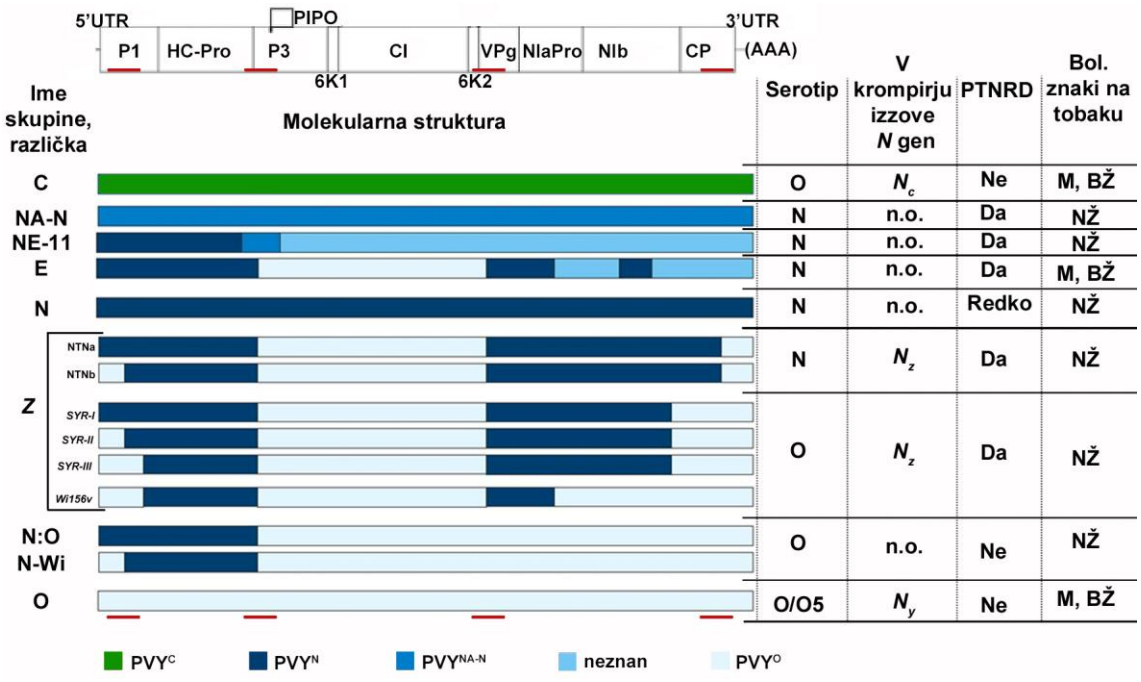
Figure 1. Filamentous virus particles of PVY (transmission electron microscopy, photo: Rupar, M.)

1.2.2 Kompleksna raznolikost različkov PVY in metode določanja

Poznamo veliko različkov virusa PVY, ki jih ločimo na podlagi molekulskih in genetskih kriterijev. Glede na njihovo zmožnost izzvati izražanje obrambnih genov *N* v različnih kultivarjih krompirja jih delimo v pet skupin (Blanchard in sod., 2008; Karasev in Gray, 2013), t.i. genetska klasifikacija. Različki iz skupin PVY^{O} sprožijo preobčutljivostno reakcijo v kultivarjih, ki imajo gen za odpornost Ny_{tbr} (pentland crown, désireé), PVY^{C} v kultivarjih z genom *Nc* (king edward), PVY^{Z} pa v kultivarjih z domnevnim genom *Nz*. Različki iz skupin PVY^{O}, PVY^{C} in PVY^{Z} v večini neodpornih sort krompirja povzročajo močne nekroze na listih, odpadanje listov in pegavost. Različki iz skupine PVY^{N} ne sprožijo preobčutljivostne reakcije proti genom Ny_{tbr}, *Nc* in *Nz*, večinoma povzročajo le mila znamenja mozaika na listih krompirja, povzročajo pa močne nekroze žil na tobaku. Bolezenske znamenja na tobaku uporabljamo le kot dodatno informacijo za pomoč pri razvrščanju. Skupina PVY^{E} ima nekaj lastnosti PVY^{O} in PVY^{N} različkov, ne povzroča nekroz žil na tobaku, premosti pa rastlinsko odpornost pogojeno z vsemi tremi geni (Ny_{tbr}, *Nc*, *Nz*) (Blanchard in sod., 2008). Genetska karakterizacija omogoča najbolj neposredno in s tem relevantno klasifikacijo različkov v skupine s podobnim biološkim vplivom na gostitelja. Prav razlikovanje med biološko različnimi izolati nam omogoča določitev optimalnih ukrepov za obvladovanje posameznih različkov. Navkljub veliki informativnosti genetskih testiranj na testnih rastlinah se tovrstne metode klasifikacije redko uporabljajo, predvsem ker so: i) dolgotrajne (priprava rastlin, inokulacija, opazovanje bolezenskih znamenj), ii) zahtevajo veliko prostora v rastlinjakih ali rastnih komorah (inokulirati moramo več rastlin (5-10), najmanj treh kultivarjev krompirja in vzporedno gojiti kontrolne/slepo inokulirane rastline), iii) pojav bolezenskih znamenj je odvisen od pogojev rasti (temperatura, vlaga, osvetlitev), zato ima tovrstna tipizacija slabo ponovljivost med

laboratoriji, iv) sama izvedba testiranja ni standardizirana med laboratoriji (uporablja se različne kultivarje z *N* geni, različne postopke inokulacije, ipd).

Razvoj hibridoma tehnologije je omogočil razvoj serološkega določanja in klasifikacije PVY z monoklonskimi protitelesi. S tem se je razvila tudi serološka klasifikacija. S pomočjo protiteles proti proteinu plašča (CP) lahko izolate razvrstimo v dve serološki skupini, O in N. Izolatov PVY s serološkimi tehnikami ne moremo natančno ločevati, saj je znotraj obeh seroloških skupin več podskupin z različnimi lastnostmi, ki jih lahko ločimo le z molekulskimi metodami. S protitelesi proti O tako zaznamo, ne pa tudi ločimo med izolati iz skupin PVY^{O}, $PVY^{N:O}$ in PVY^{N-Wi}. S protitelesi proti N pa zaznamo izolate iz skupin PVY^{N} in PVY^{NTN}. Velika večina izolatov, ki povzroča PTNRD, pripada serološki N skupini, kar pomeni, da je serološka lastnost še vedno precej dober pokazatelj za uporabo v diagnostiki. (Nikolaeva in sod., 2012). Problem seroloških testov je tudi njihova občutljivost, saj ne omogočajo zanesljivega določanja nizkih koncentracij virusa v vzorcih, npr. v primeru združenih vzorcev več gomoljev, kjer je le 1 okužen. Kljub pomanjkljivostim imajo serološke tehnike na čelu z encimsko-imunskim testom (ELISA) tudi prednosti. ELISA je enostavna za uporabo, omogoča delno avtomatizacijo in s tem visoko zmogljivost, poleg tega pa je stroškovno učinkovita. Spričo teh lastnosti se ELISA še vedno veliko uporablja za diagnostiko, npr. za določanje PVY v programih certificiranja semenskega materiala (Karasev in Gray, 2013).



Slika 2. Prikaz genomske strukture, seroloških lastnosti in pomembnejših bolezenskih znamenj vseh devetih skupin različkov virusa PVY, povzeto po Karasev in Gray (2013). Kratice: n.o., gen ne obstaja ali pa je neznan; M, mozaik; BŽ, bledenje žil; NŽ, nekroza žil.

Figure 2. Genomic structure, serological properties and symptoms induced by nine different PVY subgroups, adopted from Karasev and Gray (2013). Acronyms: n.o., gene not existing or unknown; M, mosaic; BŽ, vein clearing; NŽ, vein necrosis.

Napredek na področju sekvenciranja je omogočil določitev nukleotidnih zaporedij velikega števila različkov PVY in s tem razvoj molekularnih metod določanja in karakterizacije PVY. Glede na sorodnost nukleotidnih zaporedij razvrščamo različke v devet skupin (slika 2). Štiri skupine, katerih genom ni rekombiniran: PVY^{O}, PVY^{N}, PVY^{NA-N} in PVY^{C}. Med njimi na krompirju najpogosteje najdemo izolate iz skupin PVY^{N} in PVY^{O}, poročila o izolatih iz skupin PVY^{NA-N} in PVY^{C} pa so precej manj pogosta. Poznamo tudi pet rekombiniranh skupin različkov: PVY^{Z}, $PVY^{N:O}$, PVY^{N-Wi}, PVY^{E} in PVY^{NE-11}. V Evropi prevladujejo različki PVY^{Z}, ki so posledica rekombinacij genomov iz skupine PVY^{O} in PVY^{N} (Blanchard in sod., 2008; Karasev in Gray, 2013). Najpogostejši so različki tipa PVY^{NTN} (slika 2), ki imajo tri ali štiri mesta rekombinacije (v genih za proteine P1, P3, VPg in CP) (Boonham in sod., 2002; H. Barker in sod., 2009; Glais in sod., 2002). V pridelavi krompirja so najpomembnejši prav različki PVY^{NTN}, saj vsi povzročajo PTNRD (Rupar in sod., 2013a). Nekroze na gomoljih lahko povzročajo tudi nekateri različki, ki imajo v genomu le eno do dve mesti rekombinacije med PVY^{O} in PVY^{N} in jih imenujemo $PVY^{N:O}$ in PVY^{N-Wi} (Piche in sod., 2004; Chikh Ali in sod., 2007; Schubert in sod., 2007). Ti so manj pogosti kot PVY^{NTN}. Najdeni so bili tudi nerekombinirani različki PVY^{N} in PVY^{O}, ki povzročajo nekrozo gomoljev (Nie in Singh, 2003; Glais in sod., 2005), vendar pa so le ti zelo redki.

Za preučevanje epidemiologije in načrtovanje preventivnih ukrepov za preprečevanje širjenja nekrotičnih različkov nujno potrebujemo učinkovito določanje in karakterizacijo različkov. Serološki test ELISA je sicer hiter, cenovno dostopen, nudi določevanje in razlikovanje med izolati z O in N serotipom, vendar pa ne loči PVY^{N} in PVY^{NTN} izolatov. Obstajajo sicer monoklonska protitelesa za razlikovanje med PVY^{N} in PVY^{NTN} (Ceřovská, 1998), ki pa niso dovolj zanesljiva.

Hitra evolucija in adaptacija je ena izmed značilnosti PVY, zaradi česar je razvoj zanesljivih metod za določanje zelo otežen. Za natančnejše razlikovanje in boljše poznavanje pestrosti izolatov PVY potrebujemo njihova nukleotidna zaporedja. S pomočjo informacije o nukleotidnih zaporedjih so bila razvita molekulska orodja, ki na podlagi razlik v nukleotidnih zaporedjih zanesljivo ločijo med različki PVY. Do sedaj so bile razvite metode verižne reakcije s polimerazo z reverzno transkripcijo (RT-PCR) z enim in več pari nukleotidnih začetnikov (Boonham in sod., 2002; Nie in Singh, 2002; Moravec in Cerovska 2003; Lorenzen in sod., 2006; Schubert in sod., 2007), RT PCR v kombinaciji z analizo restrikcijskih fragmentov (Glais in sod. 1996; Rosner in Maslenin, 1999) ter AmpliDet RNA testom (Szemes in sod., 2002). Najnovejši in najnaprednejši metodi za razlikovanje pa sta SNaPshot (Rolland in sod., 2008), ki različke ločuje na podlagi točkovnih polimorfizmov preko celotnega genoma, in RT-PCR v realnem času (RT-qPCR) (Agindotan in sod., 2007; Balme-Sinibaldi in sod., 2006; Kogovšek in sod., 2008), ki poleg ločevanja med različki omogoča tudi kvantifikacijo števila kopij virusnega genoma.

Do sedaj je bilo določeno le eno zaporedje genoma virusa PVY, izoliranega v Sloveniji, zato je potrebno pridobiti več informacij o genetski pestrosti, s katerimi bi omogočili izboljšano diagnostiko. Z naborom večjega števila nukleotidnih zaporedij lahko nato raziskujemo, kateri deli genoma so vključeni v procese adaptacije na gostiteljsko rastlino, katere aminokisline so podvržene pozitivnim in negativnim evolucijskim pritiskom in tako ocenimo, kateri molekularni označevalci so bolj ali manj primerni za uporabo v molekulski diagnostiki (Cuevas in sod., 2012b). Zaporedje gena za CP je eno izmed najpogosteje raziskovanih delov genoma PVY (Moury in Simon, 2011), pogosto uporabljeno za filogenetske študije in klasifikacijo različkov (Chikh Ali in sod., 2010a; Kogovšek in sod., 2008; Lorenzen in sod., 2006; Moravec in sod., 2003; Schubert in sod., 2007), CP je direktno povezan tudi s serološkim določanjem PVY, saj lahko točkovne mutacije v CP genu spremenijo serološki značaj izolata in s tem povzročijo napačno klasifikacijo (Chikh Ali in sod., 2007; Karasev in sod., 2009). Potivirusni CP je primer večfunkcionalnega virusnega proteina, ki pomembno vpliva na povzročanje bolezenskih znamenj pri različnih gostiteljih (Andrejeva in sod., 1999; Hu in sod., 2011; Ullah in Grumet, 2002), udeležen je pri prenosu virusa z vektorjem med rastlinami (Peng in sod., 1998), pri čemer je za prenos z ušmi še posebno pomemben DAG motiv v N- terminalnem delu CP (Atreya in sod., 1991, 1995). Pokazali so tudi njegove interakcije s protivirusnim proteinom Hc-Pro (Seo in sod., 2010) in z veliko podenoto encima RubisCO (Feki in sod., 2005).

1.2.3 Čista raztopina virusnih delcev PVY: zakaj in kako?

Hitra evolucija virusnega genoma in s tem tudi proteina CP vpliva na učinkovitost seroloških metod določanja PVY. Posledica mutacij ali rekombinacij je slabše ali pa popolnoma onemogočeno določanje. V ta namen je potrebno sprotno proizvajati protitelesa proti novo nastajajočim različkom. Za postopke imunizacije pri pridobivanju novih monoklonskih protiteles potrebujemo suspenzijo očiščenih virusnih delcev. Suspenzije čistih virusnih delcev so poleg imunizacije potrebne tudi za študije interakcij virusnih delcev s protitelesi in z drugimi proteini (Gutiérrez-Aguirre in sod., 2014) ter za študije *in-vitro* prenosa z vektorji (Huet in sod., 1994; Peng in sod., 1998).

Klasična izolacija čistih virusnih delcev PVY iz rastlinskega materiala je navadno težaven proces, ki traja 4-5 dni in vključuje več korakov čiščenja: i) homogenacija rastlinskega materiala v nevtralnem pufru z dodatki za preprečevanje agregacije (EDTA, urea, citrat, Triton X-100…); ii) bistrenje homogenata s kloroformom, dietiletrom, ogljikovim tetrakloridom, centrifugiranjem, ipd.; iii) čiščenje: z obarjanjem z amonijevim sulfatom, z ultracentrifugiranjem skozi saharozni gradient ter gradient cezijevega klorida in frakcioniranje gradienta; iv) dializa in zamenjava pufra (Leiser in Richter, 1978). Proces zahteva zelo izurjeno ter specializirano osebje z veliko izkušnjami ter drago opremo, kot so ultarcentrifugirne naprave, zato je mnogim laboratorijem nedostopen.

Za premostitev zgoraj omenjenih pomanjkljivosti klasične izolacije smo v tej nalogi razvili bolj dostopno metodo čiščenja na podlagi ionsko izmenjevalne kromatografije na monolitnih nosilcih Convective Interaction Media® (CIM) (Rupar in sod., 2013b). Metakrilatni monolitni kromatografski nosilci so se izkazali kot zelo uporabni pri izolaciji in čiščenju večjih organskih molekul. Njihova prednost pred klasičnimi kromatografskimi kolonami je v tem, da imajo izjemno veliko skupno površino za vezavo in hkrati relativno velike pore/kanalčke (800-1200 μm), ki omogočajo vstop velikih biomolekul v kolono. Vsi kanalčki so med seboj povezani, kar pomeni, da je vsa površina kolone dostopna za vezavo, ne da bi bila za to potrebna difuzija delcev v »slepe ulice« kolone, kot je to v primeru klasičnih kromatografskih kolon. S tem je število dostopnih mest za vezavo (dinamična kapaciteta kolone) neodvisno od pretoka skozi kolono, kar omogoča delo z večjimi volumni vzorcev (Podgornik in Strancar, 2005). Metakrilatni monolitni kromatografski nosilci pod tržnim imenom CIM® (BiaSeparations, Slovenija) so bili že uspešno uporabljeni za koncentriranje virusa mozaika tobaka (TMV) (Kramberger in sod., 2004), za čiščenje rigidnih paličastih delcev virusa mozaika paradižnika (ToMV) iz rastlinskega materiala (Kramberger in sod., 2007), dvo-verižne RNA (dsRNS) in satelitne RNA virusa mozaika kumar (Krajacić in sod., 2007), hipovirusne dsRNA iz glive *Cryphonectria parasitica* (Perica in sod., 2009) ter viroida vretenatosti krompirjevih gomoljev (PSTVd) (Ruščić in sod., 2013). S CIM so bili uspešno koncentrirani rotavirusi iz pitne in rečne vode (Gutiérrez-Aguirre in sod., 2009, 2011) in virus hepatitisa A iz ustekleničene vode (Kovac in sod., 2009), virus gripe (Peterka in sod., 2008); bakteriofagi (Kramberger in sod., 2010; Smrekar in sod., 2008), virusom podobni delci (Urbas in sod., 2011), plazmidne in genomske DNA (Forcic in sod., 2005; Krajnc in sod., 2009) in celo ribosomi (Trauner in sod., 2011). CIM monoliti se do sedaj še niso uporabljali za izolacijo filamentoznih virusov. Izolacija s CIM monoliti močno skrajša čas izolacije (iz nekaj dni na nekaj ur), ne potrebuje korakov ultracentrifugiranja, odlikujejo pa jo tudi visoki izkoristki, visoka resolucija ločbe in enostavno povečevanje kapacitete čiščenja (scale up), saj imajo vse velikosti kolon od 0,1 ml do 8 l enake pore in enako dinamiko vezave.

1.2.4 Infektivni kloni PVY: kaj, kako in zakaj?

Poleg poznavanja nukleotidnega zaporedja je za uspešno določanje in preučevanje interakcij potrebno poznati tudi, kje se virus v rastlini nahaja in kako se po rastlini širi.

Močno raziskovalno orodje virologov so infektivni kloni, kjer je celoten genom vstavljen v enega ali več plazmidov, primernih za pomnoževanje izven gostiteljske rastline, npr. v *E. coli* ali *S. cerevisiae* (Desbiez in sod., 2012; Gao in sod., 2012; Lee in sod., 2011; López-Moya in García, 2000) pri čemer virus obdrži infektivnost v rastlini. Uporabljeni so bili tako za študije funkcionalne analize virusnih in rastlinskih genov kot tudi za spremljanje širjenja virusov po rastlini (natančneje opisano v nadaljevanju). Problem priprave infektivnih klonov PVY je predvsem v tem, da lahko pride do

translacije virusnih proteinov že v *E. coli*. Zaporedje PVY namreč vsebuje kriptične promotorje, zaradi katerih pride do izražanja proteinov, ki so za bakterijo toksični (Jakab in sod., 1997). Infektivni kloni omogočajo tarčno spreminjanje virusnega genoma in opazovanje sprememb virusnega fitnesa (patogenost, hitrost pomnoževanja in širjenja po rastlini, prenos z vektorji) zaradi sprememb genoma. Prvi infektivni klon PVY so razvili Jakab in sod., (1997). Genom PVY-N605 (Švicarski nekrotični izolat) so prepolovili v proteinu CI tako, da so dobili 5' in 3' polovico genoma, nato pa vsak del posebej vstavili v plazmid. Tako so dobili dva manjša klona, ki nista toksična za *E. coli*, se v bakteriji hitro in stabilno pomnožujeta in omogočata enostavno spreminjanje genoma PVY. Klona je potrebno pred biolistično inokulacijo v gostiteljsko rastlino ligirati tako, da dobimo celoten genom PVY. Z uporabo omenjenega infektivnega klona so Tribodet in sod. (2005) pripravili paleto kimernih PVY genomov med nekrotičnim različkom PVY-N605 (žilne nekroze na tobaku) in milejšim PVY O139, ki povzroča na tobaku le znamenja mozaika. Z uporabo kimer so ugotovili, kateri del genoma PVY je odgovoren za bolezensko znamenje nekroz žil in celo določili dve aminokislinski determinanti, odgovorni za nastanek žilnih nekroz: K_{400} in E/G_{419}, ki se nahajata v C-terminalnem delu proteina HcPro (zaporedna št. aminokisline kot v Jakab in sod., (1997)). Temu je sledilo še odkritje tretje aminokisline, odgovorne za nastanek nekroz žil v N-terminalnem delu proteina HcPro (N_{339}) (Faurez in sod., 2012). V isti študiji so identificirali še dve regiji med proteinoma CI in NIaPro, ki sodelujeta pri nastanku in modifikaciji jakosti nekroz žil v tobaku.

Kasneje je bil genom PVY-N605 različka vstavljen tudi v celoti v plazmid brez razpolovitve, pri čemer je bil stabiliziran s tremi introni v domnevno toksičnih genih i.e. klon PVY 123 (Bukovinszki in sod., 2007). Klon je bil uporabljen za pripravo kimere (PVY^O namesto PVYN zaporedja C-terminalnega dela proteina NIb, CP in 3' UTR regije), s katero so opazovali vpliv spremenjenega proteina CP na nastanek bolezenskih znamenj na *N. benthamiana, N. tabacum* cv. Xanthi, *N. glutinosa*, *Solanum tuberosum* cv. Russet Burbank, *Physalis pubescens* in *P. floridana*. Obstaja tudi infektivni klon, pri katerem je celoten PVY genom v enem plazmidu stabiliziranem preko točkovnih mutacij v regijah ki kodirajo kriptične prokariotske promotorje. Ta klon je drugačen od prej omenjenih, še v tem, da pripada rekombinantni skupini PVY^Z (PVY^{NTN} iz Sirije) (Chikh Ali in sod. 2011).

Uporaba infektivnih klonov je tudi edini način, s katerim lahko viruse označimo s fluorescentnimi in drugimi označevalci, kar omogoča sledenje premikanju virusa med celicami in sistemsko po rastlini. Poročali so že o konstrukciji prenekaterih rastlinskih virusov, označenih s fluorescentnimi proteini. Prvi med njimi je bil označen virus X krompirja (PVX) (Baulcombe in sod., 1995), ki je stabilno izražal zeleni fluorescentni protein (GFP) v *N. benthamiana* in s tem omogočil spremljanje vzorcev širjenja. Tej raziskavi je sledilo še več drugih, npr. virus mozaika tobaka (TMV) (Cheng in sod., 2000) in virus pegavosti paprike (PepMoV) (Lee in sod., 2011) sta bila označena z GFP

za sledenje sistemske okužbe v *N. benthaminana*. Virus pritlikavosti in mozaika navadnega fižola (BDMV) z GFP označevalcem je bil uporabljen za analizo procesa virusne okužbe v gostiteljskih in negostiteljskih vrstah (Wang in sod., 1999). Za infektivne klone treh tobravirusov, označenih z GFP, virus šelestenja tobaka (TRV), virus zgodnjega rjavenja navadnega graha (PEBV) in virus obročkaste pegavosti paprike (PepRSV) so poročali o zelo širokem razponu gostiteljev, v katerih je bil GFP izražen tudi v koreninah (MacFarlane in Popovich, 2000).

Fluorescentno ter z drugimi označevalci je bilo označenih tudi nekaj virusov iz rodu *Potyvirus*, kateremu pripada tudi PVY. Krompirjev virus A (PVA) (Rajamäki in sod., 2005) in virus združevanja žil in mozaika tobaka (TVBMV) (Gao in sod., 2012) sta bila označena z GFP v *N. benthamiana* . Z GFP je označen virus šarke (PPV), ki je bil uporabljen za študij interakcij med PPV in gostiteljskimi rastlinami na ravni cele rastline ter tudi na celični ravni (Lansac in sod., 2005). Virus lisavosti žil tobaka (TVMV), virus rumenih žil detelje (ClYVV) in PPV označeni z GFP in dsRed so bili uporabljeni za raziskovanje sočasne okužbe celic z različnimi virusi (Dietrich in Maiss, 2003). Virus razjed tobaka (TEV) in virus mozaika repe (TuMV) sta bila pred kratkim označena z Ros1 transkripcijskim faktorjem, ki sproži sintezo antocianov v okuženih celicah in s tem omogoča *in vivo* sledenje v *N. tabacum*, *N. benthaminana* in *Arabidopsis thaliana* (Bedoya in sod., 2012). Obstaja tudi z GFP označen PVY, ki je bil konstruiran na bazi infektivnega klona PVY 123 (Bukovinszki in sod., 2007) in je bil omenjen v študiji proteinsko-proteinskih interakcij med PVY in *N. tabacum* (Hofius in sod., 2007).V tej študiji pa ni bilo navedenih nobenih podatkov o bioloških značilnostih ali stabilnosti konstrukta.

1.3 RAZISKOVALNA VPRAŠANJA IN HIPOTEZE

Za uspešno omejevanje širjenja PVY in odpravljanja njegovih socio-ekonomskih ter okoljskih vplivov je potrebno poznavanje številnih vidikov njegove problematike.

Zanimale so nas metode določanja PVY, njihove prednosti in pomanjkljivosti v smislu specifičnosti in občutljivosti ter njihove uporabnosti za diagnostiko v oziru na hitro evolucijo PVY v naravi. V primeru PVY pa ni pomembno le določanje, temveč tudi klasifikacija različkov, saj posamezni različki povzročajo različna bolezenska znamenja in zahtevajo specifične ukrepe. Trenutno obstaja veliko število objav molekularnih in drugih orodij za določanje in klasifikacijo PVY, katerih diagnostična uporaba ni vedno jasno razvidna. V tej nalogi smo pod drobnogled vzeli dve najsodobnejši molekularni orodji (SNaPshot in RT-qPCR) ter ju preizkusili na vzorcih iz polja. Predvsem nas je zanimalo, katera je bolj uspešna v natančnem zaznavanju PVY^{NTN} različkov, ki povzročajo PTNRD.

Prva hipoteza:

Z metodo verižnega pomnoževanja z reverzno transkripcijo v realnem času (Kogovšek in sod. 2008) bomo zanesljiveje ločili med PVY^{N} in PVY^{NTN} različki. Metoda ne bo tako občutljiva za točkovne mutacije kot SNaPshot metoda (Rolland in sod. 2008), s katero bomo določili vse različke tudi v mešanih okužbah.

Ker je bilo do sedaj na voljo le eno nukleotidno zaporedje slovenskega izolata PVY, smo želeli določiti nukleotidna zaporedja dodatnih izolatov, najdenih na rastlinah v Sloveniji, da bi lažje ocenili, kakšna je njihova raznolikost na nivoju nukleotidnih zaporedij. V ta namen smo izvedli sekvenciranje in primerjavo nukleotidnih zaporedij. Z zbranimi nukleotidnimi zaporedji pa smo nato lahko raziskovali tudi evolucijo genoma PVY.

Druga hipoteza:

Z določitvijo nukleotidnih zaporedij izbranih genov virusa PVY bomo ugotovili prisotnost več različkov PVY v Sloveniji.

Klasični postopek izolacije PVY je zelo zamuden, zapleten in tudi težko dostopen veliko laboratorijem, saj nimajo vsi na razpolago naprav za ultracentrifugiranje. Zato nas je zanimalo, če lahko postopek poenostavimo z uporabo CIM monolitnih nosilcev. Uporaba le teh za ločevanje bioloških makromolekul je bila že pokazana, vendar pa še nikoli na tako velikem in gibljivem delcu kot je PVY.

Tretja hipoteza:

Monolitno tehnologijo CIM bo možno uporabiti za izboljšano čiščenje infektivnih virusnih delcev filamentoznega virusa PVY.

Do sedaj imamo podatke o razporeditvi in širjenju PVY v gostiteljskih rastlinah na podlagi opazovanja virusnih inkluzij, koncentracije virusne RNA, bolezenskih znamenj, ne moremo pa opazovati metabolno aktivnega virusa *in vivo*. V ta namen bomo razvili infektivne klone virusa PVY označene z proteinom GFP.

Četrta hipoteza

Z GFP označenimi infektivnimi kloni virusa PVY bomo opazovali razporeditev virusa PVY v gostiteljskih rastlinah in potrdili hipotezo, da metabolna aktivnost virusa ne korelira s pojavom vidnih bolezenskih znamenj.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Ugotavljanje primernosti metod SNaPshot in RT-qPCR za razločevanje skupin različkov krompirjevega virusa Y (PVY)

Assessment of SNaPshot and single step RT-qPCR methods for discriminating Potato virus Y (PVY) subgroups

Matevž Rupar, Polona Kogovšek, Maruša Pompe-Novak, Ion Gutiérrez-Aguirre, Agnes Delaunay, Emmanuel Jacquot, Maja Ravnikar

Journal of Virological Methods, 2013,vol. 189, no, 1, pp 93–100

Izvleček:

Krompirjev virus Y (PVY) je najpomembnejši virus, ki okužuje krompir (*Solanum tuberosum L*), saj povzroča obročkasto nekrozo na gomoljih krompirja (Potato Tuber Necrosis Ringspot Disease-PTNRD) in s tem močno vpliva na pridelavo semenskega krompirja. Po svetu krožijo številne skupine različkov z različno patogenostjo in posledično različnim gospodarskim vplivom. Orodja za natančno in zanesljivo odkrivanje in razlikovanje skupin PVY so zato bistvenega pomena za uspešno obvladovanje bolezni. V tej študiji smo ocenjevali dve najsodobnejši orodji za karakterizacijo, ki temeljita na določanju molekularnih označevalcev: RT-qPCR (Kogovšek in sod., 2008) in SNaPshot (Rolland in sod., 2008). Ocenjevali smo njihovo sposobnost natančnega razlikovanja in dodeljevanja različkov v pravo skupino. Rezultate smo potrdili z biološkimi testi, ELISA metodo ter *in silico* analizo nukleotidnih zaporedij. SNaPshot metoda je razlikovala med več različnimi skupinami različkov kot RT-qPCR, vendar je RT-qPCR bolj zanesljivo določal različke PVY^{NTN}, ki so po večini odgovorni za PTNRD. Razlike v določanju so bile posledica dejstva, da metodi uporabljata različne molekularne označevalce za določanje različkov. Obe orodji sicer uporabljata genotipske determinante za določanje PVY^{NTN} in ne bioloških. Nadaljnji razvoj se mora osredotočiti na ugotavljanje genomskih determinant za PTNRD. Dokler determinante ne bodo znane, pa ostajata metodi RT-qPCR in SNaPshot najmočnejši diagnostični orodji za karakterizacijo PVY različkov v Evropi.

Journal of Virological Methods 189 (2013) 93–100

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Assessment of SNaPshot and single step RT-qPCR methods for discriminating *Potato virus Y* (PVY) subgroups

Matevž Rupar[a,*], Polona Kogovšek[a], Maruša Pompe-Novak[a], Ion Gutiérrez-Aguirre[a,b], Agnes Delaunay[c], Emmanuel Jacquot[c], Maja Ravnikar[a,b]

[a] National Institute of Biology, Department of Biotechnology and Systems Biology, Večna pot 111, 1000 Ljubljana, Slovenia
[b] The Centre of Excellence for Biosensors, Instrumentation and Process Control-COBIK, Solkan, Slovenia
[c] INRA, UMR 385 BGPI, Campus de Baillarguet, 34398 Montpellier cedex 5, France

Article history:
Received 19 September 2012
Received in revised form 23 January 2013
Accepted 28 January 2013
Available online 5 February 2013

Keywords:
Potato virus Y (PVY) subgroup discrimination
Reverse transcription real time polymerase chain reaction (RT-qPCR)
Multiple single nucleotide polymorphisms interrogation assay (SNaPshot)
Bioassay
Potato tuber necrotic ringspot disease (PTNRD)

A B S T R A C T

Potato virus Y (PVY) is the most important virus infecting potato (*Solanum tuberosum*), causing potato tuber necrotic ringspot disease (PTNRD), with a great impact on seed potato production. Numerous PVY strain groups with different pathogenicity and economical impact are distributed worldwide. Tools for accurate and reliable detection and discrimination of PVY strain groups are therefore essential for successful disease management.

Two state of the art characterization tools based on detecting molecular markers – RT-qPCR (Kogovsek et al., 2008) and SNaPshot (Rolland et al., 2008) – were assessed for their ability to assign PVY accurately to the correct group. The results were validated by bioassay, ELISA and *in silico* sequence analysis. The spectrum of PVY strain groups distinguished by SNaPshot is broader than that by RT-qPCR. However, the latter was more reliable in discriminating the PVY^{NTN} group members, known for their ability to induce PTNRD on selected potato cultivars. The difference in discrimination precision was due to different molecular markers being targeted by RT-qPCR and SNaPshot. Both tools use genotypic markers for detecting PVY^{NTN} strain groups. Future development, however, should be focused on identifying the genomic determinants of the tuber necrosis property. Until then, the RT-qPCR and SNaPshot methods remain the most powerful diagnostic tools for detecting the PVY subgroup isolates found in Europe.

1. Introduction

Potato virus Y (PVY) is the type member of the genus *Potyvirus* (family Potyviridae) (King et al., 2011). The PVY genome comprises a 9.7 kb long single-stranded, positive-sense RNA molecule, with a VPg protein attached covalently to its 5′ end and a poly-A tail at its 3′ end (Dougherty and Carrington, 1988). The RNA encodes a polypeptide that is cleaved by three virus encoded proteases into ten products (P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and CP) (Urcuqui-Inchima et al., 2001). A second, short open reading frame (ORF) PIPO embedded within the above described large ORF has been reported (Chung et al., 2008), and its involvement in viral cell-to-cell movement confirmed (Vijayapalani et al., 2012). PVY is transmitted by at least 40 different aphid species in a non-persistent manner (Sigvald, 1984), the most important vector being the green peach aphid, *Myzus persicae* (Sulzer) (De Bokx and Cuperus, 1987; Radcliffe and Ragsdale, 2002). PVY has a wide host range including cultivated (*e.g.* potato, tomato, tobacco and pepper) and wild species within the Solanaceae family (Singh et al., 2008). It is distributed worldwide (Blanchard et al., 2008) and is one of the most important viruses infecting potato. Some PVY isolates are able to cause potato tuber necrosis ringspot disease (PTNRD) on sensitive potato cultivars (Beczner et al., 1984; Le Romancer et al.,1994), a disease characterized by the appearance of necrotic rings on infected tubers. These symptomatic rings may appear at harvest but often develop under storage conditions, resulting in rejection of up to 100% of the crops for table use, seed or processing (Boonham et al., 2002a). Consequently, PVY constitutes one of the most important sanitary problems for seed potato growers and has recently been classified into the group of the ten most important plant viruses from economic and scientific points of view (Scholthof et al., 2011).

PVY isolates were originally classified according to their capacity to induce necrotic lesions on indicator hosts (potato cultivars with various resistance genes). PVY isolates are classified according to the resulting pathotypes into five strain groups – PVY^{N} (Y^{N}), PVY^{O} (Y^{O}), PVY^{C} (Y^{C}), PVY^{Z} (Y^{Z}) and PVY^{E} (Y^{E}) (reviewed in Singh et al. (2008)). Due to the time and spatial requirements of potato bioassays, simpler and faster detection/characterization tools were required. The development of anti-PVY universal and

* Corresponding author. Tel.: +386 59 232 843; fax: +386 12 573 847.
E-mail address: matevz.rupar@nib.si (M. Rupar).

0166-0934/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2013.01.013

strain-specific antibodies, raised against PVY coat protein (CP), enabled faster analysis of samples and markedly improved PVY diagnostic procedures and epidemiology. Serological assays enable differentiation of Y^C, Y^O and Y^N (Ohshima et al., 1990; Sanz et al., 1990; Singh et al., 1993). However, with the emergence of new recombinant isolates, ELISA assay performance has become inadequate.

Molecular techniques based on differentiating PVY sequences offer better and more reliable differentiation of isolates. Sequencing and phylogenetic analysis initially revealed three main genotypes (Y^O, Y^N, Y^C) (Jakab et al., 1997; Schubert et al., 2007; Singh and Singh, 1996) and a variety of recombinant genotypes, such as PVY^N Wilga ($Y^{N\text{-}Wi}$) isolates, recognized as recombinants between Y^O and Y^N genotypes (Glais et al., 2002; Lorenzen et al., 2006a,b). The isolates designated as $Y^{N:O}$ (Nie and Singh, 2003a) in the US and Canada were recognized as recombinants between Y^N genotypes and Y^O-O5, a recently discovered group related to Y^O (Karasev et al., 2011). Similarly, isolates belonging to the Y^{NTN} subgroup were shown to result from Y^O/Y^N recombination events (Glais et al., 2002; Hu et al., 2009a,b) and were proposed to originate from $Y^{N:O}$ group *via* additional genetic exchanges (Karasev et al., 2011). Furthermore, other types of recombinants have been discovered, such as isolates from Syria exhibiting a mixture of $Y^{N\text{-}Wi}$ and Y^{NTN} genomic patterns, denoted as $Y^{NTN\text{-}NW}$ (Chikh Ali et al., 2007a,b), an isolate NE-11 corresponding to a recombinant between a Y^N genome and an uncharacterized PVY sequence (Lorenzen et al., 2008), and an isolate nnp in which $Y^O/Y^N/Y^C$ genetic sequences are associated (Fanigliulo et al., 2005; Schubert et al., 2007).

Based on the acquisition of sequence data, numerous methods have been developed for detecting and differentiating PVY according to their genomic organization. Characterization tools based on hybridization of viral RNA (Rosner and Maslenin, 1999, 2001) and PCR-RFLP analysis (Glais et al., 1996) were followed by the publication of numerous PVY genotype/variant specific RT-PCR primer sets (Boonham et al., 2002b; Chikh Ali et al., 2010a; Lorenzen et al., 2006a,b; Mallik et al., 2012; Moravec et al., 2003; Nie and Singh, 2003a; Rigotti and Gugerli, 2007; Schubert et al., 2007; Walsh et al., 2001; Weilguny and Singh, 1998). Besides their high sensitivity, PCR-based methods offer reliable detection and group discrimination in single and mixed infections (Lorenzen et al., 2006a,b). Implementation of reverse transcription real-time PCR techniques (RT-qPCR) further improved sensitivity and enabled fast and reliable group specific quantification of PVY, even in symptomless samples with low viral loads (Jacquot et al., 2005; Balme-Sinibaldi et al., 2006; Kogovsek et al., 2008).

The reverse genetic approach was used to identify molecular markers linked to tobacco vein necrosis phenotype. The two nucleotides in HC-Pro gene (nucleotides 2213 and 2271, that encode residues K_{400} and E_{419} (Tribodet et al., 2005)) were first included in the RT-qPCR method for distinguishing necrotic and non-necrotic strains of tobacco and for their quantification (Jacquot et al., 2005). These determinants were also included in a multiplex interrogation assay (SNaPshot) for specific detection of tobacco necrotic isolates and some of their genomic characteristics (Rolland et al., 2008).

No biologically significant determinants for the induction of PTNRD in potato are presently known. There is evidence that the great majority of PVY^{NTN} isolates, a minor portion of $PVY^{N\text{-}Wi}$ isolates and a few PVY^O isolates are able to induce PTNRD on certain potato cultivars (Gray et al., 2010; Lindner and Kellermann, 2012; Yin, 2011 personal communication). Since PTNRD molecular determinants have yet to be characterized and with the increasing occurrence of PVY^{NTN} variants in the field over the past 20 years, especially in Europe (Blanchard et al., 2008; Davie, 2012 personal communication; Lindner and Kellermann, 2012), it is necessary to evaluate the reliability of current molecular tools for PVY detection and discrimination.

In this work, two 'state of the art' methods for PVY strain discrimination have been assessed – SNaPshot (Rolland et al., 2008) and single step RT-qPCR (Kogovsek et al., 2008). The methods were chosen for their (i) high reliability for detection/identification in single and mixed infections, (ii) significant improvement in viral diagnostics and (iii) low detection thresholds. The aim of this work is to expose and exemplify the advantages and pitfalls of the two molecular tools for PVY discrimination and assess their use in diagnostics, with emphasis on reliable detection of PTNRD inducing PVY isolates.

2. Materials and methods

2.1. Plants and viruses

Leaves from symptomatic potato plants were collected from fields in the north and central parts of Slovenia. Samples were tested for the presence of PVY using the ELISA procedure described below. PVY-infected samples were used for inoculation of tobacco and potato. To do that, healthy tobacco (*Nicotiana tabacum* cv. White Burley) and potato (*Solanum tuberosum* cv. Igor) plants were produced in a growth chamber (21 ± 2 °C; 16 L/8D h photoperiod). Plants (at 3–5 leaf stage) were inoculated with the sap of PVY-infected field samples or with the reference isolate for the Y^{NTN} group NIBv1 (PVY-NIB-NTN GenBank ID: AJ585342) maintained in tissue cultures on *S. tuberosum* cv. Pentland. Two lower leaves of each test plant were dusted with carborundum powder (VWR International, Fonterey sous Bois, FR) and inoculated with homogenized PVY-infected plant material diluted (1:5, w/v) in 20 mM phosphate buffer, pH 7.6 with 2% polyvinylpyrrolidone (PVP) (M 10.000) (Sigma–Aldrich, St. Louis, CA, USA). Inoculum was left on the leaves for 10 min then gently rinsed off with tap water. After inoculation, plants were transferred to the greenhouse for the following 8 weeks (20 ± 5 °C; natural and artificial lighting, 16 h photoperiod).

Foliage symptoms were documented at 10 and 14 days post inoculation (dpi) on tobacco and at 14 dpi on potato. At 30 dpi, 1 or 2 non-inoculated fully developed leaves from each inoculated plant were sampled, lyophilized individually in glass containers and stored at −20 °C for subsequent RNA extraction. Tubers produced from inoculated potato plants were harvested at 55 dpi and stored at room temperature for six weeks (until 95 dpi) to monitor putative expression of PTNRD symptoms.

2.2. ELISA and immuno-capture

For ELISA and immuno-capture procedures, Nunc® microtitation plates (eBiosciences, San Diego, CA, USA) were used. The double antibody sandwich (DAS)-ELISA protocol consisted of coating wells of a microtitration plate with 200 μl of primary monoclonal antibodies from the following commercial assays, anti-PVY^N (1051, Adgen, Ayr, UK), anti-PVY^O (1129, Adgen, Ayr, UK) or anti-$PVY^{O/C}$ (1052, Adgen, Ayr, UK). These antibodies were diluted according to the manufacturer's recommendations in carbonate coating buffer (0.15 mM Na_2CO_3 (Merck, Darmstadt, DE), 0.35 mM $NaHCO_3$ (Merck, Darmstadt, DE), pH 9.6). Plates were coated for 4 h at 37 °C, followed by 3× 5 min washing with 1x PBST (1× PBS pH 7.4, containing 0.05% of Tween 20 (Sigma–Aldrich, St. Louis, CA, USA)).

Plant sap was obtained by grinding fresh or lyophilized leaf material using a Homex grinder (Bioreba, Nyon, Switzerland) in grinding buffer (1× PBS, 2% PVP (M 10.000), pH 7.4) at 1:5 or 1:50 ratio (w/v) for fresh and lyophilized tissue, respectively. Wells of the plate were loaded with 200 μl of plant sap and incubated overnight at 4 °C before being washed (4× 5 min, 1× PBST). Secondary alkaline

phosphatase conjugated antibodies specific to PVY^N (Adgen, UK), PVY^O (Adgen, UK) and $PVY^{O/C}$ (Adgen, UK) were diluted in conjugate buffer (1× PBS with 0.05% Tween 20 and 0.2% bovine serum albumin (Sigma–Aldrich, St. Louis, CA, USA) according to manufacturer's recommendations. Antibodies (200 μl per well) were incubated at 37 °C for 1 h. At the end of the incubation period, plates were washed 4× 5 min with PBST. Plate wells were filled with 200 μl of *p*-nitrophenylphosphate (1 mg/ml) in substrate buffer (1 M diethanolamine (Sigma–Aldrich, St. Louis, CA, USA), 2 mM $MgCl_2$ (Merck, Damstadt, DE), pH 9.8). The absorbance at 405 nm was measured after 60 min of incubation of the substrate at room temperature using a plate reader Tecan Sunrise (Tecan, Grödig, Austria). Data were processed using Magellan 6.0 software (Tecan, Grödig, Austria).

RNA was isolated for sequencing by immuno-capture in duplicate on samples NIBv151, NIBv152, NIBv155, NIBv157 and on reference isolate NIBv1. Plates were coated with PVY polyclonal antibodies (Maryse Guillet, FNPPPT, France). Antibodies (100 μl per well at 1 μg/ml in carbonate coating buffer) were incubated at 37 °C for 4 h, followed by washing steps (1× 5 min with distilled water, 3× 5 min with PBST and 1× 5 min with distilled water). Plant sap was obtained by grinding fresh or lyophilized tissue in grinding buffer in a FastPrep® 24 apparatus (MP Biomedicals, Santa Ana, CA, USA). Grinding was performed in 2 ml tubes filled with manufacturer's supplied matrix A (MP Biomedicals, Santa Ana, CA, USA) using a tissue to buffer ratio (w/v) of 1:5 for fresh or 1:50 for lyophilized tissue. One hundred microliters of plant sap was incubated at 4 °C overnight in antibody-coated wells, followed by washing steps as described above. Finally, 10 μl of RNase-free water was added to each well, sealed with adhesive film and incubated for 10 min at 70 °C. Denatured RNA present in the resulting heated fractions was transferred into standard PCR 96 well plates and stored at −80 °C for analysis.

2.3. Nucleic acid extraction and molecular-based assays

RNA was extracted for RT-qPCR and SNaPshot SV using the total RNA Isolation System® (Promega, Madison, WI, USA). Briefly, 175 μl of RLA buffer was added to approximately 30 mg of lyophilized tissue and ground with a Qiagen Tissuelyser Retsch MM301 Mixer (Retsch Technology, Haan, Germany) in 2 ml tubes in the presence of 5 mm glass beads. Subsequent extraction steps were according to the manufacturer's protocol, except that the incubation in the elution step that was prolonged to 5 min.

RNA was reverse transcribed to cDNA in 20 μl reactions containing 2 U of AMV reverse transcriptase (Promega, Madison, WI, USA), 10 pmol of the oligonucleotide $5'$-$_{9701}$GTCTCCTGATTGAAGTTTAC$_{9682}$-$3'$ (nucleotide positions according to the PVY^N-605 isolate (Jakab et al., 1997)), 10 nmol of each dNTP, 20 U of RNasin (Promega, Madison, WI, USA) and 5 μl of the total RNA. The reverse transcription reactions were run by incubation for 1 h at 42 °C.

All steps of the SNaPshot assay were performed according to Rolland et al. (2008), except for the PCR products cleaning, which was carried out with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich Life Sciences, Poole, UK). Samples were run on a 3130 Genetic Analyzer (ABI, Foster City, CA, USA). During electrophoresis, fluorescence was recorded and analyzed using GeneScan Software (ABI, Foster City, CA, USA). Genotypes were scored manually, using size standards for peak verification.

RT-qPCR assays were performed on 2 μl of isolated RNA in a total volume of 10 μl using the AgPath-ID™ One-Step RT-PCR Kit (ABI, Foster City, CA, USA) according to the manufacturer's recommendations. Primer and probe concentrations, together with PCR amplification conditions, were as in Kogovsek et al. (2008). Two dilutions of RNA were analyzed, each in duplicate. Assays were carried out in 384-well reaction plates using the ABI 7900HT Sequence Detection System (ABI, Foster City, CA, USA). Real-time PCR conditions included a reverse transcription step (10 min at 48 °C) followed by 45 amplification cycles (1 min at 60 °C, 15 s at 95 °C). Data were analyzed manually with SDS v 2.3 software (ABI, Foster City, CA, USA) using automatic baseline settings and setting the threshold to 0.15.

2.4. Sequencing, alignment and in silico analysis

Denatured RNA from samples NIBv1, NIBv151, NIBv152, NIBv155, NIBv157, obtained by immune-capture (IC), were reverse transcribed in the same way as for the SNaPshot procedure described above. Two viral genome regions were PCR amplified (Table 1) in 50 μl reactions, namely HC-Pro/P3 region (corresponding to nt 1308 – 2721) and Nib/CP region (nt 8134 – 9452)–nucleotide positions according to PVYN-605 isolate (Jakab et al., 1997). PCR reactions consisted of 0.6 mM of forward and reverse primers (Table 1), 3 mM $MgCl_2$ (Promega, Madison, WI, USA), 0.025 U/μl GO Taq polymerase (Promega, Madison, WI, USA), 1× GO Taq Flexi Buffer (Promega, Madison, WI, USA), 0.05 mM of each dNTP (Promega, Madison, WI, USA) and 2 μl of cDNA. Conditions for PCR reactions were as follows: 3 min at 95 °C denaturation, 40 amplification cycles (30 s at 95 °C, 1 min/kb at 57 °C, 1 min 30 s at 72 °C) and 10 min at 72 °C for final elongation. Products were visualized on 1% agarose gel; appropriate bands were excised from the gel, extracted with the Millipore® Gel Extraction Kit (Millipore, Billerica, MA, USA) and submitted to sequencing (Macrogen, Seoul, Korea). Each region (Table 1) was amplified from two independent IC/RT steps and each PCR product was sequenced with the forward and reverse primers used in the amplification step to give 4-fold coverage per region per sample. The Nib/CP region containing the T/C_{9259} SNP was explored in more detail. Nucleotide sequences corresponding to the different PVY subgroup representative isolates were taken from the NCBI nucleotide database (Appendix A), and aligned with ClustalW ("slow and accurate" settings) algorithm included in the CLC Main Workbench software (CLC Bio, Aarhus, Denmark). Hybridization sites of the primers and probes used by the molecular methods evaluated in this study were checked manually on the alignments (Appendix A). Alignments containing the initial 100 nt of the $5'$ end of the CP gene and the last 150 nt of the $3'$ end of the CP gene were both exported to MEGA 5.05 (Tamura et al., 2011). Neighbor-joining trees were constructed with these partial CP sequences (Bootstrap method, 1000 Bootstrap Replications, Maximum Composite Likelihood model) (Fig. 1).

3. Results

3.1. Biological and serological characterization of PVY isolates

Two bioassays and a serological assay were performed to determine biological and serological properties of field collected PVY isolates. The bioassays showed that all tested isolates induced (i) vein necrosis on tobacco cv. White Burley at 14 dpi and (ii) PTNRD symptoms on potato cv. Igor after 6 weeks of storage at room temperature (Table 2). ELISA assay assigned 22 of the 24 tested isolates to the Y^N serotype, indicating that these isolates could belong to the Y^N or the Y^{NTN} group, or correspond to Y^N/Y^{NTN} mixtures. These serological results, combined with necrogenic properties on tobacco and potato (PTNRD), these 22 isolates to be classified to the Y^{NTN} group. Isolate NIBv171 was detected with both Y^C and Y^O specific serologic assays. From the combined serological and biological results, NIBv171can be assigned either to $Y^{N\text{-}Wi}$ or to a mixture of Y^O/Y^C and $Y^{N\text{-}Wi}$ (Table 2). The sample NIBv168 was detected with

Table 1
List of primers used in PCR amplification for sequencing.

Region	Primers	Primer sequence (5′–3′)	Start[a]	End[a]	Length
HCPro/P3	PVY-R1- F	GTGCCCAACAATATGCCA	1208	2721	1513
	PVY-R1-R	TTCTGCYGCTGACACTCGTA			
Nib/CP	PVY R2-F	TCAGATCTTGGTTTGAAYTATGATT	8134	9452	1318
	PVY R2-R	ATAAAAGTAGTACAGGAAAAGCCAA			

[a] Nucleotide numbers are according to PVY^{N} 605 (Jakab et al., 1997).

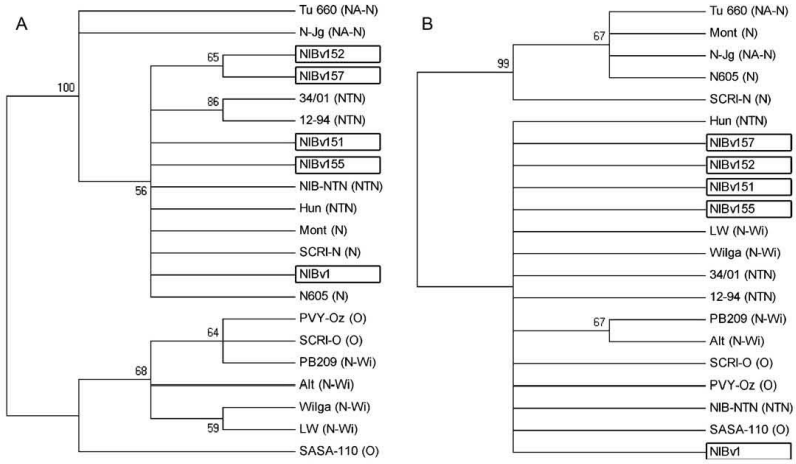


Fig. 1. Consensus topology trees constructed from partial CP sequences using the Neighbor-Joining method: (A) 100 nucleotides from the 5′ end of the CP sequence; (B) 150 nucleotides from the 3′ end of the CP sequence. The bootstrap consensus trees are inferred from 1000 replicates; branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated strains are clustered together in the bootstrap test is shown next to the branches. Samples from this study are marked with boxes.

Table 2
Main characteristics (name, symptoms and PVY subgroup assignment) of PVY isolates. ELISA, SNaPshot and RT-qPCR results are presented according to the group/subgroup assignment resulting from data analysis. n.t.: not tested; + vein necrosis on lower leaves; ++, vein necrosis on both lower and upper leaves.

Isolate name	Symptoms			PVY subgroup assignment		
	Potato	Tobacco	Tobacco	Serotype	RT-qPCR	SNaPshot
	PTNRD	Mosaic	Vein necrosis			
NIBv1[a]	Yes	Mosaics and curling of the leaves	++	N	NTN	NTN
NIBv151	Yes	Severe mosaic	++	N	NTN	N
NIBv152	Yes	Severe mosaic	++	N	NTN	N
NIBv155	n.t.	Severe vein yellowing, curling of the leaves	++	N	NTN	n.t.
NIBv156	Yes	Light mosaics	+	N	NTN	N
NIBv157	n.t.	Light mosaic	++	N	NTN	N
NIBv158	Yes	Light mosaic	+	N	NTN	N
NIBv159	Yes	Severe vein yellowing, curling of the leaves	+	N	NTN	N
NIBv160	Yes	Severe vein yellowing, curling of the leaves	++	N	NTN	NTN
NIBv161	n.t.	Mosaic	++	N	NTN	NTN
NIBv162	yes	Severe vein yellowing, curling of the leaves	+	N	NTN	N
NIBv163	Yes	Severe mosaic	++	N	NTN	N
NIBv164	Yes	Light mosaic	++	N	NTN	N
NIBv165	Yes	Severe mosaic	++	N	NTN	N
NIBv166	Yes	Severe curling of the leaves	+	N	NTN	N
NIBv167	Yes	Severe mosaic, curling of the leaves	+	N	NTN	N
NIBv168	Yes	Severe mosaic, curling of the leaves	++	Mixed O/N	O or mixed O/NTN[b]	Mixed O/N
NIBv169	Yes	Severe curling of the leaves	++	N	NTN	N
NIBv170	Yes	Mosaics and curling of the leaves	++	N	NTN	N
NIBv171	n.t.	Mosaics	+	O or mixed O/C	O or mixed O/NTN[b]	N-Wi
NIBv172	Yes	Mosaics and curling of the leaves	+	N	NTN	N
NIBv173	Yes	Mosaics and curling of the leaves	+	N	NTN	N
NIBv175	Yes	Curling of the leaves	+	N	NTN	N
NIBv176	Yes	Curling of the leaves	++	N	NTN	N

[a] Reference isolate NIB-NTN, GeneBank ID: AJ585342.
[b] Due to RT-qPCR's limitations, presence of the Y^{N-Wi} is not excluded.

all three group specific serological assays (N, O and O/C), indicating a probable mixed infected status for this sample. However, this result could also indicate an unconventional serological characteristic of an isolate possessing all antigenic domains targeted by the PVY-group specific antibodies.

3.2. Molecular characterization of PVY isolates

The performance of two 'state of the art' molecular characterization tools, RT-qPCR and SNaPshot, was compared on the 24 PVY isolates previously characterized biologically and serologically.

The RT-qPCR method (Kogovsek et al., 2008) detects PVY isolates and assigns them to groups based on the results obtained by four independent qPCR assays, one able to detect a consensus PVY sequence (present in the genome of all PVY isolates) and three to detect specific sequences from Y^O, Y^N and Y^{NTN} groups. However, as noted by Kogovšek et al. (2008), the RT-qPCR method alone does not allow Y^O to be distinguished from Y^O/Y^{NTN} mixed infection. Indeed, samples where a positive result is generated with both Y^O and Y^{NTN} probes need to be tested further by ELISA. Moreover, RT-qPCR fails to characterize $Y^{N\text{-}Wi}$ group members.

Using this procedure, 22 out of 24 samples tested were characterized as Y^{NTN} (Table 2), which is in concordance with their biological and serological characteristics (see above and Table 2). Using RT-qPCR and ELISA, isolate NIBv171 was characterized as Y^O and isolate NIBv168 as a Y^O/Y^{NTN} mixture. A positive $Y^{N\text{-}Wi}$ result cannot however be excluded in these two samples due to the above noted method limitations.

The SNaPshot technique proposed by Rolland et al. (2008) targets four SNP positions in the PVY genome (A/G_{2213}, A/C_{2271}, G/C_{8573} and T/C_{9259}). Polymorphisms at positions 2213 and 2271 (positions according to PVY^N-605 (Jakab et al., 1997) allow Y^N and Y^O strain groups to be discriminated. The two additional nucleotide positions, 8573 and 9259, target polymorphisms in the coat protein gene, enabling the recombination status of the CP to be determined. The four-digit codes produced by the association of these polymorphic nucleotides enable isolates to be assigned to the groups Y^O, Y^N, Y^{NTN} $Y^{N\text{-}Wi}$ (Rolland et al., 2008). According to SNaPshot, 18 out of 23 tested samples were characterized as Y^N. Apart from the reference isolate NIBv1, only two samples were assigned to Y^{NTN} group (NIBv160 and NIBv161). Sample NIBv171 belonged to $Y^{N\text{-}Wi}$ and sample NIBv168 was considered as a Y^O/Y^N mixture.

3.3. In silico analysis of the sites within the PVY genomic sequence targeted by RT-qPCR assays and SNaPshot

In order to clarify the differences in molecular typing obtained by the RT-qPCR and SNaPshot methods (Table 2), the HC-Pro/P3 and Nib/CP regions from some of the differentially assigned isolates (*i.e.* NIBv151, NIBv152 and NIBv157) were sequenced and analyzed. In addition, isolates that were assigned as Y^{NTN} by both methods were also sequenced (reference isolate NIBv1 and isolate NIBv155). PVY group representative isolates (16 sequences) were retrieved from the NCBI nucleotide database and aligned as references, together with the sequences generated in this study.

A procedure adapted from Boonham et al. (2002a,b) was used to determine the recombination status of the CP of the tested isolates. Two neighbor-joining trees were made, based on alignments of the first 100 nt of the 5′ end and the last 150 nt of the 3′ end of the CP gene. All five sequenced isolates clustered together with Y^N type reference strains on the CP 5′ end and with Y^O type reference strains on the CP 3′ end, clearly demonstrating the recombination status of their CP genes (Fig. 1).

The binding sites of RT-qPCR primers and probes and the SNPs targeted by SNaPshot were then checked on the sequence alignments in order to assign each PVY isolate *in silico* to its corresponding group (Appendix A).

Sequences revealed that all RT-qPCR primers and probes should hybridize optimally on the targeted positions of the samples. Some mismatches were observed in the nucleotide region corresponding to the hybridization site for the primers (mainly in the 5′end or in the middle of the primers). These mismatches, located outside the first 10 nt of the 3′ end of the primers, were less likely to compromise or alter the results produced by the RT-qPCR assay. This was proven in the case of the universal PVY RT-qPCR assay (Univ) on the sample NIBv152, where sequencing showed two mismatches in the forward primer hybridizing site (Appendix A, Fig. A.3.). Nevertheless the specificity and amplification efficiency of the assay, calculated from RT-qPCR dilution series, were not affected (data not shown). Similarly, all four RT-qPCR probes hybridize specifically on the analyzed sequences with no, or a maximum of one, mismatch. The SNaPshot method also detected the same nucleotides on the targeted SNP positions as identified *in silico* in all 5 sequenced isolates. This means that both methods are performing as designed.

In order to explain the different results obtained by RT-qPCR and SNaPshot, the SNP markers used by the SNaPshot method were examined. SNaPshot discriminates between Y^N and Y^{NTN} based on the recombination in the CP gene. Recombination is detected with one SNP at the 5′ end of the CP gene (G/C_{8573}) and one SNP in the core CP gene (T/C_{9259}). In the case of sample NIBv1, nucleotides 8573 and 9259 corresponded to G (Y^N type) and C (Y^O type), respectively, therefore the sample was characterized as an Y^{NTN} group member. In the case of the other 4 sequenced isolates, nucleotides 8573 and 9259 corresponded to G (Y^N type) and T (Y^N type) respectively, and were assigned to the non-recombinant Y^N group (Appendix A, Fig. A.2).

4. Discussion

In the last few decades significant efforts have been put into developing methods for detecting and differentiating PVY strain groups. Initially, PVY was detected and characterized by bioassays (Shukla et al., 1988). Strains were classified into groups Y^O, Y^C, Y^Z, Y^E and Y^N according to hypersensitive reactions induced on potato cultivars with different resistant genes, *e.g.* Désirée (Ny_{tbr}), King Edward (*Nc*), and Maris Bard (Ny_{tbr}, *Nc*, *Nz*), and according to induction of vein necrosis on tobacco (Kerlan et al., 2011). The advantage of bioassay is that it is to date, the only method providing clear information on both PVY biological characteristics and group assignment for tested PVY isolates. However, a few variants were found that could not be distinguished using the bioassay. In this study, bioassay was the tool that confirmed the tobacco necrosis and PTNRD inducing abilities of the tested isolates (Table 2). However, it requires space (greenhouses) and is too time consuming to deal with the diagnostic demands that are called for in actual plant protection strategies. Moreover, the bioassay provides information only on the plant reaction to the virus (symptoms), which could be very variable depending on the conditions and could also be induced by other viruses. For these reasons, bioassay remains the essential tool to assign biological properties to a virus isolate, but it is not suitable as a rapid diagnostic or screening method.

The possibility of high throughput detection and characterization of Y^O, Y^C and Y^N, together with relatively low price, time and space requirements, make serological tools such as ELISA the method of choice for many diagnostic laboratories. Serological tools enable direct detection of the PVY by detecting its coat protein rather than the plant reaction, as in bioassays. The major drawback of serological techniques however, is the lack of strict correlation between serological and biological characteristics. The limitations

of serological tools started to became apparent with the discovery of recombinant isolates, namely PVY^{NTN} and Y^{N-Wi} (often denoted as Y$^{N:O}$ in the literature from North America *e.g.* Gray et al. (2010)). Y^{N-Wi} isolates are able, like Y^{N}, to induce vein necrosis on tobacco, but are recognized serologically as Y^{O} (Chrzanowska, 1991). For example, the isolate NIBv171 from this study showed the characteristics of Y^{N-Wi} and reacted serologically in O and O/C assays (Table 2). PVY^{NTN} isolates are described serologically as Y^{N} group members and have the ability to induce PTNRD. This type of PVY was discovered in Hungary in the 1980s (Beczner et al., 1984) and is now found globally (Bouhachem et al., 2008; Crosslin et al., 2002; Djilani-Khouadja et al., 2009; Gray et al., 2010; Hu et al., 2008; Ibaba and Gubba, 2011; Kus, 1995; Le Romancer et al., 1994; Lorenzen et al., 2006a,b; McDonald and Singh, 1996; Milosevic and Djalovic, 2002; Nie and Singh, 2003a; Ohshima et al., 2000; Ramírez-Rodríguez et al., 2009; Tomassoli et al., 1998). In this study all tuber necrotic isolates have been defined as Y^{N} using ELISA (Table 2). Furthermore, several isolates with unconventional serological/biological characteristics, *e.g.* Y^{O}-O5 (Karasev et al., 2011) and Y^{NTN-NW} (Chikh Ali et al., 2007a,b), were described recently. These results further stress the limitations of serological markers for an accurate description of PVY diversity.

Knowledge of the PVY genomic sequences enabled the development of molecular tools such as multiplex RT-PCR, RT-qPCR techniques and the SNaPshot technique for detection/characterization of all the known PVY genotypes. Most molecular tools assign isolates according to selected molecular markers (*e.g.* recombination junctions or SNPs), which are not necessarily linked to the biological properties originally used to define PVY groups. The lack of connection between biological and molecular characterization was recently addressed by Kehoe and Jones (2011), who proposed a new nomenclature based on both biological and molecular characteristics. The exception is the RT-qPCR assay of Jacquot et al. (2005) in which two SNPs in the Hc-Pro gene responsible for tobacco vein necrosis (Tribodet et al., 2005) are targeted. However, the method can only distinguish between tobacco necrotic and non-necrotic strains. The later discovery of isolate L26 suggested a more complex molecular background behind the tobacco necrotic phenotype (Hu et al., 2009a,b). Knowledge of the PVY tobacco necrotic determinants has been updated with the discovery of a third SNP position by Faurez et al. (2012).

The search for PTNRD inducing molecular determinants has so far been unsuccessful. One reason is that development of PVY induced PTNRD symptoms is highly variable and dependent on both environmental conditions and potato cultivar (summarized in Singh et al., 2008). At first, the Y^{NTN} isolates most commonly associated with PTNRD in Europe and North America (in literature also denoted as EU-NTN) were characterized by necrotic phenotype in tobacco, Y^{N} serotype and presence of three recombination sites located in the HC-Pro, NIa and CP genes (Boonham et al., 2002a,b; Boonham, 2003; Nie and Singh, 2003b; Piche et al., 2004). However, non-recombinant Y^{N} isolates able to induce PTNRD have been reported in Japan, Denmark, North America and New Zealand (in literature also denoted as PVY^{NA-NTN}), but they are considered rare in the field (Boonham et al., 2002a; Nie and Singh, 2003a; Ohshima et al., 2000; Schubert et al., 2007). These non-recombinant PTNRD inducing isolates could not be distinguished from Y^{N} with the current serological and molecular tools. In addition, it has been proposed that all Y^{N} isolates have the potential to induce PTNRD under certain environmental conditions (biotic and abiotic factors) (Kerlan and Tribodet, 1996). The isolates from the Y^{N-Wi} and Y^{NTN-NW} groups, together with recombinant isolates belonging to the Y^{E} group, were also reported to cause PTNRD (Baldauf et al., 2006; Chikh Ali et al., 2007a,b; Chikh Ali et al., 2010b; Galvino-Costa et al., 2012; Piche et al., 2004; Schubert et al., 2007). Under greenhouse conditions, even a few Y^{O} strains from North America produced PTNRD symptoms (Gray et al., 2010). All these reports stress the need to find biologically relevant molecular determinants for PTNRD. Comparative studies, performed using genome sequence data from isolates with known biological properties, show that it is unlikely that PTNRD molecular markers will be found within the CP (Glais et al., 2002; Ohshima et al., 2000) or HC-Pro coding sequences (Schubert et al., 2007). The current idea is that determinants responsible for symptoms development originate from a number of amino acid changes present in several gene products, while none of them alone are essential (Barker et al., 2009).

In this study two of the latest molecular methods for PVY strain determination were investigated, the SNaPshot method (Rolland et al., 2008) and RT-qPCR (Kogovsek et al., 2008). Their ability to describe groups and variants accurately and to assign each tested isolate to the appropriate group/variant was examined, with the focus on validating the detection of Y^{NTN}. Both methods were able to detect and discriminate PVY isolates, however differences in group classification resulted from the use of these two methods. In terms of detecting PTNRD inducing Y^{NTN} strains, both methods rely on detecting a recombinant CP region, which has been typically associated with the PTNRD-causing strains (Boonham et al., 2002a). So far, strains that do not induce PTNRD but have recombinant CP have not been reported. Nonetheless, RT-qPCR was shown to be the more accurate method for assigning recombinant PTNRD-inducing isolates in the Y^{NTN} group (20/20 PTNRD inducing samples (Table 2)). SNaPshot assigned only 3/20 to the Y^{NTN} group (Table 2). On the other hand, SNaPshot has been shown to detect reliably all strains causing vein necrosis on tobacco (Table 2), on the basis of biological determinants in HC-Pro region (Tribodet et al., 2005), while RT-qPCR was not able to differentiate between Y^{O} and Y^{N-Wi} (Table 2), as already noted (Kogovsek et al., 2008). Moreover, 7 out of 24 samples were additionally tested with multiplex PCR (Lorenzen et al., 2006a,b) (data not shown), giving results that correlated with RT-qPCR and SNaPshot results in 7/7 and in 2/7 of the tested samples, respectively.

When primer/probe hybridization sites were checked on the alignment of CP sequences of differentially characterized samples (Appendix A), it became apparent that the reason for poorer performance of the SNaPshot method in detecting Y^{NTN} lay in the T/C$_{9259}$ SNP. Because SNaPshot detects single nucleotide positions, one mutation could significantly affect its result, which is not the case for RT-qPCR. In differentially characterized samples, the SNP T/C$_{9259}$ did not indicate the true overall sequence type of the region. In order to improve the SNaPhot method, markers targeting the G/A SNP in the NTN probe of the RT-qPCR (position 9216 according to PVY^{N}-605 (Jakab et al., 1997)) could be developed, where a G and an A would represent N-type and O-type sequences, respectively (based on an *in silico* evaluation of 83 full PVY genomes from public databases (data not shown)). On the other hand, the assay of Kogovsek et al. (2008) could be upgraded by an additional amplicon, overlapping one of the HC-Pro SNP positions of the SNaPshot assay (Jacquot et al., 2005). This would, in addition, enable fast and reliable detection of Y^{N-Wi}, easier discrimination between Y^{O} and Y^{NTN} and detection of mixed infection.

5. Conclusions

The methods assessed in this study are state of the art and are, at present, the most useful and reliable tools for PVY group discrimination. Furthermore, RT-qPCR can readily be implemented in diagnostic laboratories. Nevertheless, both methods, and all other current molecular tools, use only genotypic markers for PTNRD detection and not specific molecular determinants for tuber necrosis. However, they still supersede methods available previously.

Future developments should be focused on identification of all genomic determinants of the tuber necrosis property followed by the design of reliable methods targeting them. Until then, RT-qPCR and SNaPshot, qualified by awareness of the issues described in this study, remain the most powerful diagnostic techniques for detection of PVY subgroup isolates found in Europe.

Acknowledgements

The antibodies used in immune-capture procedure were kindly provided by Maryse Guillet, FNPPPT, France. Thanks to Dr. Roger Pain for improving this manuscript's style and grammar. This work was supported financially through the SLO-FR bilateral project PROTEUS (BI-FR/10-11-Proteus) and also by the Slovenian Research Agency through the grant L1-2278. Matevž Rupar is a recipient of a PhD research grant from the Slovenian Research Agency (contract nr. 1000-10-310196).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2013.01.013.

References

Baldauf, P.M., Gray, S.M., Perry, K.L., 2006. Biological and serological properties of potato virus Y isolates in Northeastern United States potato. Plant Dis. 90, 559–566.
Balme-Sinibaldi, V., Tribodet, M., Croizat, F., Lefeuvre, P., Kerlan, C., Jacquot, E., 2006. Improvement of potato virus Y (PVY) detection and quantitation using PVY(N)- and PVY(O)-specific real-time RT-PCR assays. J. Virol. Methods 134, 261–266.
Barker, H., Mcgeachy, K.D., Toplak, N., Gruden, K., Žel, J., Browning, I., 2009. Comparison of genome sequence of PVY isolates with biological properties. Am. J. Potato Res. 86, 227–238.
Beczner, L., Horváth, J., Romhányi, I., Förster, H., Box, P.O., 1984. Studies on the etiology of tuber necrotic ringspot disease in potato. Potato Res. 27, 339–352.
Blanchard, A., Rolland, M., Lacroix, C., Kerlan, C., Jacquot, E., 2008. Potato virus Y: a century of evolution. Curr. Top. Virol. 7, 21–32.
Boonham, N., 2003. Detection of potato viruses using microarray technology: towards a generic method for plant viral disease diagnosis. J. Virol. Methods 108, 181–187.
Boonham, N., Walsh, K., Hims, M., Preston, S., North, J., Barker, I., 2002a. Biological and sequence comparisons of potato virus Y isolates associated with potato tuber necrotic ringspot disease. Plant Pathol. 51, 117–126.
Boonham, N., Walsh, K., Preston, S., North, J., Smith, P., Barker, I., 2002b. The detection of tuber necrotic isolates of potato virus Y, and the accurate discrimination of PVY(O), PVY(N) and PVY(C) strains using RT-PCR. J. Virol. Methods 102, 103–112.
Bouhachem, S., Khamassy, N., Glais, L., 2008. Occurrence in Tunisia of potato tuber necrotic ringspot disease (PTNRD) caused by variant PVYNTN of potato virus Y. Plant Pathol. 57, 388.
Chikh Ali, M., Maoka, T., Natsuaki, K.T., 2007a. The occurrence and characterization of new recombinant isolates of PVY displaying shared properties of PVY N W and PVY NTN. J. Phytopathol. 155, 409–415.
Chikh Ali, M., Maoka, T., Natsuaki, K.T., Natsuaki, T., 2010a. The simultaneous differentiation of potato virus Y strains including the newly described strain PVY(NTN-NW) by multiplex PCR assay. J. Virol. Methods 165, 15–20.
Chikh Ali, M., Maoka, T., Natsuaki, T., Natsuaki, K.T., 2010b. PVY NTN-NW, a novel recombinant strain of potato virus Y predominating in potato fields in Syria. Plant Pathol. 59, 31–41.
Chikh Ali, M., Maoka, T., Natsuaki, K.T., 2007b. A point mutation changes the serotype of a potato virus Y isolate; genomic determination of the serotype of PVY strains. Virus Genes 35, 359–367.
Chrzanowska, M., 1991. New isolates of the necrotic strain of potato virus Y (PVYN) found recently in Poland. Potato Res. 34, 179–182.
Chung, B.Y.-W., Miller, W.A., Atkins, J.F., Firth, A.E., 2008. An overlapping essential gene in the Potyviridae. PNAS 105, 5897–5902.
Crosslin, J.M., Hamm, P.B., Eastwell, K.C., Thornton, R.E., Brown, C.R., Corsini, D., Shiel, P.J., 2002. First report of the necrotic strain of potato virus Y (PVYN) on potatoes in the Northwestern United States. Plant Dis. 86, 1177.
De Bokx, J.A., Cuperus, C., 1987. Detection of potato virus Y in early-harvested potato tubers by cDNA hybridization and three modifications of ELISA. EPPO Bull. 17, 73–79.
Djilani-Khouadja, F., Glais, L., Tribodet, M., Kerlan, C., Fakhfakh, H., 2009. Incidence of potato viruses and characterisation of potato virus Y variability in late season planted potato crops in Northern Tunisia. Eur. J. Plant Pathol. 126, 479–488.
Dougherty, W.G., Carrington, J.C., 1988. Expression and function of potyviral gene products. Annu. Rev. Phytopathol. 26, 123–143.
Fanigliulo, A., Comes, S., Pacella, R., Harrach, B., Martin, D.P., Crescenzi, A., 2005. Characterisation of Potato virus Y nnp strain inducing veinal necrosis in pepper: a naturally occurring recombinant strain of PVY. Arch. Virol. 150, 709–720.
Faurez, F., Baldwin, T., Tribodet, M., Jacquot, E., 2012. Identification of new Potato virus Y (PVY) molecular determinants for the induction of vein necrosis in tobacco. Mol. Plant Pathol. 13, 948–959.
Galvino-Costa, S.B.F., Reis Figueira, A., Camargos, V.V., Geraldino, P.S., Hu, X.-J., Nikolaeva, O.V., Kerlan, C., 2012. A novel type of Potato virus Y recombinant genome, determined for the genetic strain PVYE. Plant Pathol. 61, 388–398.
Glais, L., Kerlan, C., Tribodet, M., Tordo, V.M.-J., Robaglia, C., Astier-Manifacier, S., 1996. Molecular characterization of potato virus YN isolates by PCR-RFLP. Eur. J. Plant Pathol. 102, 655–662.
Glais, L., Tribodet, M., Kerlan, C., 2002. Genomic variability in Potato potyvirus Y (PVY): evidence that PVY(N)W and PVY(NTN) variants are single to multiple recombinants between PVY(O) and PVY(N) isolates. Arch. Virol. 147, 363–378.
Gray, S.M., DeBoer, S.H., Lorenzen, J., Karasev, A.V., Whitworth, J., Nolte, P., Singh, R.P., 2010. Potato virus Y: a significant and evolving threat to potato crops in the United States and Canada. Plant Dis. 94, 1384–1397.
Hu, X., Karasev, A.V., Brown, C.J., Lorenzen, J.H., 2009a. Sequence characteristics of potato virus Y recombinants. J. Gen. Virol. 90, 3033–3041.
Hu, X., Meacham, T., Karasev, A.V., Whitworth, J., Gray, S.M., Olsen, N., Nolte, P., 2008. Identification of Potato virus Y strains associated with tuber damage during a recent virus outbreak in potato in Idaho. Plant. Dis. 92, 1371.
Hu, X., Meacham, T., Ewing, L., Gray, S.M., Karasev, A.V., 2009b. A novel recombinant strain of Potato virus Y suggests a new viral genetic determinant of vein necrosis in tobacco. Virus Res. 143, 68–76.
Ibaba, J.D., Gubba, A., 2011. Diversity of potato virus Y isolates infecting solanaceous vegetables in the province of KwaZulu-Natal in the Republic of South Africa. Crop Prot. 30, 1404–1408.
Jacquot, E., Tribodet, M., Croizat, F., Balme-Sinibaldi, V., Kerlan, C., 2005. A single nucleotide polymorphism-based technique for specific characterization of YO and YN isolates of Potato virus Y (PVY). J. Virol. Methods 125, 83–93.
Jakab, G., Droz, E., Brigneti, G., Baulcombe, D., Malnoë, P., 1997. Infectious in vivo and in vitro transcripts from a full-length cDNA clone of PVY-N605, a Swiss necrotic isolate of potato virus Y. J. Gen. Virol. 78, 3141–3145.
Karasev, A.V., Hu, X., Brown, C.J., Kerlan, C., Nikolaeva, O.V., Crosslin, J.M., Gray, S.M., 2011. Genetic diversity of the ordinary strain of Potato virus Y (PVY) and origin of recombinant PVY strains. Phytopathology 101, 778–785.
Kehoe, M.A., Jones, R.A.C., 2011. A proposal to help resolve the disagreement between naming of potato virus Y strain groups defined by resistance phenotypes and those defined by sequencing. Arch. Virol. 156, 2273–2278.
Kerlan, C., Tribodet, M., 1996. Are all PVY-N isolates able to induce tuber necrosis ringspot disease? In: Proceeding of 13th Triennial Conference of the European Association of Potato Research, pp. 65–66.
Kerlan, C., Nikolaeva, O.V., Hu, X., Meacham, T., Gray, S.M., Karasev, A.V., 2011. Identification of the molecular make-up of the potato virus Y strain PVY(Z): genetic typing of PVY(Z)-NTN. Phytopathology 101, 1052–1060.
King, A., Adams, M.J., Lefkowitz, E., Carstens, E.B., 2011. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier.
Kogovsek, P., Gow, L., Pompe-Novak, M., Gruden, K., Foster, G.D., Boonham, N., Ravnikar, M., 2008. Single-step RT real-time PCR for sensitive detection and discrimination of Potato virus Y isolates. J. Virol. Methods 149, 1–11.
Kus, M., 1995. The epidemic of the tuber necrotic ringspot strain of potato virus Y PVY NTN and its effect on potato crops in Slovenia. In: Proceedings of the 9th European Association on Potato Res, Virology Section, Bled, Slovenia, pp. 159–160.
Le Romancer, M., Kerlan, C., Nedellec, M., 1994. Biological characterisation of various geographical isolates of potato virus Y inducing superficial necrosis on potato tubers. Plant Pathol. 43, 138–144.
Lindner, K., Kellermann, A., 2012. Analyse des aktuellen PVY Stammspektrums in Bayern sowie dessen Virulenz und Symptomausprägung (Analyses of the actually strain spectrum in Bavaria and its variation in virulence and symptoms. Julius-Kühn-Archiv 438, 301.
Lorenzen, J.H., Meacham, T., Berger, P.H., Shiel, P.J., Crosslin, J.M., Hamm, P.B., Kopp, H., 2006a. Whole genome characterization of Potato virus Y isolates collected in the western USA and their comparison to isolates from Europe and Canada. Arch. Virol. 151, 1055–1074.
Lorenzen, J.H., Piche, L.M., Gudmestad, N.C., Meacham, T., Shiel, P., 2006b. A multiplex PCR assay to characterize potato virus Y isolates and identify strain mixtures. Plant Dis. 90, 935–940.
Lorenzen, J., Nolte, P., Martin, D., Pasche, J.S., Gudmestad, N.C., 2008. NE-11 represents a new strain variant class of Potato virus Y. Arch. Virol. 153, 517–525.
Mallik, I., Anderson, N.R., Gudmestad, N.C., 2012. Detection and differentiation of potato virus Y strains from potato using immunocapture multiplex RT-PCR. Am. J. Potato Res. 89, 184–191.
McDonald, J., Singh, R., 1996. Host range, symptomology, and serology of isolates of potato virus Y (PVY) that share properties with both the PVY N and PVY O strain groups. Am. J. Potato Res. 73, 309–315.
Milosevic, D., Djalovic, I., 2002. The intensity of potato virus Y (PVY) infection in different localities of West Serbia and its importance to seed potato production. Acta Agric. Serb. 7, 61–70.
Moravec, T., Cerovska, N., Boonham, N., 2003. The detection of recombinant, tuber necrosing isolates of Potato virus Y (PVYNTN) using a three-primer PCR based in the coat protein gene. J. Virol. Methods 109, 63–68.

Nie, X., Singh, R.P., 2003a. Evolution of North American PVY(NTN) strain Tu 660 from local PVY(N) by mutation rather than recombination. Virus Genes 26, 39–47.
Nie, X., Singh, R.P., 2003b. Specific differentiation of recombinant PVYN: O and PVYNTN isolates by multiplex RT-PCR. J. Virol. Methods 113, 69–77.
Ohshima, K., Inoue, A.K., Ishikawa, Y., Shikata, E., Hagita, T., 1990. Production and application of monoclonal antibodies specific to ordinary strain and necrotic strain of potato virus Y. Japan J. Phytopathol. 56, 508–514.
Ohshima, K., Sako, K., Hiraishi, C., Nakagawa, A., Matsuo, K., Ogawa, T., Shikata, E., 2000. Potato tuber necrotic ringspot disease occurring in Japan: Its association with potato virus Y necrotic strain. Plant Dis. 84, 1109–1115.
Piche, L.M., Singh, R.P., Nie, X., Gudmestad, N.C., 2004. Diversity among potato virus Y isolates obtained from potatoes grown in the United States. Phytopathology 94, 1368–1375.
Radcliffe, E.B., Ragsdale, D.W., 2002. Aphid-transmitted potato viruses: the importance of understanding vector biology. Am. J. Potato Res. 79, 353–386.
Ramírez-Rodríguez, V.R., Frías-Treviño, G., Silva-Rosales, L., Aviña-Padilla, K., Martínez-Soriano, J.P., 2009. Presence of necrotic strains of Potato virus Y in Mexican potatoes. Virol. J. 6, 48.
Rigotti, S., Gugerli, P., 2007. Rapid identification of potato virus Y strains by one-step triplex RT-PCR. J. Virol. Methods 140, 90–94.
Rolland, M., Glais, L., Kerlan, C., Jacquot, E., 2008. A multiple single nucleotide polymorphisms interrogation assay for reliable Potato virus Y group and variant characterization. J. Virol. Methods 147, 108–117.
Rosner, A., Maslenin, L., 2001. Differentiating PVY(NTN) from PVY(N) by annealing to reference RNA transcripts. J. Virol. Methods 97, 125–131.
Rosner, A., Maslenin, L., 1999. Transcript conformation polymorphism: A novel approach for differentiating PVYNTN. J. Phytopathol. 147, 661–664.
Sanz, A., Cambra, M., Roman, C.P., Miguet, J.G., Cortes, E., Gorris, M.T., Vela, C., 1990. Preparation of additional monoclonal antibodies for detection and discrimination of potato virus Y isolates infecting potato. Potato Res. 33, 365–375.
Scholthof, K.G., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., 2011. Top 10 plant viruses in molecular Plant Pathology. Mol. Plant Pathol. 12, 938–954.
Schubert, J., Fomitcheva, V., Sztangret-Wiśniewska, J., 2007. Differentiation of Potato virus Y strains using improved sets of diagnostic PCR-primers. J. Virol. Methods 140, 66–74.
Shukla, D.D., Thomas, J.E., McKern, N.M., Tracy, S.L., Ward, C.W., 1988. Coat protein of potyviruses. 4. Comparison of biological properties, serological relationships, and coat protein amino acid sequences of four strains of potato virus Y. Arch. Virol. 102, 207–219.
Sigvald, R., 1984. The relative efficiency of some aphid species as vectors of potato virus Y O (PVYO). Potato Res. 27, 285–290.
Singh, M., Singh, R.P., 1996. Nucleotide sequence and genome organization of a Canadian isolate of the common strain of potato virus Y (PVYO). Can. J. Plant Pathol. 18, 209–2234.
Singh, R.P., Boucher, A., Somerville, T.H., Dhar, A.K., 1993. Selection of a monoclonal antibody to detect PVYN and its use in ELISA and DIBA assays. Can. J. Plant Pathol. 15, 293–300.
Singh, R.P., Valkonen, J.P.T., Gray, S.M., Boonham, N., Jones, R.A.C., Kerlan, C., Schubert, J., 2008. The naming of Potato virus Y strains infecting potato. Arch. Virol. 153, 1–13.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Tomassoli, L., Lumia, V., Cerato, C., Ghedini, R., 1998. Occurrence of potato tuber necrotic ringspot disease (PTNRD) in Italy. Plant Dis. 82, 350-350.
Tribodet, M., Glais, L., Kerlan, C., Jacquot, E., 2005. Characterization of Potato virus Y (PVY) molecular determinants involved in the vein necrosis symptom induced by PVYN isolates in infected *Nicotiana tabacum* cv. Xanthi. J. Gen. Virol. 86, 2101–2105.
Urcuqui-Inchima, S., Haenni, A.L., Bernardi, F., 2001. Potyvirus proteins: a wealth of functions. Virus Res. 74, 157–175.
Vijayapalani, P., Maeshima, M., Nagasaki-Takekuchi, N., Miller, W.A., Nagy, P.D., 2012. Interaction of the trans-frame potyvirus protein P3N-PIPO with host protein PCaP1 facilitates potyvirus movement. PLoS Pathog. 8.
Walsh, K., North, J., Barker, I., Boonham, N., 2001. Detection of different strains of Potato virus Y and their mixed infections using competitive fluorescent RT-PCR. J. Virol. Methods 91, 167–173.
Weilguny, H., Singh, R.P., 1998. Separation of Slovenian isolates of PVY(NTN) from the North American isolates of PVY(N) by a 3-primer PCR. J. Virol. Methods 71, 57–68.

2.1.2 Molekularna evolucija in filogeografija PVY na osnovi gena za protein plašča (CP)

Molecular evolution and phylogeography of potato virus Y based on the CP gene

José M. Cuevas, Agnes Delaunay, Matevž Rupar, Emmanuel Jacquot, Santiago F. Elena

Journal of General Virology, 2012, vol: 93, pp 2496–2501

Izvleček:

Krompirjev virus Y (PVY) je pomemben rastlinski virus s široko paleto gostiteljskih rastlin, med drugim okužuje krompir, tobak, paradižnik in papriko. Protein plašča (CP) PVY se pogosto uporablja v filogenetskih študijah za razvrščanje izolatov. V tej študiji smo uporabili nabor 292 CP nukleotidnih zaporedij iz izolatov, zbranih po vsem svetu. Po določanju in odstranjevanju rekombinantnih zaporedij smo uporabili bayesianske tehnike za preučevanje vpliva geografskega porekla in vrste gostiteljske rastline na strukturo populacije CP in njeno dinamiko. Opravili smo tudi analizo selekcije in kovariacije specifičnih aminokislin, ki sodelujejo pri prilagajanju. Naši rezultati kažejo, da je pestrost proteina CP virusa PVY signifikantno odvisna tako od geografskih adaptacij, kot tudi od prilagoditev na gostiteljske rastline. Pozicije aminokisline podvržene pozitivni selekciji so bile zgoščene v N-terminalni regiji proteina. Nekatere izmed teh pozicij omogočajo razlikovanje med skupinami PVY različkov in v veliko manjši meri med izolati, najdenimi na krompirju, in tistimi, najdenimi na drugih gostiteljskih rastlinah.

Journal of General Virology (2012), **93**, 2496–2501 DOI 10.1099/vir.0.044347-0

Short Communication

Molecular evolution and phylogeography of potato virus Y based on the CP gene

José M. Cuevas,[1] Agnès Delaunay,[2]† Matevz Rupar,[3] Emmanuel Jacquot[2]† and Santiago F. Elena[1,4]

Correspondence
José M. Cuevas
cuevast@uv.es

[1]Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-Universidad Politécnica de Valencia, València 46022, Spain

[2]INRA-Agrocampus Ouest-Université Rennes 1, UMR1099 BiO3P (Biology of Organisms and Populations Applied to Plant Protection), Le Rheu 35653, France

[3]NIB, Department of Biotechnology and Systems Biology, Večna pot 111, 1000 Ljubljana, Slovenia

[4]The Santa Fe Institute, 1399 Hyde Park Road, Santa Fe, NM 87501, USA

Received 16 May 2012
Accepted 24 July 2012

Potato virus Y (PVY) is an important plant pathogen with a wide host range that includes, among others, potato, tobacco, tomato and pepper. The coat protein (CP) of PVY has been commonly used in phylogenetic studies for strain classification. In this study, we used a pool of 292 CP sequences from isolates collected worldwide. After detecting and removing recombinant sequences, we applied Bayesian techniques to study the influence of geography and host species in CP population structure and dynamics. Finally, we performed selection and covariation analyses to identify specific amino acids involved in adaptation. Our results show that PVY CP diversification is significantly accounted for by both geographical and host-driven adaptations. Amino acid positions detected as positively selected concentrate in the N-terminal region of the protein. Some of these selected positions may discriminate among strains, and to a much lesser extent, between potato and non-potato isolates.

Potato virus Y (PVY) is responsible for serious diseases in potato, tobacco, pepper and tomato crops. PVY was originally classified into strain groups (e.g. PVY^N, PVY^O and PVY^C) according to their biological properties, serological characteristics and/or genome sequences (Moury *et al.*, 2002; Singh *et al.*, 2008). Recombination is highly pervasive in PVY and additional genomic organizations have been described (Lorenzen *et al.*, 2008; Schubert *et al.*, 2007).

Molecular evolution studies are useful tools to shed light on the molecular bases of virus geographical spread and adaptation to new hosts and for designing better epidemic control strategies (Elena *et al.*, 2011; Jones, 2009). We recently studied the phylogeography and molecular evolution of PVY whole-genomes (Cuevas *et al.*, 2012), showing that host and geographical origin influenced PVY diversification, and detecting positively selected sites. Here, we revisit these topics but focus on coat protein (CP). Novelties of this study are: (i) a much larger dataset is available for CP, which is expected to allow a more robust characterization of phylogenetic and selection patterns, (ii) CP plays an important role in host adaptation for many plant viruses, and (iii) CP is the most diverse and well-studied gene in PVY and other potyviruses (Moury & Simon, 2011; Ogawa *et al.*, 2008; Rohožková & Navrátil, 2011; Visser & Bellstedt, 2009).

A detailed description of the methods employed in this study can be found elsewhere (Cuevas *et al.*, 2012). For this study, we retrieved 198 PVY CP sequences from GenBank, plus 94 additional sequences from worldwide isolates (PVYwide Organization, http://www.inra.fr/pvy_organization) (Table S1, available in JGV Online). This dataset was aligned with MUSCLE (Edgar, 2004) as implemented in MEGA 5 (Tamura *et al.*, 2011). We ran recombination analyses to remove its effect from subsequent analyses. Bayesian Markov chain Monte Carlo (MCMC) coalescent analyses were performed with non-recombinant isolates to study the effect of local adaptation and host species in the observed diversity. Finally, we performed selection analyses to identify regions from the CP cistron that may be more likely involved in PVY adaptation dynamics.

Seventy-five of the 292 isolates (Table S1) showed a breakpoint, indicating ancestral recombination between PVY^N and PVY^O strains at position 9170 (considering the full genome) in CP (Schubert *et al.*, 2007) and worldwide distributed. Five other isolates showed uncommon breakpoints detected by at least three of the methods implemented in RDP3 (Martin *et al.*, 2010). N Nysa isolate

†Present address: INRA-CIRAD-Supagro, UMR385 BGPI (Biology and Genetics of Plant-Pathogen Interactions), Montpellier 34398, France

Supplementary material is available with the online version of this paper.

showed a newly described breakpoint at position 8896 (Cuevas *et al.*, 2012). IAC and v951204-N isolates showed a breakpoint at position 8735 (Mont and SASA-110 being the major and minor parents, respectively), almost coincident with other previously described breakpoints (Moury *et al.*, 2002). Finally, S-RB96 and NN-UK-N isolates showed a new recombination point at position 8947 (SASA-110 and Mont are the major and minor parents, respectively). All recombinants were excluded, reducing the dataset to 212 isolates.

Phylogenetic analyses were performed using the GTR+Γ_4+I substitution model in the Bayesian MCMC framework, as implemented in BEAST 1.6 (Drummond & Rambaut, 2007). Substitution rates were estimated using the relaxed uncorrelated exponential clock model. The three typical PVY strain groups (PVY^C, PVY^O and PVY^N) could be observed (Fig. S1), although the differentiation between PVY^C and PVY^O strains was poorly supported. Chile3 occupies a basal position in the tree, outside any of the strain groups, supporting its ancestry (Moury, 2010). Within the PVY^C clade, 17 of 22 isolates were collected from five different non-potato hosts. However, host species did not account for clustering within this clade, since most of the isolates from a given host were dispersed along the clade or closely grouped with isolates from other hosts. Only isolates PVY-MN and NC57 (from tobacco) formed a differentiated cluster, as observed previously (Kehoe & Jones, 2011; Mascia *et al.*, 2010). PVY^C clade has been subdivided into PVY^{C1} and PVY^{C2} subgroups depending on their ability to infect pepper (Blanco-Urgoiti *et al.*, 1998). In our phylogenetic tree, only isolates PVY-C-CM and Adgen-C were of pathotype PVY^{C2}, forming a differentiated cluster. Isolate CAA82, collected from pepper, grouped outside the PVY^{C1} subgroup. More isolates from subgroup PVY^{C2} are thus necessary to check the relative distance of isolate CAA82 to those from the non-pepper subgroup PVY^{C2}. Most isolates in our dataset belong to PVY^O. The globally low branch supports suggests a very genetically homogeneous group, compatible with a recent origin with minimal selection (Pagán *et al.*, 2006; Roossinck *et al.*, 1999). In fact, well-supported clusters within the PVY^O clade included isolates with common geographical origins. Finally, a similar trend was observed in the PVY^N clade, although internal branches close to the basis of the tree were usually well supported, thus differentiating several monophyletic clusters. Our study supports the classification proposed by Ogawa *et al.* (2008) into two PVY^N main groups (i.e. N-Europe and N-North America). Some well-supported clusters were observed into each PVY^N group, although this differentiation was not strictly associated with geographical origin.

A visual inspection of the maximum clade credibility (MCC) phylogeny did not show a clear structure in terms of geographical origin at the continent level (Fig. S1 and Table S1). For commercial and geographical reasons, North African and Middle East isolates were included in the European group. For the same reason, the only isolate from New Zealand was not included in any continental group. We used BATS 1.0b2 (Parker *et al.*, 2008) to calculate three statistics (AI, association index; PS, parsimony score and MC, maximum monophyletic clade size) describing the correlation between the geographical and phylogenetic relationships. Significant signatures for geographical structure in the diversity of the CP cistron were observed when grouped by geographical origins (Table 1), as shown by the significant AI and PS values. Asian, European,

Table 1. Analysis of the geographical and host effect on the population structure of PVY isolates

Analyses	# Isolates	Association statistics	Test value	*P*
Geographical regions		PS	106.985	<0.001
		AI	17.979	<0.001
Asia	30	MC	1.911	0.0099
Europe	88	MC	3.623	0.0099
South Africa	47	MC	2.451	0.0099
North America	43	MC	2.272	0.0099
South America	3	MC	1.004	1
New Zealand	1	MC	NA*	
Host species		PS	31.480	<0.001
		AI	6.651	<0.001
Potato	180	MC	13.145	0.0199
Tobacco	14	MC	1.286	0.0400
Pepper	10	MC	1.136	0.0099
Tomato	4	MC	1.005	1
Black nightshade	2	MC	1.001	1
Ají	1	MC	NA*	
Tamarillo	1	MC	NA*	

*Insufficient sample size (n<2).

South African, and North American groups showed differentiated subpopulations (significant MC values). The South American group did not show a significant association, which is accounted for by the small sample size, and no inference was possible for the single New Zealand isolate.

Host-driven adaptation could also be tested using host as a grouping variable, and a significant signature was also observed (Table 1). In this case, the differentiation was due to three subpopulations of isolates derived from potato, tobacco and pepper. For tomato and black nightshade no significant association was detected, whereas no inference was possible for single isolates from ají and tamarillo. Since most of the samples in our dataset are potato isolates, the significance of AI and PS values could be a consequence of the global distribution of the same state across most of the branches in the tree (Parker *et al.*, 2008). However, host structure explained the phylogeny quite well, since clade PVY^{C} predominantly included non-potato isolates (17 of 22), whereas the remaining main clades only included 14 non-potato isolates (of 189). Twelve of the 14 non-potato isolates falling outside the PVY^{C} clade were collected from tobacco. In this sense, tobacco infection could accidentally take place from potato crops early in the year, thus leading to misidentification of some tobacco isolates (M. Chrzanowska, personal communication). Besides, it is not surprising either that the tomato isolate GR_PVY12 fell outside clade PVY^{C}, since tomato can be infected with most PVY potato isolates (Singh *et al.*, 2008), and thus a recent introduction from potatoes cannot be excluded. Finally, the inclusion of black nightshade isolate SYR-Sn into the PVY^{O} clade is surprising, although the biological properties of this isolate are not yet available.

Selective pressures at a codon level were estimated using FEL, IFEL and MEME methods (www.datamonkey.org). Intramolecular covariation analyses were carried out using CAPS 1 (Fares & Travers, 2006), as described previously (Cuevas *et al.*, 2012). Table 2 shows the distribution of codon positions under purifying, neutral and positive selection, and covarying positions. As previously shown, most of the codons evolve neutrally, whereas purifying selection is the main force driving the evolution of CP (Cuevas *et al.*, 2012). Negatively selected positions are scattered along the ORFs, suggesting that no domain is particularly constrained. FEL and IFEL predicted codon 1 as positively selected, whereas MEME detected three additional codons (68, 193 and 216) to be under episodic diversifying selection (Table 2). Finally, a covariation group of nine codons was also detected, all located in the first half of CP. Selected codon 1 was involved in this covariation group.

Table 2. Results of the codon selection and covariation analyses at the CP gene

For selection methods (FEL, IFEL and MEME), the number of codons detected to be under negative, neutral or positive selection are given. The last column indicates the location of positively selected sites besides those positions showing covariation (CAPS).

Method	Negative	Neutral	Positive	Location
FEL	113	153	1	1
IFEL	76	190	1	1
MEME	NA*	NA*	4	1, 68, 193, 216
CAPS	–	–	–	1, 11, 17, 24, 26, 29, 31, 99, 138

*NA, Not applicable.

Previous phylogenetic studies showed that non-potato isolates mainly fell into the PVY^{C} clade (Ogawa *et al.*, 2008; Schubert *et al.*, 2007), highlighting the importance of host-driven adaptation. Our study, which included a significantly larger number of non-potato isolates, clearly showed that, in spite of the global consideration of non-potato isolates as belonging to the PVY^{C} clade, several other non-potato isolates were dispersed in the phylogeny. In fact, the analysis of amino acid composition for positively selected and covarying positions showed no clear differences between potato and non-potato isolates (Tables S2 and S3). Globally, both groups, except for positions 24, 138 and 193, shared the same predominant amino acid at a given position. Whereas similar amino acid composition between both groups was found for positions 24 and 193, the main difference was found at position 138, since the predominant amino acid for non-potato isolates was absent in potato isolates (Table S3). Besides, with the exception of position 138, specific residues of potato and non-potato isolates were always present at low frequencies. We also obtained the amino acid composition of positively selected and covarying codons, but grouping in this case for the PVY^{C}, PVY^{O} and PVY^{N} strains, which allowed us to check if selective forces were strain-specific (Tables S4 and S5). Globally, the same predominant amino acid at a given position was usually shared by the three strains. For those cases showing differences in the predominant amino acid, these predominant residues for a given strain were also usually present at low frequencies in at least one of the alternative strains. We observed positions 24 and 193 wherein the predominant amino acid for the PVY^{O} strain was different from that of the PVY^{C} and PVY^{N} strains. Besides, the predominant amino acid from the PVY^{N} strain was different from that observed in the PVY^{C} and PVY^{O} strains for positions 1, 11, 17, 26, 29 and 31. Finally, positions 99 and 138 showed different predominant residues for the three strains. Interestingly, the predominant residue for the PVY^{C} strain at these two positions was absent in the other two strains, although the predominant amino acids from the PVY^{O} and PVY^{N} strains were also present at low frequencies. Consequently, the analysis of amino acid composition at selected and covarying positions showed more partially discriminant residues among strains than among potato and non-potato isolates, which indicates that selective forces are mainly acting independently of the

potato/non-potato distinction. In this sense, as mentioned before, PVY does not have a narrow host range, which would account for the lack of association between selected positions and host usage.

Selection analyses at a branch level were performed using SWAPSC (Fares, 2004) to check the potential association between selective events and the phylogeny. Thirty-four branches showed evidence of positive selection (18 internal and 16 terminal branches; Fig. S1), and this selective signature was detected in 13 regions, often overlapping (Table 3). Most of them fell into the N-terminal region, congruently with the above selection and covariation analyses (Tables 2 and 3). With respect to the distribution of the selected branches in the phylogeny, we could differentiate between internal and terminal branches (Fig. S1). The frequency of selected internal branches was different among clades (20, 3.7 and 15.8 % for PVY^{C}, PVY^{O} and PVY^{N} clades, respectively; Fisher's exact test, P=0.003), but not for terminal branches (with frequencies of 9.1, 6.1 and 10.5 % for PVY^{C}, PVY^{O} and PVY^{N} clades, respectively; Fisher's exact test, P=0.568). These results suggest that selective forces are stronger into the PVY^{C} and PVY^{N} clades and milder into PVY^{O}. It is worth mentioning that one selected internal branch lead to the PVY^{C} clade (named as b2 in Table 3 and Fig. S1), except for the tamarillo isolate falling outside the selected cluster. We obtained the amino acid composition of the region involved in this branch specific selection event (codons 187–194) for the PVY^{C}, PVY^{O} and PVY^{N} clades (Table S6). This region included the selected site 193, which have been discussed above. Besides, the predominant amino acid for the PVY^{N} clade was different from that observed in the PVY^{C} and PVY^{O} clades at position 187. Finally, position 194 clearly discriminated between the PVY^{O} and PVY^{N} clades, but the two fixed residues present in these strains were also observed in the PVY^{C} strain. In conclusion, branch selection analyses showed evidence of the differential effect of selective events among strains, but did not provide particular positions accounting for these differences at a strain level.

The role of CP in the pathology of potyviruses has been previously confirmed (Andrejeva *et al.*, 1999; Hu *et al.*, 2011; Ullah & Grumet, 2002) and symptom determinants may be different even between strains of PVY in a particular host (Bukovinszki *et al.*, 2007). The N-terminal part of CP is a clear example of multifunctionality. It is exposed on the virion surface (potential function in binding ligands), besides being involved in vector transmission (Peng *et al.*, 1998) and systemic plant colonization (Andersen & Johansen, 1998; López-Moya & Pirone, 1998), becoming a potential target of selection at both vector and plant levels. In addition, CP from PVY interacts with different chloroplast proteins (Feki *et al.*, 2005). Consequently, it is not easy to discern if a given amino acid position is involved in one or more functions.

Regarding biological functions of CP, several commonalities were found when comparing our results with those described by Moury & Simon (2011). All positions showing positive selection in this previous study are within the N-terminal region of the CP cistron. In particular, positions 11, 24, 26, 68 and 138, were also detected to be under positive selection or covariation in our study. Position 11 is close to the DAG conserved motif involved in aphid transmission (Atreya *et al.*, 1991, 1995), and it has been

Table 3. Results of branch selection analysis

First column indicates all regions (codons) showing evidence of positive selection and second column shows the branches associated with the selection event for a given region. For terminal branches, the name of the corresponding isolate is shown. Internal branches are numbered as indicated in Fig. S1 and marked in bold. Positively selected and covarying positions falling into the regions providing a positive selection signature are shown in the last two columns, respectively.

Region	Branch	FEL-IFEL-MEME	Covariation
7–11	SASA-110, **b3**		11
8–13	PN-82		11
23–28	**b12**		24, 26
23–29	**b14**		24, 26, 29
25–28	PB_707, US05_30, SYR-NB-16		26
25–29	**b15, b16, b17**		26, 29
26–29	**b5, b7, b11**		26, 29
29–33	CAA141, PB_707, PB_602, PB_752, SC143, SC61, US05_30, US05_7, NN71_111, SYR-NB-16, 605, **b4, b8, b9, b13, b18**		29, 31
62–65	German_45, US06_55, **b6**		
135–138	**b1**		138
187–193	Nicola	193	
187–194	**b2, b3, b10**	193	
214–217	German_45, **b6**	216	

shown that mutations in a neighbouring residue can reduce transmissibility substantially (Atreya *et al.*, 1995). Furthermore, position 25 was shown to affect virus accumulation in host plants (Moury & Simon, 2011), and covarying positions detected in the vicinity could have some influence in this respect. Regarding position 68, it is worth mentioning that a mutation in this codon promoted differences in viral accumulation and transmissibility by aphids (Moury & Simon, 2011). Finally, the region spanning amino acid positions 133–148 of CP from soybean mosaic virus (positions 136–151 of PVY CP) is involved in binding to HC-Pro (Seo *et al.*, 2010), and then a potential influence for the included covarying position 138 could be postulated.

Acknowledgements

This research was supported by the Spanish Ministry of Science and Innovation grant BFU2009-06993 to S. F. E. and J. M. C was supported by the JAE-Doc program from CSIC. We thank Mario A. Fares for fruitful bioinformatics guidance. PVY isolates used in this work were available thanks to PVYwide Organization members (Carole Balmelli, Peter Dedic, Stewart Gray, Christophe Lacomme, Kestin Lindner, Benoit Moury, Maja Ravnikar, Laura Tomassoli, René Van der Vlugt, and Christina Varaveri). The authors do not have a commercial or any other association that might pose a conflict of interest.

References

Andersen, K. & Johansen, I. E. (1998). A single conserved amino acid in the coat protein gene of pea seed-borne mosaic potyvirus modulates the ability of the virus to move systemically in *Chenopodium quinoa*. *Virology* **241**, 304–311.

Andrejeva, J., Puurand, U., Merits, A., Rabenstein, F., Järvekülg, L. & Valkonen, J. P. (1999). Potyvirus helper component-proteinase and coat protein (CP) have coordinated functions in virus-host interactions and the same CP motif affects virus transmission and accumulation. *J Gen Virol* **80**, 1133–1139.

Atreya, P. L., Atreya, C. D. & Pirone, T. P. (1991). Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. *Proc Natl Acad Sci U S A* **88**, 7887–7891.

Atreya, P. L., López-Moya, J. J., Chu, M., Atreya, C. D. & Pirone, T. P. (1995). Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids. *J Gen Virol* **76**, 265–270.

Blanco-Urgoiti, B., Sánchez, F., Pérez de San Román, C., Dopazo, J. & Ponz, F. (1998). Potato virus Y group C isolates are a homogeneous pathotype but two different genetic strains. *J Gen Virol* **79**, 2037–2042.

Bukovinszki, A., Götz, R., Johansen, E., Maiss, E. & Balázs, E. (2007). The role of the coat protein region in symptom formation on *Physalis floridana* varies between PVY strains. *Virus Res* **127**, 122–125.

Cuevas, J. M., Delaunay, A., Visser, J. C., Bellstedt, D. U., Jacquot, E. & Elena, S. F. (2012). Phylogeography and molecular evolution of potato virus Y. *PLoS ONE* **7**, e37853.

Drummond, A. J. & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* **7**, 214.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792–1797.

Elena, S. F., Bedhomme, S., Carrasco, P., Cuevas, J. M., de la Iglesia, F., Lafforgue, G., Lalić, J., Pròsper, A., Tromas, N. & Zwart, M. P. (2011). The evolutionary genetics of emerging plant RNA viruses. *Mol Plant Microbe Interact* **24**, 287–293.

Fares, M. A. (2004). SWAPSC: sliding window analysis procedure to detect selective constraints. *Bioinformatics* **20**, 2867–2868.

Fares, M. A. & Travers, S. A. (2006). A novel method for detecting intramolecular coevolution: adding a further dimension to selective constraints analyses. *Genetics* **173**, 9–23.

Feki, S., Loukili, M. J., Triki-Marrakchi, R., Karimova, G., Old, I., Ounouna, H., Nato, A., Nato, F., Guesdon, J.-L. & other authors (2005). Interaction between tobacco ribulose-1,5-biphosphate carboxylase/oxygenase large subunit (RubisCO-LSU) and the PVY coat protein (PVY-CP). *Eur J Plant Pathol* **112**, 221–234.

Hu, X., Nie, X., He, C. & Xiong, X. (2011). Differential pathogenicity of two different recombinant PVY(NTN) isolates in *Physalis floridana* is likely determined by the coat protein gene. *Virol J* **8**, 207.

Jones, R. A. (2009). Plant virus emergence and evolution: origins, new encounter scenarios, factors driving emergence, effects of changing world conditions, and prospects for control. *Virus Res* **141**, 113–130.

Kehoe, M. A. & Jones, R. A. (2011). A proposal to help resolve the disagreement between naming of potato virus Y strain groups defined by resistance phenotypes and those defined by sequencing. *Arch Virol* **156**, 2273–2278.

López-Moya, J. J. & Pirone, T. P. (1998). Charge changes near the N terminus of the coat protein of two potyviruses affect virus movement. *J Gen Virol* **79**, 161–165.

Lorenzen, J., Nolte, P., Martin, D., Pasche, J. S. & Gudmestad, N. C. (2008). NE-11 represents a new strain variant class of potato virus Y. *Arch Virol* **153**, 517–525.

Martin, D. P., Lemey, P., Lott, M., Moulton, V., Posada, D. & Lefeuvre, P. (2010). RDP3: a flexible and fast computer program for analyzing recombination. *Bioinformatics* **26**, 2462–2463.

Mascia, T., Finetti-Sialer, M. M., Cillo, F. & Gallitelli, D. (2010). Biological and molecular characterization of a recombinant isolate of potato virus Y associated with a tomato necrotic disease occurring in Italy. *J Plant Pathol* **92**, 131–138.

Moury, B. (2010). A new lineage sheds light on the evolutionary history of potato virus Y. *Mol Plant Pathol* **11**, 161–168.

Moury, B. & Simon, V. (2011). dN/dS-based methods detect positive selection linked to trade-offs between different fitness traits in the coat protein of potato virus Y. *Mol Biol Evol* **28**, 2707–2717.

Moury, B., Morel, C., Johansen, E. & Jacquemond, M. (2002). Evidence for diversifying selection in potato virus Y and in the coat protein of other potyviruses. *J Gen Virol* **83**, 2563–2573.

Ogawa, T., Tomitaka, Y., Nakagawa, A. & Ohshima, K. (2008). Genetic structure of a population of potato virus Y inducing potato tuber necrotic ringspot disease in Japan; comparison with North American and European populations. *Virus Res* **131**, 199–212.

Pagán, I., Del Carmen Córdoba-Sellés, M., Martínez-Priego, L., Fraile, A., Malpica, J. M., Jordá, C. & García-Arenal, F. (2006). Genetic structure of the population of pepino mosaic virus infecting tomato crops in Spain. *Phytopathology* **96**, 274–279.

Parker, J., Rambaut, A. & Pybus, O. G. (2008). Correlating viral phenotypes with phylogeny: accounting for phylogenetic uncertainty. *Infect Genet Evol* **8**, 239–246.

Peng, Y. H., Kadoury, D., Gal-On, A., Huet, H., Wang, Y. & Raccah, B. (1998). Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions. *J Gen Virol* **79**, 897–904.

Rohožková, J. & Navrátil, M. (2011). P1 peptidase – a mysterious protein of family *Potyviridae*. *J Biosci* **36**, 189–200.

Roossinck, M. J., Zhang, L. & Hellwald, K. H. (1999). Rearrangements in the 5′ nontranslated region and phylogenetic analyses of cucumber

mosaic virus RNA 3 indicate radial evolution of three subgroups. *J Virol* **73**, 6752–6758.

Schubert, J., Fomitcheva, V. & Sztangret-Wiśniewska, J. (2007). Differentiation of potato virus Y strains using improved sets of diagnostic PCR-primers. *J Virol Methods* **140**, 66–74.

Seo, J. K., Kang, S. H., Seo, B. Y., Jung, J. K. & Kim, K. H. (2010). Mutational analysis of interaction between coat protein and helper component-proteinase of soybean mosaic virus involved in aphid transmission. *Mol Plant Pathol* **11**, 265–276.

Singh, R. P., Valkonen, J. P., Gray, S. M., Boonham, N., Jones, R. A., Kerlan, C. & Schubert, J. (2008). Discussion paper: the naming of potato virus Y strains infecting potato. *Arch Virol* **153**, 1–13.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Ullah, Z. & Grumet, R. (2002). Localization of Zucchini yellow mosaic virus to the veinal regions and role of viral coat protein in veinal chlorosis conditioned by the *zym* potyvirus resistance locus in cucumber. *Physiol Mol Plant Pathol* **60**, 79–89.

Visser, J. C. & Bellstedt, D. U. (2009). An assessment of molecular variability and recombination patterns in South African isolates of potato virus Y. *Arch Virol* **154**, 1891–1900.

2.1.3 Hitro čiščenje filamentoznega krompirjevega virusa Y z monolitnimi kromatografskimi nosilci

Fast purification of the filamentous Potato virus Y using monolithic chromatographic supports

Matevž Rupar, Maja Ravnikar, Magda Tušek-Žnidarič, Petra Kramberger, Laurent Glais, Ion Gutiérrez-Aguirre

Journal of Chromatography A, 2013, vol. 1272, no. 11, pp33– 40

Izvleček:

Pridobivanje čiste raztopine virusnih delcev je bistven korak v mnogih aplikacijah, kot so proizvodnja cepiv, proizvodnja protiteles, priprava z virusi obogatenih vzorcev za *in vitro* karakterizacijo in drugih. Postopki čiščenja so običajno sestavljeni iz dolgotrajnih in zapletenih protokolov, ki vključujejo več korakov ultracentrifugiranja. Kompleksnost čiščenja je še posebej očitna v primeru rastlinskih virusov, kjer je potrebno virusne delce izolirati iz zelo kompleksnega rastlinskega homogenata. Monolitni kromatografski nosilci Convective Interaction Media (CIM) so bili uspešno uporabljeni za čiščenje velikih biomolekul, kot so virusi, virusom podobni delci in plazmidi iz različnih vzorcev. V tej študiji smo razvili metodo za hitro čiščenje nitastega krompirjevega virusa Y (PVY) (velikost virusnih delcev 740 nm × 11 nm) iz rastlinskega tkiva na osnovi CIM monolitnih nosilcev. PVY je eden od najbolj pomembnih rastlinskih virusov in povzroča veliko gospodarsko škodo v pridelavi krompirja. Preizkušene so bile različne mobilne faze, kemije vezave in tehnike priprave vzorca. Prisotnost virusa v kromatografskih frakcijah smo spremljali preko kvantifikacije virusne RNA (RT-qPCR), čistost virusnih proteinov smo ocenili z metodo SDS–PAGE, integriteto delcev pa smo spremljali s presevno elektronsko mikroskopijo. Optimiziran postopek čiščenja vključuje začetne korake bistrenja, ki jim sledijo kromatografija na CIM kvartarni amin (QA) monolitni koloni. Razvita metoda dosega primerljiv donos, koncentracijo in čistost v primerjavi s klasičnim postopkom čiščenja, ki vključuje ultracentrifugiranje v gradientu saharoze in cezijevega klorida. Rastlinske nukleinske kisline so bile prav tako uspešno odstranjene. Nova metoda je dobro ponovljiva in poleg tega skrajša čas čiščenja iz štirih delovnih dni, potrebnih za klasično čiščenja, na dan in pol. To je prva študija, kjer je bil filamentozni virus uspešno očiščen z uporabo CIM monolitnih nosilcev. Zaradi svojih prednosti je postopek privlačen v proizvodnji seroloških diagnostičnih orodij, ki zahteva čisto raztopino virusnih delcev v koraku imunizacije. Rezultati te študije so izhodišče za izboljšanje metod čiščenja drugih pomembnih nitastih virusov.

Journal of Chromatography A, 1272 (2013) 33–40

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Fast purification of the filamentous *Potato virus Y* using monolithic chromatographic supports☆

Matevž Rupar[a,*], Maja Ravnikar[a,d], Magda Tušek-Žnidarič[a], Petra Kramberger[b,d], Laurent Glais[c,e], Ion Gutiérrez-Aguirre[a,d]

[a] *National Institute of Biology (NIB), Department for Biotechnology and Systems Biology, Večna Pot 111, 1000 Ljubljana, Slovenia*
[b] *BIA Separations, d.o.o., Mirce 21, 5270Ajdovščina, Slovenia*
[c] *INRA, UMR1349 IGEPP, F-35653 Le Rheu, France*
[d] *The Centre of Excellence for Biosensors, Instrumentation and Process Control-COBIK, Solkan, Slovenia*
[e] *Fédération Nationale des Producteurs de Plants de Pomme de Terre et des semences (FN3PT), 43-45 rue de Naples, 75008 Paris, France*

ARTICLE INFO

Article history:
Received 13 September 2012
Received in revised form
14 November 2012
Accepted 21 November 2012
Available online 29 November 2012

Keywords:
Virus purification
Monolith chromatography
Filamentous virus
Potato virus Y (PVY)
CIM-QA ion exchange chromatography

ABSTRACT

Obtaining pure virus suspensions is an essential step in many applications, such as vaccine production, antibody production, sample preparation for procedures requiring enrichment in viruses and other *in vitro* characterizations. Purification procedures usually consist of complex, long lasting and tedious protocols involving several ultracentrifugation steps. Such complexity is particularly evident in the case of plant viruses, where the virus needs to be isolated from the complex plant tissue matrix. Convective Interaction Media (CIM) monoliths are chromatographic supports that have been successfully utilized for the purification of large bio-molecules such as viruses, virus like particles and plasmids from various matrixes. In this study a CIM monolith based procedure was developed for the fast purification from plant tissue of the filamentous *Potato virus Y* (PVY) (virion size, 740 nm × 11 nm), which is one of the most important plant viruses causing great economical losses in potato production. Different mobile phases, chemistries and sample preparation strategies were tested. The presence of the virus in the chromatographic fraction was monitored with viral RNA quantification (RT-qPCR), viral protein purity estimation (SDS-PAGE) and viral particle integrity observation (transmission electron microscopy). The optimized procedure involves initial clarification steps, followed by chromatography using CIM quaternary amine (QA) monolithic disk column. In comparison to classical purification procedure involving ultracentrifugation through sucrose and caesium chloride, the developed CIM-QA purification achieved comparable yield, concentration and purity. Plant nucleic acids were successfully removed. Purification showed good reproducibility and moreover it reduced the purification time from four working days required for classic purification to a day and a half. This is the first study where a filamentous virus was purified using CIM monolithic supports. The advantages of this new purification procedure make it an attractive method in serological diagnostic tool production, which requires purified viruses for the immunization step. Moreover, the outcome of this study could serve as starting point for the improvement of the purification methods of other important filamentous viruses.

1. Introduction

Obtaining purified virus suspensions is very important for performing the immunizations required to produce antibodies for diagnostic purposes and also in vaccines production. Pure plant virus suspensions are also important for in *in vitro* vector transmission studies [1,2] and any other application where it is essential to separate the virus particle from the plant tissue. Plant viruses are usually purified with classical methods *i.e.* Leiser and Richter [3], involving clarification steps and multiple ultracentrifugation steps. Such procedures take several days to complete and require the use of ultracentrifuges. Faster and simpler virus purification methods would positively contribute to the applications where very clear virus suspension is needed such as next generation sequencing (NGS) for the characterization of viruses. In NGS a fast pre-purification of viruses, that removes the host plant nucleic acid, might considerably simplify the data analysis required after the NGS experiment [4].

In the past 20 years, monolithic supports [5] with the trade name CIM Convective Interactive Media® have proven to be a very versatile tool for purification of large biomolecules such as viruses and

☆ Presented at the MSS 2012, Ajdovščina, Portorož, Slovenia, 1–6 June 2012.
* Corresponding author. Tel.: +386 59 232 843; fax: +386 12 573 847.
E-mail address: matevz.rupar@nib.si (M. Rupar).

0021-9673/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2012.11.058

plasmid DNA. The improved performance of methacrylate monoliths for virus purification and concentration in contrast to classic bead-based chromatographic supports resides in their structure, which consists of large flow-through channels that enable convective mass transport of molecules, leading to flow-independent dynamic binding capacity and separation [6,7]. CIM monoliths enabled quicker and simpler purification of plant viruses such as *Tomato mosaic virus* (ToMV) [8,9], genomic DNA [10], plasmids [11], double stranded RNA (dsRNA) [12,13], bacteriophages [14,15] and mycobacteriophages [16], concentration or/and purification of human viruses such as influenza [17], hepatitis virus A [18], rotaviruses [19,20], rubella virus [21], virus like particles [22,23] and even ribosomes [24]. The first experiments of bindbinding pure filamentous bacteriophage M13 to CIM monoliths were also done by Smrekar et al. [25]. However, the potential of CIM monoliths has not been fully exploited.

Potato virus Y (PVY) is the type member of the genus *Potyvirus* (family Potyviridae) [26]. It is aphid transmitted and is one of the most important plant viruses infecting numerous wild and cultivated species from *Solanaceae* family worldwide [27,28]. Most importantly PVY can cause potato tuber necrosis ringspot disease (PTNRD) [29,30] resulting in effective crop losses of up to 100% [31]. Consequently, it has recently been classified into the group of the ten most important plant viruses from economic and scientific points of view [32]. The PVY virions are filamentous flexuous rods with helical structure and average dimensions of about 740 nm long and 11 nm wide [26]. The viral 9.7 kB long positive sense single stranded RNA is enclosed with ≈2000 molecules of coat protein (CP) and with one copy of viral genome-linked protein (VPg) covalently bound to the 5′ end of the RNA [33]. The isoelectric point of the CP has been described to range from 6.1 to 7.4, depending on the isolate [34].

In this study a fast and efficient CIM monolith based method for the purification of PVY was developed. The method is comparable with the classical method in terms of yield and purity and enables purification in less than 2 days. This is to our knowledge the first time that CIM monoliths have been applied to the purification of a filamentous virus.

2. Materials and methods

2.1. Virus and plant material

Infected plant material was prepared using PVY strain NIB-NTN (GenBank ID: AJ585342). Three different plant materials were used in this study. For classical purification PVY was propagated on *Nicotiana tabacum* cv. Xanthii. Healthy tobacco plants were mechanically inoculated with PVY at 3–4 leaf stage. Two lower leaves of each plant were dusted with carborundum powder (0.062 mm) (VWR International) and inoculated by gently rubbing with homogenized PVY infected plant material diluted (1:5, w/v) in inoculation buffer (20 mM sodium phosphate buffer, pH 7.6 with 2% PVP (M 10 000) (Sigma)). The inoculum was left on the leaves for 10 min and then gently rinsed off with tap water.

For optimization of the conditions for monolithic chromatography-based PVY purification, two plant sources were used. In initial runs, leaves from mechanically inoculated *N. tabacum* cv. White Burley collected at different days post inoculation were used. In following runs, whole green parts (leaves and stems) from PVY infected *Solanum tuberosum* cv. Pentland plants grown in tissue cultures were used.

Infectivity of the eluted viruses was tested on healthy *N. tabacum* cv. White Burley. Plants (3–4 leaf stage) were mechanically inoculated with 100 μl of pure virus suspension diluted in inoculation buffer (100×). Two lower leaves of each plant were dusted with carborundum powder (0.062 mm) and inoculated by gentle rubbing. The inoculum was left on the leaves for 10 min and then gently rinsed off with tap water. Symptoms (mosaic and vein necrosis) were observed at 18 days post inoculation (DPI) and compared with mock inoculated and plants inoculated with the sap from PVY NIB-NTN infected potato leaves (positive control).

2.2. Classical purification of PVY

Classic purification was based partially on the procedure reported by Leiser and Richter [3]. In details: apical non symptomatic leaves and stems of infected tobacco cv. Xanthii were harvested at 17 DPI and homogenized in a glass cup blender (Philips HR2170) with chilled (4 °C) grinding buffer (0.5 M trisodium citrate (Kemika), 0.005 M ethylenediaminetetraacetic acid (EDTA) (Sigma), 0.02 M sodium diethyldithiocarbamate (DIECA) (Sigma–Aldrich), pH 7.4) in 1:3 (w:v) ratio. The resulting crude homogenate was then passed through a cloth and centrifuged at 4600 × *g*, 10 min, 4 °C in the Avanti J-25 centrifuge with a JA 18 rotor (Beckman, Palo alto, CA, USA) to remove plant debris. Triton X-100 was slowly added to the collected supernatant to a final concentration of 3% (v/v). The mixture was kept at 4 °C with constant, slow stirring for 30 min. The detergent-treated supernatant was centrifuged at 18 000 RPM, 2.5 h, 4 °C in an Avanti J-25 centrifuge with a JA 18 rotor (Beckman, Palo Alto, CA, USA). The supernatant was discarded and the pellet was soaked overnight in 2 ml of resuspending buffer 1 (RB1: 0.01 M trisodium citrate (Kemika), 1 M urea (Fluka), 0.1% mercaptoethanol (Aldrich), pH 7.4). The second day the pellet was resuspended in 22 ml of RB1 with the help of Wheaton tissue handhomogeniser (Neolab) and centrifuged at 4360 × *g*, 10 min, 4 °C, in a Sigma 3k 18 centrifuge (Sigma, St Louis, MO, USA), to remove non-resuspended debris. The clear supernatant (20 ml) was ultracentrifuged at 35 000 × *g*, 2 h, 4 °C in a Beckman L8-80M ultracentrifuge with a 70 Ti rotor (Beckman, Palo Alto, CA, USA) through a 10 ml sucrose pillow (20% sucrose (Kemika) (w/v) in 0.01 M trisodium citrate (Kemika), pH 7.4). The supernatant was discarded and the pellet was soaked overnight in 100 μl of the resuspending buffer 2 (RB2: 0.005 M disodium tetraborate (Merck), pH 8). Next day the pellet was resuspended in a total volume of 440 μl of RB2 and centrifuged (4360 × *g*, 15 min, 4 °C) to remove the non-resuspended material. Exactly 5.875 g of CsCl (Sigma) was added to 12.50 ml of RB2 to prepare the CsCl solution. Two 6 ml CsCl gradients were prepared, and then 200 μl of clear supernatant was added on top of each gradient followed by ultracentrifugation at 110 000 g, 5 h, 12 °C, in a Beckman L8-80M ultracentrifuge with a 70.1 Ti rotor. Afterwards, gradients were fractionated, and fractions analyzed spectrophotometrically with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Virus rich fractions were collected and dialysed overnight against 1 l of RB2 at 4 °C with slow mixing. The dialysed virus suspension was diluted to a final volume of 30 ml in RB2 and ultracentrifuged at 110 000 × *g*, 3 h, 4 °C in a Beckman L8-80M ultracentrifuge with a 70 Ti rotor. Supernatant was discarded; the pellet was soaked in 100 μl of RB2 for 60 min and then resuspended to a final volume of 200 μl to obtain the final suspension of pure PVY. Concentration of pure virus suspension was determined spectrophotometrically. Absorption intensity at the maximum was divided with the factor 2.4 for a 1 cm path length, to obtain PVY concentration [mg/ml], modified from Leiser and Richter [3].

2.3. Chromatography columns and instrumentation

Most of the experiments were done using CIM disk monolithic columns, quaternary amine (QA) and diethylamine (DEAE) chemistries (BIA Separations d.o.o., Ajdovščina, Slovenia), with a bed volume of 0.34 ml and standard pore size (≈1 μm). One run

was performed with CIM QA-1 mL tube monolithic column with large pore size (≈6 μm). CIM disk monolithic column was placed in the peek housing and connected to a FPLC system ÄKTA Purifier (GE Healthcare, Upssala, Sweden) equipped with P-900 pumps and a UV/conductivity detector UPC-900. HPLC hardware and software from GE Healthcare (data acquisition and control station Unicorn) were used in all experiments. All experimentation was done at flow rates 3 or 4 ml/min.

2.4. Sample preparation and optimization of chromatography conditions for purification of PVY

The samples loaded on chromatography columns in optimization experiments were prepared in two different manners (Fig. 1). The first and more simple preparation consisted of homogenization of plant material with loading buffer in 1:10 ratio (w:v) in a Bioreba extraction bag (Bioreba, Reinach, Switzerland). The crude homogenate was centrifuged (1500 × *g*, 10 min). The obtained clear supernatant was further diluted 10× in loading buffer and centrifuged again (1500 × *g*, 10 min). This last supernatant was taken as a loading material. For the second loading material the initial steps of classic purification until the sucrose gradient step were performed (homogenization, centrifugation, detergent treatment, high speed centrifugation; for details see Section 2.2).

Optimization of the separation conditions included testing CIM DEAE (diethylaminoethyl) disk monolithic column and CIM QA (quaternary amine) disk monolithic column, several loading buffer compositions (0.18 M citrate–phosphate buffer, pH 7.1; 0.01 M sodium citrate buffer, pH 7.4; 0.05 M HEPES (Sigma), pH 7.1) with or without urea, different starting material (tobacco grown in soil and potato tissue cultures), and different monolith pore size (summarized in Fig. 1). Washing buffer was always the same as the loading buffer and loading buffer with different NaCl (Merck) concentration was used as an elution buffer for stepwise elution (250 mM, 500 mM, 750 mM, 1000 mM NaCl).

2.5. Chromatography based PVY purification

The final chromatographic purification, based on the conditions optimized in previous runs, was repeated twice with independent starting material. Eight to nine weeks old PVY infected plants of potato cv. Pentland in tissue cultures were harvested. Roots were discarded; leaves and stems (19 g for run A and 38 g for run B) were used to prepare the loading material according to the initial steps of classical purification (homogenization, centrifugation, detergent treatment, high speed centrifugation, resuspension of the pellet and removal of non resuspended material; for details see Section 2.2). Ten microliters of clarified load was then injected into a CIM QA disk monolithic column (0.34 ml bed volume). Loading buffer was composed of 0.01 M trisodium citrate (Kemika), pH 7.4, with 1 M urea (Fluka). Upon loading, the column was further washed with loading buffer until the UV signal decreased to baseline values. Elution was done in two parts, an initial stepwise elution with 250 mM NaCl in the loading buffer followed by a linear gradient from 250 mM to 1000 mM of NaCl in 30 ml (cca. 100 column volumes). Flow through (FT), wash (W) and 250 mM elution fractions were collected in their totality while the gradient was fractionated in 1 ml fractions. In case the purified virus suspension needs to be stored for a long time, or if subsequent downstream applications require it, a buffer exchange step can be included. Such step can be done by conventional dialysis, by using commercial desalting columns, or by ultracentrifugation. In this study, fractions corresponding to the peak enriched in pure virus particles (Fig. 2A) were pooled and sedimented by ultracentrifugation using the same conditions as for the classical isolation procedure (110 000 × *g*, 3 h, 4 °C, with a Beckman 70 Ti rotor). The pellet was resuspended in 200 μl of RB2 and virus suspension stored at 4 °C.

2.6. Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) and western blotting

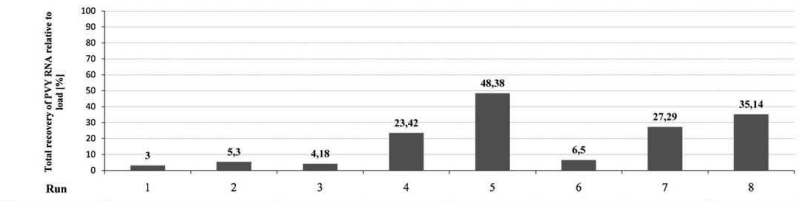
Protein purity in chromatographic fractions was evaluated on discontinuous polyacrilamide gels; 4% stacking gel and 10% resolving gel (Acryl amide/Bis-acrylamide 37:1 (Sigma)) Samples (25 μl) were mixed with 5 μl of loading buffer (0.225 M Tris–HCl (Sigma), 50% glycerol (Kemika), 5% SDS (Sigma), 0.05 bromophenol blue (Sigma), 0.25 M dithiothreitol (Sigma)), then 25 μl of the mixture was loaded on the gel. Proteins were stained with PageBlue™ Protein Staining Solution (Fermentas). For confirmation of the protein specificity, electrophoretically separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (BioRad, USA), incubated in blocking buffer overnight (25 mM Tris–HCl, 1 mM EDTA (Sigma) 150 mM NaCl, 4% BSA, pH 7.6) and washed with washing buffer (25 mM Tris–HCl, 1 mM EDTA (Sigma), 150 mM NaCl, pH 7.6). The membrane was incubated with PVY specific antibodies with alkaline phosphatase (Bioreba, Reinach, Switzerland) (dilution 1:1000), washed with washing buffer and incubated in detection buffer (washing buffer with 0.015% 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Sigma) and 0.03% nitroblue tetrazolium (NBT) (Sigma)). PVY specific proteins were coloured purple after a few minutes.

2.7. Nucleic acid extraction and RT qPCR quantification of viral and plant nucleic acids

In all experiments RNA was extracted from 140 μl of sample using QIAamp Viral RNA Mini Kit (QIAGEN, USA) according to manufacturer's instructions. Final elution was done with 45 μl of RNAse free H_2O pre warmed at 65 °C. Luciferase control RNA (Promega, Madison, WI, USA) (2 ng per sample) was added as an external control for monitoring variations in nucleic acid extraction and the presence of inhibitors in different fractions.

Relative quantification of PVY in chromatographic fractions was done with specific PVY-Uni RT-q PCR assay developed by Kogovsek et al. [35]. Due to its abundance and uniform distribution in plant cells, plant cytochrome oxidase gene (COX) usually used as an internal control [36] was used as indicator of the presence of plant nucleic acids in each sample. COX was targeted with the modified RT-qPCR assay by Weller et al. [37]. RNA from the highly concentrated samples used as loading material was isolated and 10-fold serially diluted with RNAse free water (Sigma), from non-diluted until 1×10^{-7} dilution. All dilutions were applied to PVY-Uni (threshold value 0.15) and COX (threshold value 0.25) assays. Quantification cycles values (Cq) were calculated and used to build a standard curve with theoretical log concentration on *x*-axis and Cq value on *y*-axis. In addition to PVY-Uni and COX assays, the RNA from chromatographic fractions was also applied to luciferase specific RT-qPCR assay (LUC) [38] (threshold value 0.5). All samples and standard curve were applied to RT-qPCR assays in triplicates (10 μl reactions) and average Cq values were used in subsequent calculations. LUC Cq values were used to normalize the PVY and COX specific Cq in order to account for variations in the RNA isolation and or RT-qPCR procedure [18]. Normalized Cq values were used to calculate theoretical concentrations of PVY and COX in all samples. Recoveries were calculated as the percentage of PVY and COX present in each fraction in relation to the load.

AgPath-ID One-Step RT-PCR Reagents (Life Technologies, Carlsbad, CA, USA) and Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster city, USA) were used in all experiments. Amplification conditions for all assays were 10 min at 48 °C, 10 min at 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C.



CIM chemistry[a]	DEAE	QA	DEAE	QA	QA	QA	QA large pores	QA
Plant material	Leaves of *Nicotiana tabacum* cv. White Burley				Leaves and stem of *Solanum tuberosum* cv. Pentland tissue cultures			
Load preparation	Simple[b]							Initial steps of classic isolation
Loading buffer[c]	CP	CP	CP + 0.5 M UREA	CP + 0.5 M UREA*	CP	HEPES	CP	CT + 1 M UREA

[a] 0.34 ml CIM discs with standard pore size (1 μm) were used in all runs apart from run 7 where 1 mL tube monolithic column with larger pore size (6 μm) was used
[b] the simple load preparation consisted of homogenisation, short centrifugation and dilution in loading buffer
[c] CP, 0.18 M citrate phosphate buffer, pH 7.1; HEPES 0.05 M, pH 7.1; CT, 0.01 M sodium citrate; pH 7,4
* 0.5 M urea + 0.01 M DIECA + 0.01 M EDTA

Fig. 1. Optimization of the binding/elution of PVY using CIM monolithic supports. Total recovery of PVY RNA relative to the load is shown in grey columns for each run. The exact percentage of recovered RNA is denoted above the columns. The conditions used in each optimization run are presented in the table below the columns.

Quantification cycle was determined after automatic adjustment of the baseline using SDS v 2.2 software (Applied Biosystems, Foster city, USA).

2.8. Transmission electron microscopy (TEM)

Different samples including chromatographic fractions were examined under TEM, using the negative-staining method. Twenty-five microliters of chromatographic fractions were applied to Formvar-coated (Agar Scientific), carbone-stabilized copper grids and stained with 2% aqueous solution of uranyl acetate (SPI Supplies). All samples were examined at magnification ranging from 19 000× to 92 000× with TEM (Philips CM 100; Amsterdam, The Netherlands) operating at 80 kV. The images were recorded with a Bioscan CCD camera using Digital Micrograph Software (Gatan Inc., Washington, USA).

3. Results and discussion

3.1. Optimization of PVY binding and elution conditions

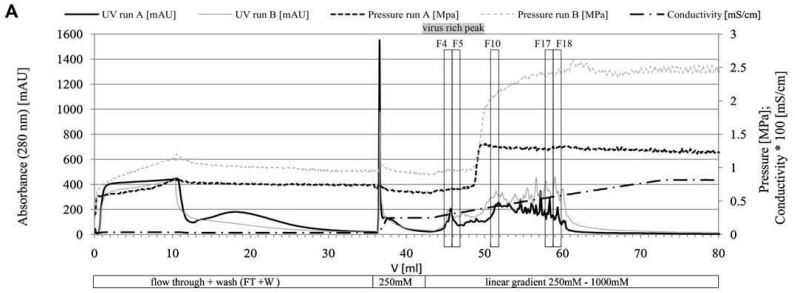
Initial steps were focused on optimizing the binding/elution conditions of PVY using CIM monoliths, with a special emphasis on increasing the virus recovery in the elution fractions relative to the load. Virus recovery was monitored by following the viral RNA presence with RT-qPCR. A simple load preparation procedure from homogenized tobacco leaves (Section 2.4) was first used to prepare starting material and citrate phosphate 0.18 M, pH 7.1 as a loading buffer. Due to the filamentous shape of PVY (740 nm × 11 nm) and aggregation tendency of potyviruses [39,40], potential loses of the virus due to its accumulation in the disk housing and frits could be expected, but preliminary tests using only housing and frits with no disk showed such loses to be negligible (data not shown).

Next the different CIM anion exchange chemistries were tested. Runs done with QA and DEAE resulted in very low PVY RNA recoveries, being slightly better the ones obtained with CIM QA (Fig. 1, runs 1 and 2). Due to the above-mentioned tendency for protein induced aggregation of potyviruses, which was also observed under the TEM (data not shown), the effect of the chaotropic agent urea on PVY recovery was evaluated. Urea is known to prevent protein aggregation [41,42]. In runs 3 and 4 (Fig. 1) 0.5 M urea was present in the grinding and chromatographic buffers, which resulted in slight recovery improvement when using CIM DEAE and greater recovery improvement in the case of CIM QA. Therefore the CIM QA was used in further optimization steps.

The mature plant tissue is known to be a complex matrix containing proteins, cell wall compounds, pigments and poly-phenols in high abundance. Virus particles may interact with all these molecules, resulting in aggregation and consequently decreased recoveries. In order to decrease such abundance, a less complex starting material, such as plants grown in tissue culture, was used in next runs [43]. In runs 5–7 the same simple procedure for load preparation was maintained but green parts of PVY infected potato cv. Pentland grown in tissue cultures were used instead of tobacco leaves. The PVY RNA recovery was greatly improved by using potato tissue cultures as a starting material (Fig. 1, run 5 in comparison with run 2). However none of the elution fractions obtained in runs 1–5 contained pure PVY coat protein. Next, the effect of the widely used chromatographic buffer HEPES (pH 7.1) (Fig. 1, run 6), and of CIM QA disk monolithic column with larger pore diameter (Fig. 1, run 7) on the recovery were evaluated. None of these modifications significantly improved the recovery (Fig. 1); furthermore the use of HEPES buffer reduced the total recovery significantly in comparison to citrate phosphate buffer (Fig. 1, runs 5 and 6). Summarized, using CIM QA, urea and tissue culture for loading material preparation, increased the recovery of PVY RNA up to 50%. A possible reason for not recovering the totality of the loaded PVY RNA lays on the virus replication cycle. Plant RNA viruses are present in the plant in two simultaneous forms, encapsidated and non-encapsidated (free replicative RNA form). In the loaded material, both these populations are detected with the RT-qPCR assay after RNA isolation. However, the interactions between the highly negatively charged 9.7 kb long free viral RNA and the positively charged CIM QA resin are expected to be much stronger than the interactions between the CP protein of encapsidated particles and CIM QA. Therefore free RNA might bind so strongly to the monolith as to prevent an efficient elution in our buffer conditions. This could contribute to underestimated RNA recoveries in the eluted fractions relative to the load.

To further optimize the purification and to address the purity issue, initial steps from classic purification procedure were introduced for load preparation (Fig. 1, run 8). A similar strategy was previously applied to *Tomato mosaic virus* (ToMV), where coupling initial steps from classic purification with CIM chromatography resulted in an efficient virus purification [9]. To prepare the load of run 8, potato tissue cultures were homogenized, debris removed, detergent treated and centrifuged according to classic purification. The loading buffer used in this run was 0.01 M citrate with 1 M urea, pH 7.4 (as in classic purification). The change in the sample preparation procedure slightly reduced the recovery from 48.38%

A

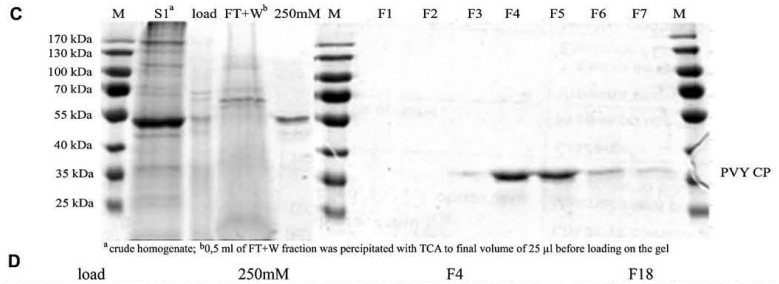


B

			load	FT+W	250mM	F4	F5	F10	F17	TOTAL [a]
Volume [ml]			10	36	6	1	1	1	1	72
Recovery (%) [b]	run A	PVY	/	0	0.38	22.49	21.54	0.24	0.28	53.19
		COX[d]	/	0	0	0	0	0	2.47	2.47 [c]
	run B	PVY	/	0	1.88	16.49	6.49	0.47	0.6	34.84
		COX[d]	/	0	0	0	0	0.01	23.81	86.71
TEM[e]			+	-	+	+++	+++	+	+	/

a) sum of all collected fractions;
b) recovery of RNA relative to the load
c) In run A, only gradient fractions F1 - F10 and F17 were applyed to COX assay, thus the low total recovery
d) cytochrome oxidase (COX) gene represents plant nucleic acids
e) -, no viral particles observed; +, 1-10; +++, more than 100 viral particles per field of view (25 000x magnification)

C



[a] crude homogenate; [b] 0,5 ml of FT+W fraction was percipitated with TCA to final volume of 25 µl before loading on the gel

D

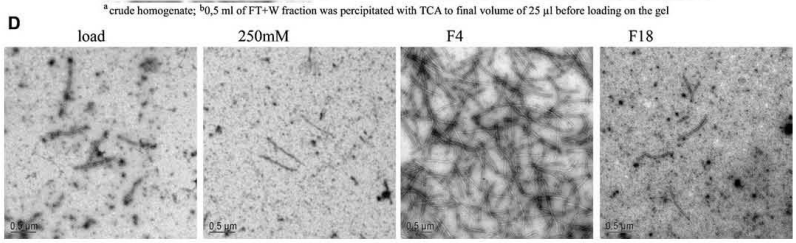


Fig. 2. Results of chromatographic PVY purification. (A) Chromatograms from runs A and B on CIM QA. (B) RT-qPCR analysis of representative fractions from runs A and B. (C) SDS-PAGE analysis from run A. (D) TEM micrographs from representative fractions (magnification: 25 000×).

to 35.14% (Fig. 1, runs 5 and 8). However the SDS-PAGE protein profile of the loading material showed less complex and less contaminant bands (data not shown). Despite less complex load, still none of the collected fractions consisted of pure PVY.

3.2. Purification of PVY using CIM QA disk monolithic column

Based on the optimization data, the following parameters were chosen to be used in the CIM based PVY purification: CIM QA disk monolithic column with standard pore size, 0.01 M citrate buffer (pH 7.4) with 1 M urea as a loading buffer, initial steps of classical purification for loading material preparation and infected potato tissue cultures as a starting plant material. Since in most of the optimization runs the highest PVY RNA recoveries were observed in the 250 mM and 500 mM NaCl fractions, elution steps were modified accordingly. To determine the exact viral elution position an initial stepwise elution with 250 mM NaCl was performed, followed by a linear gradient until 1000 mM NaCl.

Purification of PVY using such conditions was performed twice using independent batches of PVY infected potato tissue cultures as starting material (Fig. 2A; run A, 19 g and run B, 38 g of plant material) with good reproducibility. The UV signal was very similar in both runs, while the generated backpressure was significantly higher in run B, probably due to twice the amount of starting material in run B in comparison to run A (Section 2.5).

Chromatographic fractions (load, flow through pooled together with wash, 250 mM elution and gradient fractionated in 1 ml fractions) were analyzed for the presence of PVY and plant nucleic acids with both PVY-Uni [35] and COX [37] RT-qPCR assays. Recoveries relative to the load were calculated (Fig. 2B). In both runs the great

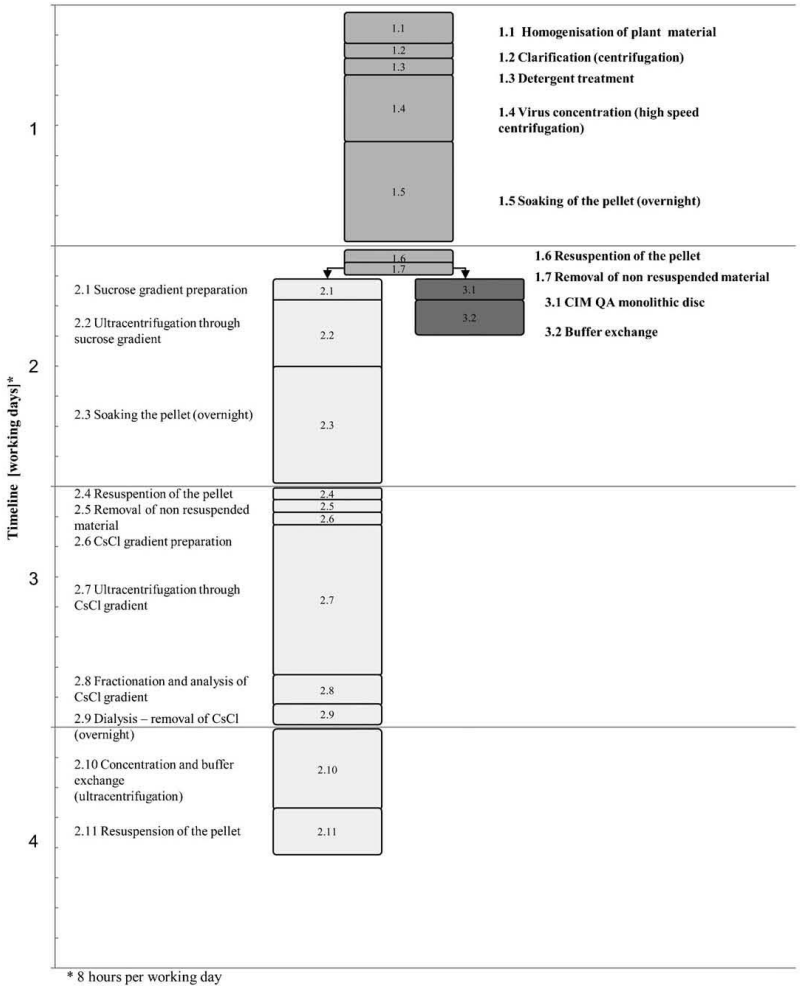


Fig. 3. Comparison between classic and CIM QA purification procedures. Numbered boxes correspond to the purification step with the same number. The size of the boxes is proportional to the approximate time needed for each step.

majority of PVY RNA (44.03% from a total recovery of 53.19% in run A and 22.98% from a total recovery of 34.84% in run B) was recovered in the first eluted peak during the gradient (Fig. 2A) which was collected in fractions F4 and F5 (Fig. 2A and B). The combined flow through and wash fraction contained only traces of PVY RNA with Cq values close to the limit of detection of the assay. Low amount of PVY RNA was recovered in the 250 mM elution fraction and minute amounts were present in the rest of the gradient fractions (Fig. 2B, F10, F17). Plant nucleic acids, represented by cytochrome oxidase (COX) gen, eluted much later in the gradient. The majority was recovered in fractions from F10 to F20 (Fig. 2B, F10, F17). Importantly, only traces of plant RNA were detected in fractions F4 and F5. Probably, some plant nucleic acids bound too stably to the column to be eluted in our conditions. Therefore the total recovery of COX RNA did not reach 100% under used conditions. In a similar way, PVY RNA total recovery reached a maximum of 53% (run A) and 35% (run B), indicating that close to half of the virus RNA (presumably corresponding mainly to the uncapsidated RNA population) stayed in the column. The decrease of 20% in the RNA recovery obtained in run B could be related to a higher presence of free virus RNA in the load from that particular run. This is supported by the fact that all other methods, TEM, SDS-PAGE and spectrophotometric measurements, showed very similar results in both runs A and B, and points to the importance of having different characterization techniques (targeting RNA, proteins and particles) for such complex system (RNA virus in plant).

In correlation with what was observed with the RNA recovery, the SDS-PAGE results showed that most of the PVY CP was eluted in fractions F4 and F5, and more importantly that no contaminant proteins co-eluted in those fractions (Fig. 2C). The specificity of the suspected PVY CP band was confirmed by Western blot using anti PVY antibodies conjugated with alkaline phosphatase (data not

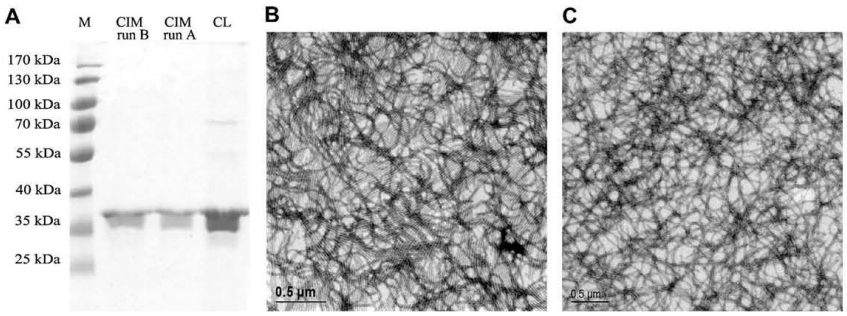


Fig. 4. Comparison of purity and integrity of purified PVY particles (isolate NIB-NTN) purified classically and with CIM chromatography. (A) SDS-PAGE showing final virus suspensions from CIM QA purifications (run A and run B) and classic purification (CL). (B) TEM micrograph showing final virus suspension after classical purification. (C) TEM micrograph showing final virus suspension after CIM QA purification (run A). Both TEM micrographs were taken at 25 000× magnification.

shown). Traces of PVY CP were present in all gradient fractions and 250 mM fraction but not in combined flow through and wash fraction (FT + W) (data not shown). As expected, using the initial steps of the classic purification effectively reduced the protein abundance observed in the load (Fig. 2C, load) compared to crude homogenate (Fig. 2C, S1).

TEM was used to evaluate particle integrity and quantity. Results correlated with RT-qPCR and SDS-PAGE analysis. As expected, the largest amount of PVY particles were found in the fractions F4 and F5, where great majority were intact (cca. 740 nm long) (Fig. 2D). Few particles were also found in all subsequent gradient fractions, however particles were often broken (represented in Fig. 2D, F18). Low amount of intact particles was found in the 250 mM fraction (Fig. 2D); no particles were observed in FT + W (data not shown).

Immediately after the elution of the virus rich peak, an acute increase in the pressure was observed in both runs (up to 1.25 MPa in run A and up to 2.5 MPa in run B). After this point faint CP bands were still observed in the SDS PAGE, disrupted virus particles under the TEM and lower RNA recoveries with the RT-qPCR assay. It could be speculated that the elution of the PVY filamentous particles, promoted an accumulation of those within the monolith pores that resulted in a partial clogging with the consequent pressure increase. Such pressure increase may have induced the disruption of particles, stabilizing the pressure and resulting in the above-described observations.

Infection of the test plants *N. tabaccum* cv. White Burley with chromatographically purified PVY from run B was successful. Mosaic, hyperplasia and vein necrosis symptoms were developed on the leaves by the 18 DPI, same as in plants inoculated with the plant sap from PVY infected potato leaves (positive control) indicating that the infectivity of the particles was not affected by the purification on CIM QA.

Depending on the requirements of the downstream application, virus particles might be kept in the elution buffer or exchanged to alternative buffer appropriate for chosen application. In present study the buffer from pooled fractions 4 and 5 was exchanged to RB2, using ultracentrifugation. However, conventional dialysis or commercially available buffer exchange columns may have been used instead, in case of an ultracentrifuge would not have been available.

The concentration of the purified virus solution was determined with the same procedure as for classically isolated virus (Section 2.2) then isolation yield was calculated. The isolation yields of run A and run B were comparable being 1.4 and 1.8 µg of pure PVY per g of fresh tissue.

We have also preformed preliminary experiments with PVY infected *N. tabacum* cv. Xanthii grown in soil. Experiments resulted in similar recoveries as with potato tissue cultures as starting material.

3.3. Comparison between classic and CIM based purification

PVY purification schemes using classic and CIM QA based procedures are presented in Fig. 3. Classical isolation requires 4 days, while CIM QA based purification could be achieved in less than 2 days. In the classical isolation the throughput is usually limited with centrifugation steps (rotors and tube volume). However, if larger amount of purified virus is needed CIM based purification enables easily scalable purification without considerable increase of time consumption. This could be achieved by applying larger load volumes (provided that the dynamic binding capacity of the column is not reached), or by scaling up from 0.34 ml disks to 1 ml or even 8 ml columns. In addition, with the CIM purification procedure the use of ultracentrifuges, which are costly equipment that are not available in many laboratories, can be avoided. The purity of the purified PVY is comparable between both isolation procedures. SDS-PAGE profiles of the purified virus suspensions after buffer exchange from both classic and CIM purifications are comparable (Fig. 4A). The concentration of the classically isolated PVY NIB-NTN (0.58 mg/ml) was slightly higher than the same isolate purified with CIM (0.17 mg/ml and 0.26 mg/ml for CIM run A and run B, respectively) (Fig. 4A). However, the purification yield was comparable between both methods, being 2.23, 1.4 and 1.8 µg of pure PVY per gram of fresh tissue for classical, CIM run A and CIM run B purification, respectively. The 3× higher concentration of the classically purified NIB-NTN may explain the presence of a faint CP dimer band (70 kDa) in lane CL (Fig. 4A), which was confirmed by western blot with CP specific primers. In addition, a faint band at around 55 kDa can be seen in the classic isolation of NIB-NTN isolate (Fig. 4A, CL). This band could correspond to traces of the highly abundant plant protein Rubisco (55 kDa) large chain, or to the viral helper component protein (Hc-Pro, 58 kDa), which has been described to interact with virus particles [44,45]. The band is not seen in the lanes of CIM based purifications. The 260 nm/280 nm absorbance ratio is another parameter often used to estimate the purity of the viral suspensions. In the case of potyviruses the 260/280 ratio of pure virion solution should ideally be 1.21–1.22 [3] (equivalent to 95% protein and 5% ss RNA). In our case, both methods reached sufficient purity, with 1.29, 1.16 and 1.3 ratios for classical, CIM run A and CIM run B, respectively. Both methods successfully removed plant nucleic acids (above 99.99% of COX was removed with both

procedures, as observed by RT-qPCR), preserved the PVY particle integrity (Fig. 4B) and infectivity.

4. Conclusions

This study is the first case of monolith based chromatographic method for purification of a filamentous virus. The interactions of particles of such dimensions (700 nm × 11 nm [26]) and flexibility as the ones of PVY with the stationary phase (CIM monolithic column) make the purification more difficult than in the case of rod shaped [9] or spherical viruses [18,19,22] and bacteriophages [14]. In addition, the complexity of plant homogenate, which includes structural elements of the cell wall, pigments, poly-phenols and a variety of endogenous plant proteins in large concentrations, further increases the difficulty of the purification. The use of different methods (RT-qPCR, SDS-PAGE and TEM) was proven to be crucial for proper analysis of chromatographic runs. The described CIM QA based PVY purification method showed good reproducibility of purification with viral yields and purity comparable to classic method performed on the same virus isolate. Furthermore, it enables fast isolation in less than 2 days, with the possibility of simple scale up. The developed method could enable faster and easier PVY antibody production, due to easier obtainable pure virus suspensions for immunization. The method could also be used in sample preparation for next generation sequencing, to get a clear view over the viral population structure and dynamics, without the interference of host nucleic acid, which negatively affects the data analysis. Potyviruses are the largest family of plant viruses and since they have similar structures, the proof of principle established in this study could enable faster development of purification methods for other economically important potyviruses such as *Plum pox virus* and also human viruses of similar shape and dimensions.

Acknowledgements

This work was financially supported by Slovenian Research Agency through the Grant L1-2278. Matevž Rupar is a recipient of a PhD research grant from Slovenian Research Agency (Contract No. 1000-10-310196).

References

[1] H. Huet, A. Gal-On, E. Meir, H. Lecoq, B. Raccah, J. Gen. Virol. 75 (1994) 1407.
[2] Y.H. Peng, D. Kadoury, A. Gal-On, H. Huet, Y. Wang, B. Raccah, J. Gen. Virol. 79 (1998) 897.
[3] R.-M. Leiser, J. Richter, Arch. Phytopathol. Pflanzenschutz 14 (1978) 337.
[4] D.J. Studholme, R.H. Glover, N. Boonham, Annu. Rev. Phytopathol. 49 (2011) 87.
[5] A. Strancar, A. Podgornik, M. Barut, R. Necina, in: S. Belkin, I. Endo, M.B. Gu, W.S. Hu, B. Mattiasson, J. Nielsen, G.N. Stephanopoulos, R. Ulber, A.P. Zeng, J.-J. Zhong, W. Zhou (Eds.), Advances in Biochemical Engineering/Biotechnology, vol. 76, 2002, p. 49.
[6] A. Jungbauer, R. Hahn, J. Chromatogr. A 1184 (2008) 62.
[7] A. Podgornik, A. Strancar, Biotechnol. Annu. Rev. 11 (2005) 281.
[8] P. Kramberger, N. Petrovic, A. Strancar, M. Ravnikar, J. Virol. Methods 120 (2004) 51.
[9] P. Kramberger, M. Peterka, J. Boben, M. Ravnikar, A. Strancar, J. Chromatogr. A 1144 (2007) 143.
[10] D. Forcic, K. Branovic-Cakanic, J. Ivancic, R. Jug, M. Barut, A. Strancar, J. Chromatogr. A 1065 (2005) 115.
[11] N.L. Krajnc, F. Smrekar, J. Cerne, P. Raspor, M. Modic, D. Krgovic, A. Strancar, A. Podgornik, J. Sep. Sci. 32 (2009) 2682.
[12] M. Krajačič, J. Ivancic-Jelecki, D. Forcic, A. Vrdoljak, D. Škorić, J. Chromatogr. A 1144 (2007) 111.
[13] M.C. Perica, I. Sola, L. Urbas, F. Smrekar, M. Krajacić, J. Chromatogr. A 1216 (2009) 2712.
[14] F. Smrekar, M. Ciringer, M. Peterka, A. Podgornik, A. Strancar, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 861 (2008) 177.
[15] P. Kramberger, R.C. Honour, R.E. Herman, F. Smrekar, M. Peterka, J. Virol. Methods 166 (2010) 60.
[16] K. Liu, Z. Wen, N. Li, W. Yang, L. Hu, J. Wang, Z. Yin, X. Dong, J. Li, J. Virol. Methods 186 (2012) 1.
[17] M. Peterka, A. Strancar, M. Banajc, P. Kramberger, E. Maurer, T. Muster, U.S. Patent WO2007EP56890 20070706, 2008.
[18] K. Kovac, I. Gutiérrez-Aguirre, M. Banjac, M. Peterka, M. Poljsak-Prijatelj, M. Ravnikar, J.Z. Mijovski, A.C. Schultz, P. Raspor, J. Virol. Methods 162 (2009) 272.
[19] I. Gutiérrez-Aguirre, M. Banjac, A. Steyer, M. Poljsak-Prijatelj, M. Peterka, A. Strancar, M. Ravnikar, J. Chromatogr. A 1216 (2009) 2700.
[20] I. Gutiérrez-Aguirre, A. Steyer, M. Banjac, P. Kramberger, M. Poljšak-Prijatelj, M. Ravnikar, J. Chromatogr. A 1218 (2011) 2368.
[21] D. Forcic, M. Brgles, J. Ivancic-Jelecki, M. Santak, B. Halassy, M. Barut, R. Jug, M. Markušič, A. Strancar, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 879 (2011) 981.
[22] C.S. Burden, J. Jin, A. Podgornik, D.G. Bracewell, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 880 (2011) 82.
[23] L. Urbas, B.L. Jarc, M. Barut, M. Zochowska, J. Chroboczek, B. Pihlar, E. Szolajska, J. Chromatogr. A 1218 (2011) 2451.
[24] A. Trauner, M.H. Bennett, H.D. Williams, PLoS ONE 6 (2011) e16273.
[25] F. Smrekar, M. Ciringer, A. Štrancar, A. Podgornik, J. Chromatogr. A 1218 (2011) 2438.
[26] A. King, M.J. Adams, E. Lefkowitz, E.B. Carstens, Virus Taxonomy Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier, 2011.
[27] R.P. Singh, J.P.T. Valkonen, S.M. Gray, N. Boonham, R.A.C. Jones, C. Kerlan, J. Schubert, Arch. Virol. 153 (2008) 1.
[28] A. Blanchard, M. Rolland, C. Lacroix, C. Kerlan, E. Jacquot, Curr. Top. Virol. 7 (2008) 21.
[29] L. Beczner, J. Horváth, I. Romhányi, H. Förster, P.O. Box, Potato Res. 27 (1984) 339.
[30] M. Le Romancer, C. Kerlan, M. Nedellec, Plant Pathol. J. 43 (1994) 138.
[31] N. Boonham, K. Walsh, M. Hims, S. Preston, J. North, I. Barker, Plant Pathol. 51 (2002) 117.
[32] K.G. Scholthof, S. Adkins, H. Czosnek, P. Palukaitis, E. Jacquot, T. Hohn, B. Hohn, K. Saunders, T. Candresse, P. Ahlquist, C. Hemenway, G.D. Foster, Mol. Plant Pathol. 12 (2011) 938.
[33] S. Urcuqui-Inchima, a.L. Haenni, F. Bernardi, Virus Res. 74 (2001) 157.
[34] N. Watts, R.P. Singh, Phytopathology 84 (1994) 991.
[35] P. Kogovsek, L. Gow, M. Pompe-Novak, K. Gruden, G.D. Foster, N. Boonham, M. Ravnikar, J. Virol. Methods 149 (2008) 1.
[36] M.M. López, P. Llop, A. Olmos, E. Marco-Noales, M. Cambra, E. Bertolini, Curr. Issues Mol. Biol. 11 (2009) 13.
[37] S.A. Weller, J.G. Elphinstone, N.C. Smith, N. Boonham, D.E. Stead, Appl. Environ. Microbiol. 66 (2000) 2853.
[38] N. Toplak, V. Okršlar, Plant Mol. Biol. Rep. (2004) 237.
[39] M. Bashir, R.O. Hampton, Pak. J. Bot. 27 (1995) 243.
[40] C.S. Luciano, J.F. Murphy, R.E. Rhoads, J.G. Shaw, J. Gen. Virol. 72 (1991) 205.
[41] S.E. Bondos, A. Bicknell, Anal. Biochem. 316 (2003) 223.
[42] F. Edwin, Y.V. Sharma, M.V. Jagannadham, Biochem. Biophys. Res. Commun. 290 (2002) 1441.
[43] N. Petrovič, K. Gruden, M. Ravnikar, Acta Chim. Slov. 42 (1995) 425.
[44] D.W. Thornbury, G.M. Hellmann, R.E. Rhoads, T.P. Pirone, Virology 144 (1985) 260.
[45] G. Roudet-Tavert, S. German-Retana, T. Delaunay, B. Delécolle, T. Candresse, O. Le Gall, J. Gen. Virol. 83 (2002) 1765.

2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Delo v postopku objavljanja: Fluorescentno označeni krompirjev virus Y: vsestransko orodje za funkcionalno analizo interakcij med rastlino in virusom

Fluorescently labelled Potato virus Y: a versatile tool for functional analysis of plant – virus interaction

Matevž Rupar, Florence Faurez, Michel Tribodet, Ion Gutiérrez-Aguirre, Agnes Delaunay, David Dobnik, Kristina Gruden, Emmanuel Jacquot, Maja Ravnikar

Delo je trenutno v fazi vstavljanja popravkov vseh soavtorjev pred pošiljanjem v objavo v revijo Molecular Plant-Pathogen Interactions.

Izvleček:

Krompirjev virus Y (PVY) je gospodarsko pomemben virus, ki okužuje poljščine iz družine *Solanaceae*, kot so tobak, krompir in paprika. Do zdaj so bile študije o širjenju in lokalizaciji PVY v rastlinah omejene na določanje virusne RNA ali beljakovin *ex vivo* . V tej študiji je bil z zelenim fluorescentnim proteinom (GFP) označen sev PVY N605. Temeljita karakterizacija na rastlini *Nicotiana tabacum* je pokazala, da je PVY N605-GFP konstrukt biološko primerljiv z neoznačenim virusom. Izkazal se je kot stabilen v vsaj treh pasažah iz rastline na rastlino in 4 mesece v okuženi rastlini. Signal GFP je bila zaznan 2 dni pred pojavom bolezenskih znamenj, intenzivnost signala pa je korelirala s koncentracijo RNA. PVY N605-GFP je omogočil *in vivo* sledenje širjenju virusa med celicami in sistemsko. Še več, s pomočjo PVY N605-GFP smo lahko ocenili hitrost širjenja PVY na inokuliranem listu. Poleg *N. tabacum* je PVY N605 GFP uspešno okužil tudi vrsto *Nicotiana benthamiana*, različne kultivarje krompirja (*Solanum tuberosum* L.) ter divje sorodnike krompirja. PVY N605-GFP je torej močno orodje za prihodnje študije interakcij rastlin z virusom, npr. funkcionalno analizo virusnih in rastlinskih genov, ki sodelujejo pri širjenju virusa po rastlini, kjer je sledenje in/ali točna lokalizacija virusa ključnega pomena za poglabljanje razumevanja molekulskih mehanizmov med rastlino in virusom.

V procesu objavljanja. Rupar in sod., 2014

Fluorescently labelled Potato virus Y: a versatile tool for functional analysis of plant – virus interaction

Rupar Matevž [1], Faurez Florence [2], Tribodet Michel [2], Gutierrez-Aguirre Ion [1,3], Delaunay Agnes [4], Dobnik David [1], Kristina Gruden [1,3], Jacquot Emmanuel [4], Ravnikar Maja [1,3]

[1] **National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia**
[2] INRA, UMR1349 IGEPP, F-35653 Le Rheu, France
[3] **The Centre of Excellence for Biosensors, Instrumentation and Process Control-COBIK, Solkan, Slovenia**
[4] CIRAD, UMR-BGPI TA A-54/K, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France

Abstract:
Potato virus Y (PVY) is an economically important plant virus infecting crops of *Solanaceae* such as tobacco, potato and pepper. Until now, studies on the movement and localisation of PVY in plants were limited to the detection of viral RNA or proteins ex vivo. In this study PVY N605 strain was tagged by a green fluorescent protein (GFP). Thorough characterisation in *Nicotiana tabacum* indicated that the PVY N605 GFP construct is biologically comparable to the non-labelled one. It is stable at least through three plant-to-plant passages and for 2 months in the inoculated plant. The GFP signal was detected 2 days before any symptoms were observed and the intensity correlated with PVY RNA concentration. The PVY N605 GFP enabled in vivo tracking of the viral cell-to-cell and long distance movement in inoculated and systemically infected leaves. Moreover, the PVY N605 allowed the estimation of the cell-to-cell movement rate of the PVY. In addition to *N. tabacum* PVY N605 GFP is also able to infect *Nicotiana benthamiana*, different potato (*Solanum tuberosum*) cultivars and wild potato relatives. The developed PVY N605 GFP is therefore a powerful tool for future studies of plant-virus interactions such as functional analysis of viral and plant genes involved in viral movement where tracking and/or accurate localisation of the virus is crucial to advance towards a deeper understanding of the molecular interplay of the plant and virus.

V procesu objavljanja. Rupar in sod., 2014

Introduction

The replication and movement of plant viruses within their hosts has been subject of many investigations using a wide range of different techniques. One approach is based on the detection of a combination of viral components such as viral proteins (ELISA, Western blotting, immuno-localisation), viral nucleic acids (in situ hybridisation, leaf blot and PCR techniques) or virions (transmission electron microscopy) in different plant organs (Leisner et al. 1992; Mas and Pallas 1996; Mehle et al. 2004; Ghanim et al. 2009; Kogovšek et al. 2011; Rusetsky and Blotskaya 2001; Lucas 2006; Nass et al. 1998; Rajamäki and Valkonen 2003; Rasheed et al. 2006; Tilsner et al. 2009; Simón-buela and García-arenal 1999) In the 1990's the introduction of infectious cDNA clone strategy has enabled the construction of plant viruses labelled with fluorescent proteins (Baulcombe et al. 1995; Oparka et al. 1996;) and non fluorescent tags. The advantage of fluorescently labelled viruses over the detection of nucleic acids or proteins is the non-invasive monitoring of cell-to-cell movement and systemic spread in live plants (Cohen et al. 2000; Tilsner et al. 2013). However, there are two main concerns associated to the tagged virus approach: i) the stability of the inserted transgene. The inserted gene is often instable i.e. deleted, recombined or modified, resulting in loss of function due to the rapid, error prone multiplication of viral genomes in the plant (Seaberg et al. 2012; Avesani et al. 2007). ii) the effect of the inserted gene on virus multiplication, movement and pathogenicity (Cruz et al. 1996).

Potato virus Y (PVY), type member of the family *Potyviridae* (single-stranded positive sense RNA)(King et al. 2011) was recently classified as one of the ten most important plant viruses (Scholthof et al. 2011). PVY infects a wide range of plant species, especially *Solanaceae*, and it is present worldwide (Blanchard et al. 2008; Gray et al. 2010). Most important symptoms caused by PVY are vein necrosis on tobacco and tuber necrosis on potato, known also as Potato tuber necrosis ringspot disease, which represents the most important viral potato disease (Kerlan 2006).

In this study the *gfp* gene was inserted into a PVY infectious clone derived from strain N605 (Jakab et al. 1997) in such a way that the GFP is excised from the polyprotein upon translation. The resulting construct was thoroughly characterised. The pathogenicity as well as viral RNA accumulation throughout the plant was compared between PVY N605-GFP and non labelled PVY N605-wt. A reverse transcription real time PCR (RT-qPCR) approach, including a newly developed assay specific for the PVY N605-wt was used to assess the stability of the *gfp* inserted in PVY genome. The PVY N605-GFP allowed a detailed observation of the viral movement within tobacco plants. We were able to estimate the rate of viral cell-to-cell movement by using an *in vivo* tracking approach. In addition to *Nicotiana tabacum* and *Nicotiana benthamiana* the PVY N605-GFP infected also different potato (*Solanum tuberosum*) cultivars and *Solanum* species. The developed clone represents a powerful tool for studying the interaction of PVY with its host plants and will serve for complementing and broadening previous knowledge on the movement of PVY.

V procesu objavljanja. Rupar in sod., 2014

Results

In order to construct a GFP labelled PVY N605 three different cloning strategies were attempted, however only one yielded infective GFP labelled PVY clone (illustrated in the Appendix A). Moreover, the infectivity of the clone using a gene gun bombardment procedure was very low. We detected only 3 infected plants out of 60. However, virus was successfully multiplied further mechanically.

Comparing the pathogenicity and fitness of PVY N605-GFP *and PVY N605-wt*

The effect of the inserted *gfp* gene on the PVY pathogenicity and fitness was investigated first to verify that the fluorescently labelled and non-labelled PVY N605 were biologically comparable. Parallel inoculations of *Nicotiana tabacum* (Nt) cv. Xanthii with calibrated inoculums showed that both PVY N605-GFP and PVY-N605-wt cause comparable symptoms at the same day post inoculation (DPI) (Figure 1). Both caused vein clearing on systemic leaves at 4 DPI, hyperplasia at 6 DPI and vein necrosis on systemic leaves at 9 DPI. GFP fluorescence was detected in all PVY N605-GFP inoculated plants in all top systemic leaves at 14 DPI. Moreover, in the case of both viruses (PVY N605-GFP and PVY N605-wt) all inoculated plants were symptomatic at 14 DPI. The PVY N605-GFP genome concentrations were quantified in systemic leaves at different DPI and compared to PVY N605-wt infected ones (Appendix B). The concentration of PVY N605-GFP was comparable to PVY N605-wt in spite of the addition *gfp* gene. At 5 DPI both isolates reached concentrations up to 10^8 copies per 10^6 copies of cytocrome oxydase gene (COX) in systemic leaves. Additionally no significant differences in genome concentrations between labelled and non-labelled PVY were observed in systemic leaves at 14 DPI (Appendix B).

V procesu objavljanja. Rupar in sod., 2014

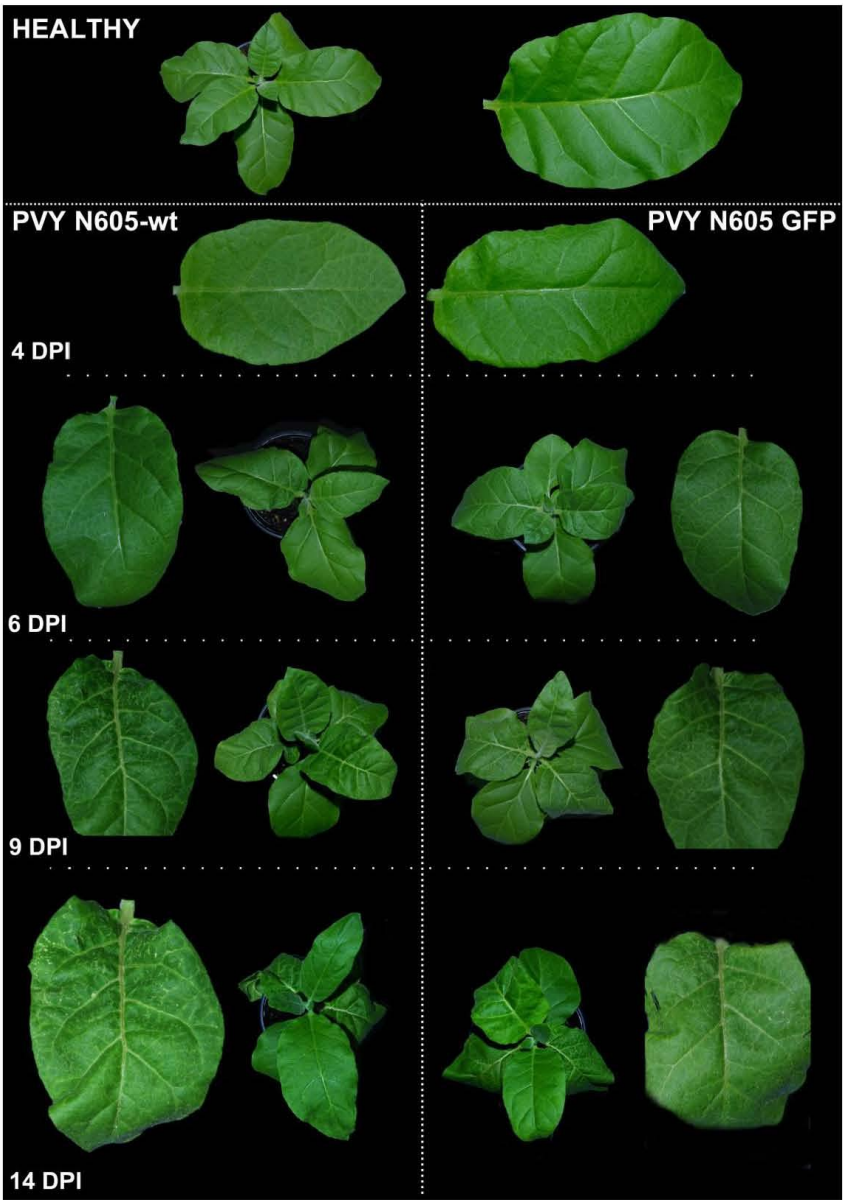


Figure 1: Symptoms on systemically infected leaves of Nicotiana tabacum cv. Xanthii, caused by PVY N605 GFP in comparison to PVY N605-wt and healthy plant. Fluorescently labelled and non-labelled PVY cause the same symptoms at the same time post inoculation.

V procesu objavljanja. Rupar in sod., 2014

The stability of the *gfp* gene insert in the PVY N605 genome was estimated by i) GFP fluorescence detection with fluorescent microscope and ii) a specific detection of potential genomes with deleted *gfp* gene (PVY N605-wt genomes) by newly developed RT-qPCR. First the stability of the insert through passages from plant to plant was estimated. Three plant-to-plant passages were done on Nt cv. Xanthii. GFP fluorescence was detected after 14 DPI in systemic leaves in all passages.
The stability of the *gfp* gene in the samples was additionally confirmed by RT-qPCR with four assays: universal PVY (UNI) (Kogovsek et al. 2008), amplifying all PVY genomes; PVY–wt, specifically amplifying PVY N605-wt genomes and the ones with *gfp* gene deleted; PVY-*gfp* (Joshi et al. 2008), amplifying only the *gfp* gene and COX (Weller et al. 2000), amplifying plant endogene for cytochrome oxydase, used for cross sample normalisation.
The inoculums used for passing PVY N605-GFP from Nt cv. Xanthii to Nt cv. Xanthii were tested and contained no genomes with eliminated *gfp* gene i.e. PVY N605-wt genomes. In the PVY N605-GFP samples, the measured concentrations of the *gfp* gene (PVY-*gfp* assay) were equivalent to the concentrations of the total PVY population in the sample (UNI assay). This is illustrated by the 0.97 to 1 ratio between the theoretical copy number (TCN) measured by PVY-*gfp* and UNI assays respectively (Figure 2). The equimolar increase in the concentration of the *gfp* gene and all PVY genomes in all the samples, indicates a stable insertion and multiplication of the *gfp* gene with the rest of the PVY genes. In addition, the ratio between the TCN measured by PVY-wt and UNI assays is 0.0058 in all of the samples and it did not increase with increased concentration of total PVY genomes (Figure 2). Both ratios are implicating that *gfp* gene was stably multiplied with the rest of PVY genes at a comparable rate and was not deleted in substantial amounts during viral multiplication. More precisely, out of 164 tested samples, 90 were free of PVY N605-wt genomes (all quantification results are listed in the Appendix I), 65 samples contained less than 0.05 % of PVY N605-wt genomes in the whole PVY population. Only 5 samples with PVY N605-wt concentrations above 0.05 % of all PVY population were found. The amount of PVY N605-wt in the samples did not increase with time. Samples with minute background amounts of PVY N605-wt were sporadically present throughout the timeline (Figure 3), however the PVY N605-wt did not outcompete the GFP labelled PVY in the plant. In fact no PVY-wt genomes were detected in plants infected with PVY N605-GFP at 120 DPI (Figure 3, Appendix I).

V procesu objavljanja. Rupar in sod., 2014

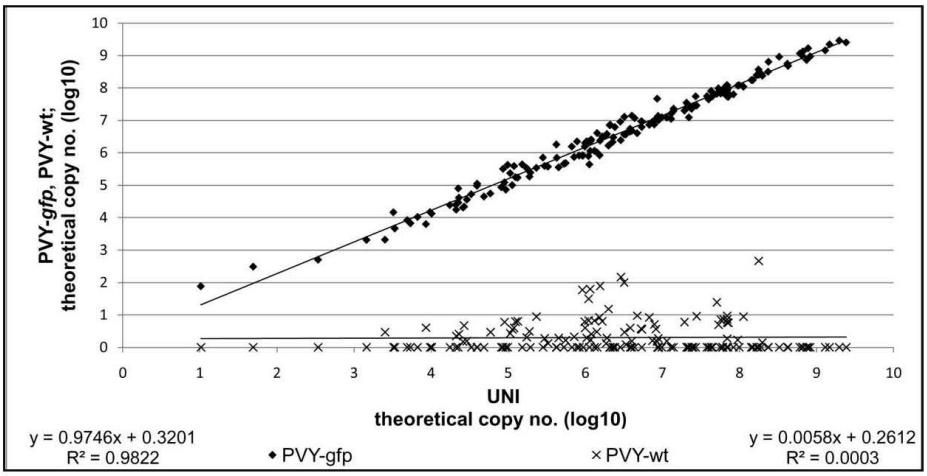


Figure 2. Stability of PVY N605-GFP shown as the correlation between theoretical copy no. (TCN) of all PVY genomes (quantified by UNI RT- qPCR assay) and TCN for *gfp* gene / PVY-wt genomes in PVY N605-GFP inoculated samples (1-5, 7 10 14 120 DPI). The concentration of *gfp* is increasing linearly with concentration of UNI, while concentration of PVY-wt genomes is less than 10 copies in most of the samples (max to 1000 copies) and is not increasing with concentration all PVY genomes. The exact concentrations along with raw Cq values of all assays are listed in the Appendix I.

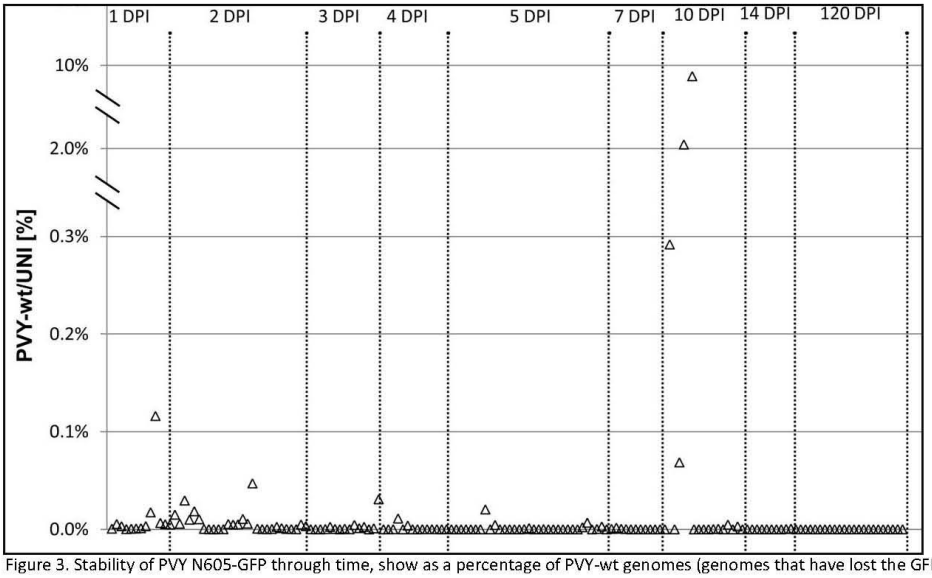


Figure 3. Stability of PVY N605-GFP through time, show as a percentage of PVY-wt genomes (genomes that have lost the GFP gene) of all PVY population (quantified with UNI assay) in PVY N605-GFP inoculated samples. The PVY-wt is detected sporadically in almost all DPI, however it did not increase with time after inoculation. Majority of the samples contained no PVY-wt genomes or their concentration was below 0.05 % of the whole PVY population. The exact concentrations and percentage in every sample is listed in Appendix I.

V procesu objavljanja. Rupar in sod., 2014

Monitoring of PVY movement

Confirmation that the introduced *gfp* gene is stable over time and that it does not affect the biology and the fitness of the resulting virus, enabled the use of PVY N605-GFP clone for observation of the movement of PVY in inoculated and systemically infected leaves of Nt cv. Xanthii. In parallel to GFP fluorescence detection, PVY genomic RNA was quantified and symptoms were observed.

High background fluorescence was present on inoculated leaves in green and red channels during the first few days after inoculation and was gradually decreasing with time (Figure 4). Nonetheless, a detailed fluorescence microscopy inspection (Figure 4, ii) enabled detection of an increased GFP fluorescence signal specific to infected cells as soon as at 2 DPI, well before any symptoms were observed. At later DPI the cell-to-cell movement of the PVY N605-GFP in the inoculated leaf is indicated by enlarged fluorescent foci. Interestingly, the GFP fluorescence was also detected along the veins e.g. at 7 DPI (Figure 4) demonstrating that PVY proteins are being actively translated in the phloem and/or companion cells.

Inoculated and systemically infected leaves were sectioned based on fluorescence detection (Figure 5), then concentration of PVY was quantified with UNI assay in each section separately. The increase in GFP signal correlated with the increase in PVY RNA concentration quantified with UNI qPCR assay on both inoculated and systemically infected leaves (Figure 5, graph in Appendix C). Furthermore, PVY RNA was present in the regions with no GFP signal as well. On the inoculated leaf the concentration of PVY RNA in the GFP foci (Figure 5, A) was approximately 10x higher than the background PVY concentration in the neighbouring tissue without GFP signal where PVY RNA originated primarily from the mechanical inoculation and amplification of genomic RNA prior to protein synthesis. A gradient of PVY RNA concentration was observed on systemic leaves. Concentrations were higher in the tip compared to the base of the leaf in all the cases (Figure 5 and Appendix C). In the case of systemically infected leaf the quantity of GFP signal linearly correlated with the of RNA concentrations (Appendix C).

With PVY N605-GFP we were able to observe that the PVY unloads from class I, II, III and IV veins in the systemically infected leaf (Figure 6).

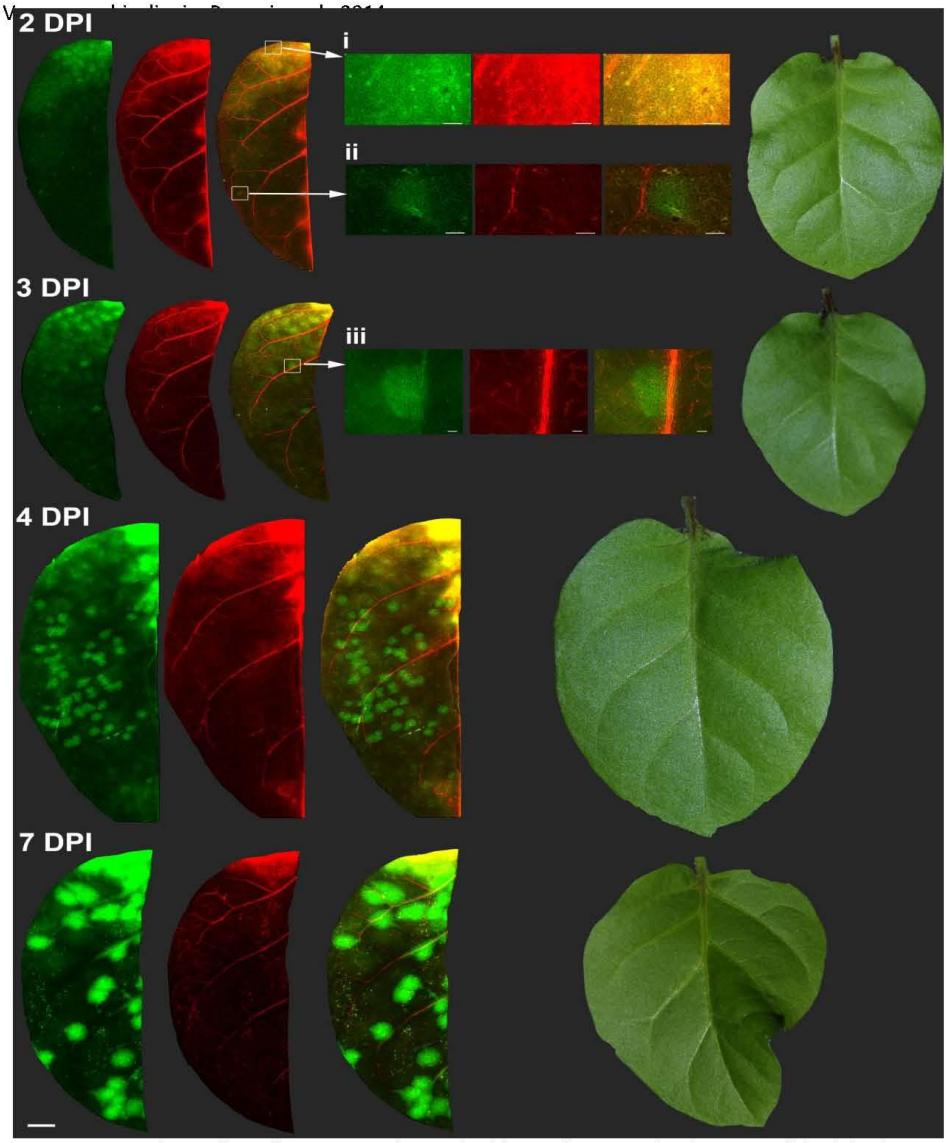


Figure 4. Detection of PVY cell-to-cell movement in the inoculated leaves of Nt cv. Xanthii. Fluorescence (left side) and symptom observation (right side) on Nt cv. Xanthii, inoculated leaves at different DPI. The same leaf is shown for each given DPI. Fluorescence observations in green and red are shown together with the overlay of green and red channels (pixels with signal in both red and green channel are presented yellow on the overlay). Detailed sections of: i) unspecific fluorescent signal (the same patterns and intensities observed in red and green channel); ii) first PVY N605GFP infection foci detected at 2 DPI (distinct signal only in green channel); iii) PVY N605-GFP foci infecting the nearby vein. The fluorescent foci grew larger at later DPI indicating the spread of the virus. At 7 DPI some GFP fluorescence is detected along the veins as well. No symptoms were observed on inoculated leaves. The images are identically scaled to accurately present the size of the GFP fluorescence foci (the bar at the left bottom corner represents 5 mm). Bars on detailed sections represent 250µm.

- 8-

V procesu objavljanja. Rupar in sod., 2014

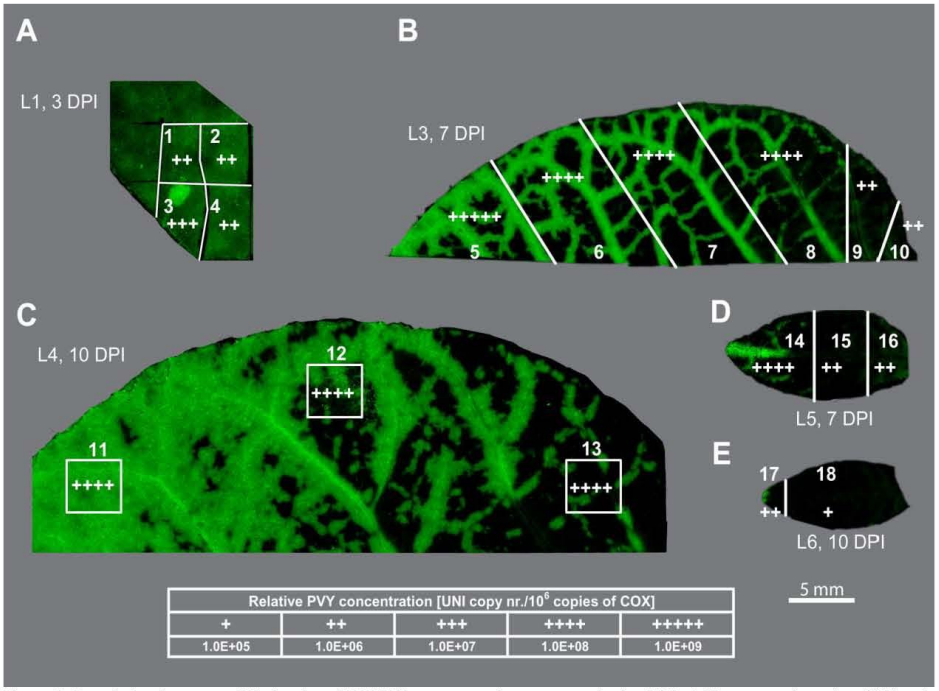


Figure 5. Correlation between GFP signal and PVY RNA concentration measured using UNI qPCR assay on inoculated (A) and systemic leaves (B, C, D, E).The sections of the leaves used in the qPCR analysis are separated with white lines. On the systemically infected leaves (B, C, D, E) the intensity of GFP signal and RNA concentration were both higher at the tip of the leave in comparison to the base. The same was observed in leaf 4 at 10 DPI (C), in which quantified 7.3 x 10^8, 2,6 x 10^8, 1.9 x $10^8$and PVY genome copies per 10^6 COX copies in sections 11, 12 and 13, respectively. The measured concentrations of PVY RNA in every region (numbered from 1 – 18) are in the Appendix C. The images are identically scaled, thus the 5mm bar applies to all parts of the figure. All leaves on the figure are oriented left (tip) to right (base). No background signals were detected in red channel.

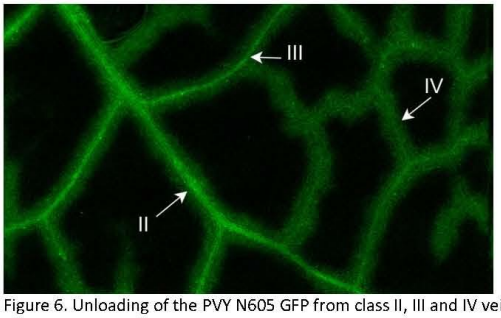


Figure 6. Unloading of the PVY N605 GFP from class II, III and IV veins of Nt cv. Xanthii.

V procesu objavljanja. Rupar in sod., 2014

Long distance movement of PVY using in vivo tracking technique

Adaptation of the plant growing technique (Appendix D) allowed us *in vivo*, non-invasive tracking the movement of PVY in the same plant at different DPI (Figure 7). The first distinct GFP foci on L1 appeared at 3-4 DPI, while the first GFP fluorescence in systemic leaves was detected from 4-5 DPI. The GFP signal was most intense in the inoculated leave and the systemic leaves L3 and L4. Interestingly, on L2 (the first leaf above inoculated) only some faint GFP signal was detected in the veins while the majority of the leaf remained free of GFP fluorescence signal. Similar result was obtained when the experiment was repeated (Appendix E).

V procesu objavljanja. Rupar in sod., 2014

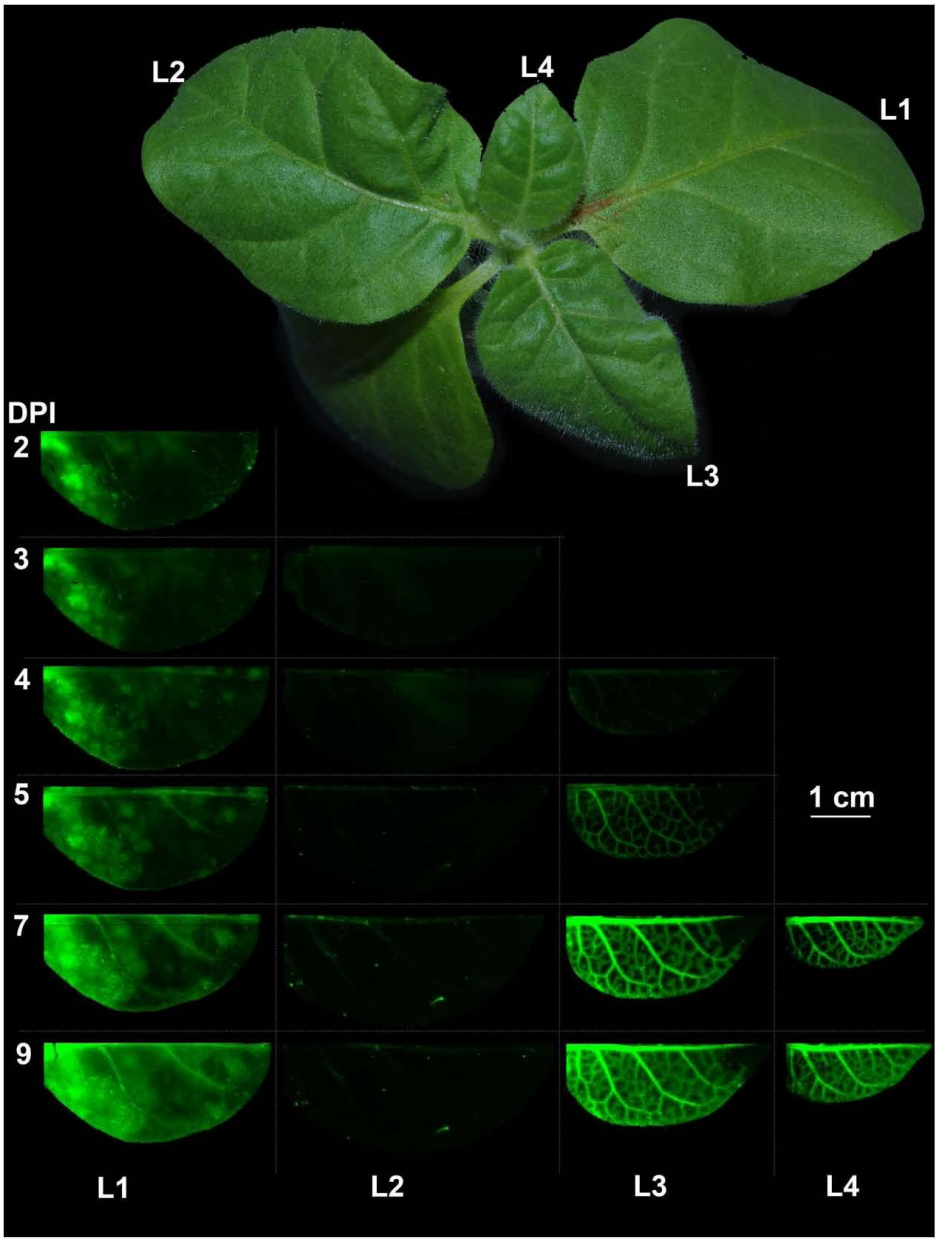


Figure 7. Tracking the long distance movement of the PVY *in vivo*. Symptoms at 9 DPI (upper part), GFP fluorescence on inoculated (L1) and systemic leaves (L2, L3, L4) of the same plant at different DPI (lower part). A certain amount of unspecific fluorescence was detected on the inoculated leaf (indicated by overlay with red channel (data not shown)). Independent repetition of the experiment is presented in the Appendix E.

V procesu objavljanja. Rupar in sod., 2014

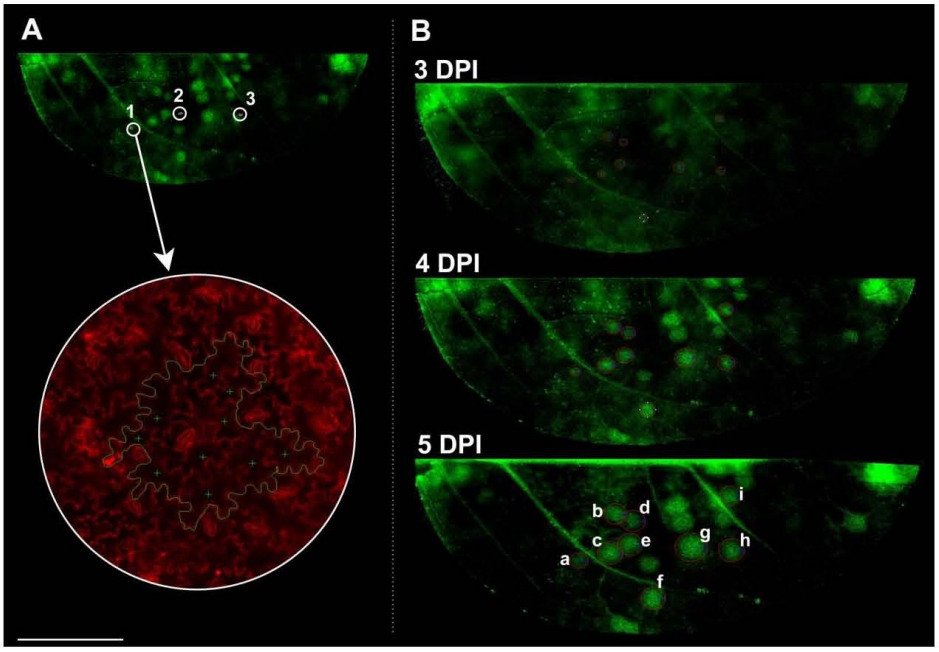


Figure 8. Measuring the rate of cell-to-cell movement of PVY N605-GFP in the inoculated leaf. A) To account for the natural increase in the size of cells over time, 3 regions on the leaf near the GFP fluorescent foci were chosen at each DPI (upper image). In each region 10 cells were counted (marked by a green cross) and their area measured (lower image). The values were used to estimate the area of the average cell at each DPI. B) The increase in size of GFP fluorescent foci over time is presented. The diameter of each foci (a-i) was measured at 3, 4 and 5 DPI (exact 27 h interval). The area of the foci was calculated and then the number of infected cells in each foci estimated using the average cell area calculated at each DPI as shown in in A).

Rate of viral cell-to-cell *movement*

Utilising the *in vivo* PVY N605-GFP tracking technique it was possible to estimate the rate of the movement of PVY on the inoculated leaf by measuring the growth of the fluorescent foci area (measurement prosess illustrated in Figure 8). On average, foci increased by 6.5 cells/ hour in the first day and 9 cells/hour in the second day (Table 1). Thus, in these initial stages the PVY cell-to-cell spread seems to accelerate with time after infection.

V procesu objavljanja. Rupar in sod., 2014

Table 1. Measurements of the PVY cell-to-cell movement rate in the inoculated leaf. The measurements were done in 27 h intervals, no. of cells in foci and the spread rate was estimated separately for each foci (a-i) at each time point (0, 27 and 54 h after 3 PDI). The measured average cell areas used for calculation of no. of cells per foci were 7630.5 μm^2 (at 3 DPI), 8070.9 μm^2 (at 3 DPI + 27 h) and 9259.7 μm^2 (at 3 DPI + 54 h). Exact position of the foci on the leaf is denoted on Figure 8.

Foci	Cell-to-cell movement rate [newly infected cells per hour]			Hours after 3 DPI	Calculated area of the fluorescent foci [x10^6 μm^2]	Calculated no. of infected cells per foci
	0 - 27 h	27 - 54 h	Average, 0 - 54 h			
a	2	7	4	0	0.20	26
				27	0.65	80
				54	2.42	261
b	5	7	6	0	0.64	83
				27	1.80	224
				54	3.84	415
c	8	9	8	0	0.45	59
				27	2.17	268
				54	4.75	513
d	5	10	7	0	0.39	51
				27	1.44	179
				54	4.17	450
e	7	8	7	0	0.75	98
				27	2.30	285
				54	4.52	488
f	5	13	9	0	0.46	61
				27	1.89	204
				54	4.48	556
g	11	16	13	0	0.88	115
				27	3.24	402
				54	7.60	821
h	7	9	8	0	0.58	76
				27	2.17	269
				54	4.86	525
i	5	6	6	0	0.35	46
				27	1.55	191
				54	3.37	364
Average[a]:	**6.5**	**9.0**	**7.8**			

a) PVY cell-to-cell movement rate over all measured foci [newly infected cells per hour

V procesu objavljanja. Rupar in sod., 2014

To investigate the potential use of the tagged GFP PVY on other plant species, a range of *Solanum* and *Nicotiana* species and cultivars were screened for the susceptibility to PVY N605-GFP infection (Table 2). *Nicotana benthamiana* (systemically infected with PVY N605-GFP at 6-8 DPI) was tested since it is a model plant to investigate the plant-virus interactions. To study the movement of PVY in *Solanum tuberosum*, four cultivars with different sensitivity to PVY were chosen: Désirée, NahG-Désirée, Bintje and Bea. On Désirée PVY N605 induces mild or no symptoms, in comparison to NahG-Désirée, where symptoms are vein necrosis with leaf drop. The NahG-Désirée is highly susceptible to PVY infection due to the disrupted salicylic acid signalling pathway (Halim et al. 2004). PVY N605 causes hyperplasia but no PTNRD symptoms on Bintje; a yellowing mosaic, vein necrosis and PTNRD symptoms on Bea. The PVY N605-GFP was detected as small fluorescent foci in the inoculated leaves of Désirée, NahG-Désirée and Bintje already at 6-8 DPI. Discreet fluorescent patches were detected in systemically infected leaves of all four cultivars at 18-20 DPI. In addition different wild potato relatives, chosen based on their potential use as a model to study the interactions of PVY were challenged with PVY N605-GFP. In majority of the tested wild *Solanum* species the GFP signal was observed at 14 DPI in the inoculated leaves. However, PVY N605-GFP was detected systemically (at 14 DPI) in *Solanum mochiquense*, *Solanum papita* and in 3 out of 6 tested *Solanum venturii* accessions.

V procesu objavljanja. Rupar in sod., 2014

Table 2. The susceptibility of different *Nicotiana* and *Solanum* species and cultivars to PVY N605-GFP infection. Detection of GFP in *Nicotiana* species and *Solanum tuberosum* was done at 11 and 18 DPI on inoculated and systemically infected leaves, respectively. Other *Solanum* species were examined at 14 DPI. Accession no. according to SolRgene database: http://www.plantbreeding.wur.nl/SolRgenes/.

Species	SolRgene accession no.	GFP signal detected [a]	
		inoculated leaves	systemic leaves
Nicotiana tabacum cv. Xanthii	/	+	+
Nicotiana benthamiana	/	+	+
Solanum tuberosum cv. Désirée [b]	/	+	+
Solanum tuberosum cv. NahG-Désirée [b]	/	+	+
Solanum tuberosum cv. Bea [b]	/	n.t.	+
Solanum tuberosum cv. Bintje [b]	/	+	+
Solanum bulbocastanum	331-2	+	-
Solanum hjertingii	349-3	+	-
Solanum jamesii	355-1	+	-
Solanum lesteri	358-4	+	-
Solanum mochiquense	186-1	+	+
Solanum okadae	970-3	+	-
Solanum polytrichon	378-2	+	-
Solanum papita	767-8	+	+
Solanum species	278-2	+	-
Solanum venturii	283-1	+	+
Solanum venturii	366-2	+	+
Solanum venturii	365-1	+	+
Solanum venturii	741-1	+	-
Solanum venturii	250-2	+	-
Solanum venturii	896-4	+	-

a) +, GFP fluorescence present; -, GFP fluorescence absent; n.t., not tested
b) inoculated with nondiluted sap from infected Nt cv. Xanthii

V procesu objavljanja. Rupar in sod., 2014

Discussion

A number of fluorescently labelled potyviruses were already reported e.g. *Potato virus A* (PVA) (Rajamäki et al. 2005) and *Tobacco vein banding mosaic virus* (TVBMV) (Gao et al. 2012) were s tagged with GFP in *N. benthamiana*. GFP-tagged *Plum pox virus* (PPV) was utilized to study Prunus–PPV interactions at the whole plant and cellular levels (Lansac et al. 2005). *Tobacco vein mottling virus* (TVMV), *Clover yellow vein virus* (ClYVV) and PPV labelled with GFP and DsRed were used to investigate co-infection of the cells with different viruses or identical viruses with different tags (Dietrich and Maiss 2003). *Bean common mosaic virus* (BCMV) was tagged with UidA gene encoding β-glucuronidase (GUS) and green fluorescent protein (GFP) for resistance studies in bean (*Phaseolus vulgaris L.*) (Naderpour and Johansen 2011). *Tobacco etch virus* (TEV) and *Turnip mosaic virus* (TuMV) were recently tagged with Ros1 transcription factor for *in vivo* tracking in *Nicotiana tababum, Arabidopsis thaliana* and *Nicotiana benthaminana* (Bedoya et al. 2012). A GFP labelled PVY clone was already mentioned in the study by Hofius et al. (2007), however no data on the biological characteristics was shown.

In this study, a GFP labelled PVY strain N605 was constructed to create a tool for expanding the knowledge on the multiplication and movement of PVY in the host plants. The *gfp* gene was inserted between the potyviral NIb and CP genes, since this site has been described to yield very high transgene protein levels (Chen et al. 2007). However, the construction of PVY N605-GFP was not successful in the first attempt, denoting that production of fluorescently labelled PVY is still not a straightforward process. GFP was flanked with NIa protease recognition sites (PRS) in order to be excised from the polyprotein and released in the cytoplasm after translation. Before using the fluorescently tagged PVY for monitoring the movement in host plants he stability of the inserted *gfp* gene and its biological effect on the virus fitness were thoroughly characterised. To ensure higher stability, the PRS at the 5' of *gfp* gene was modified with the use of alternative codons to decrease the possibility of recombination due to sequence repetitions. First, it was shown that GFP tag did not impair PVY symptoms development on the host plant *N. tabacum*. Symptoms of comparable severity were shown on PVY N605-GFP and PVY N605-wt (control) inoculated plants in the different experiments done during the study (Figure 1). All PVY N605-GFP inoculated tobacco plants were systemically infected and GFP fluorescence was detected in all systemic leaves of all plants. Additionally, equivalent concentrations of PVY RNA were detected in lower leaves at the beginning of the infection (L1 and L2, 1-5 DPI) and in upper systemic leaves after 14 DPI indicating a comparable multiplication and long distance movement of nucleic acid between PVY N605-GFP and PVY N605-wt (Appendix B).

When used for localisation and spread studies, the stability of the labelled virus is another essential aspect. Since the potyviral genome is highly prone to recombination (Chare and Holmes 2006) the *gfp* gene could likely be deleted or recombined during the PVY N605-GFP infection cycle. To confirm that leaf regions with no GFP signal were not the result of the *gfp* gene deletion, a combination of qPCR assays specifically quantifying total PVY (UNI) (Kogovsek et al. 2008), *gfp* gene (PVY-*gfp*) (Joshi et al. 2008) and PVY N605-wt was used (results presented in Appendix I and Appendix J). The insert proved to be very stable in the PVY genome. Fluorescence was detected systemically in all inoculated plants in all passages, even up to 120 DPI. In PVY N605-GFP infected plants, a hypothetical excision of the *gfp* gene from the PVY N605-GFP genome would be reflected as a marked decrease in the *gfp* gene copy number (due to exclusion of the excised gene from the virus replication cycle and consequent degradation) and a simultaneous increase in the PVY N605-wt concentration. RT-qPCR analysis showed that background levels of PVY N605-wt genomes were present, however in less than half of the samples, in very low concentrations and these low concentrations did not increase either with the increased total PVY concentration or with the time after inoculation (Figure 3). Moreover, the concentrations of *gfp* gene were almost identical as total PVY concentration in all the PVY N605-GFP infected samples (Figure 2), regardless of the GFP signal being detected in the sample or not

(Appendix I). Taken altogether, we can conclude that the PVY N605-GFP was not outcompeted by the PVY N605-wt and is stably amplifying in host plants.

To our knowledge this is the first example of thorough characterisation of the stability of the inserted gene and biological properties of a fluorescently labelled plant virus in comparison to wild type. There are studies where the pathogenicity of viral infective clones was compared to the natural isolates (Gao et al. 2012; Lee et al. 2011), or where the symptoms of fluorescently labelled plant virus were evaluated (Masuta et al. 2000), but a comprehensive comparison of labelled and non-labelled viruses was not done. Stable expression of the *gfp* gene through several passages enabled the use of simple, efficient and inexpensive propagation of PVY N605-GFP with mechanical inoculation from plant sap, instead of bombardment with two ligated subclones, which yielded less than 5% infection. Fluorescently labelled PVY was used for observation of the virus spread in both inoculated and systemic leaves of Nt cv. Xanthii. On the inoculated leaves of Xanthii the PVY infection is difficult to detect with conventional molecular methods, since the detection of increased viral RNA or proteins levels is obscured by inoculum. The PVY N605-GFP proved to be a good PVY infection indicator, since the GFP signal could be detected undoubtedly on L1 already at 2 DPI (Figure 4) before any symptoms were observed. Similarly, on systemic leaves the GFP signal was detected already at 4-5 DPI, at least 2 days before any symptoms appeared. Following the inoculated leaf the PVY N605-GFP fluorescence was first detected in younger, developing systemically infected leaves, while fully developed L2 remained fluorescence free or the fluorescence was detected only around the base of the leaf (Figure 7, Appendix E). The presence of the RNA in the sections with no detectable GFP signal (Figure 5) could indicate that in the infected cell the viral RNA is first multiplied to high concentrations and then translocated to neighbouring cells before detectable amount of GFP and other viral proteins are produced. A lower sensitivity of the microscopy based fluorescence detection in comparison to qPCR could also explain this result.

Unloading of the PVY N605-GFP in the systemically infected "sink" leaves was also observed (Figure 6). PVY unloaded from class I, II and III veins as reported for PVX in N. benthamiana (Roberts et al. 1997). In this study high resolution microscopy images revealed unloading of PVY from class IV leaves as well in Nt cv. Xanthii (Figure 6). The lamina and the smaller veins were infected subsequently by cell-to-cell movement. In some cases class I and II veins were not completely fluorescent from the base till the edge of the leaf (Figure 5, C, D, E) indicating that PVY genomes have been relocated from inoculated leaf to the tip of the systemic leaf without producing detectable amount of GFP on the way, e.g. without translation of viral polyprotein. *In vivo* tracking enabled the estimation of the cell-to-cell movement rate of PVY in Nt cv. Xanthii (Figure 8, Table 1) expressed as number of cells infected per hour. The measured rate is accelerating with time after infection (cca 6.5 cell/h and 9 cells/h in first and second day, respectively). A possible explanation is that as the virus spreads, an increasing number of non infected neighbouring cells are available for infection. The observed rate actually denotes the increase in the number of cells actively producing viral polyprotein to detectable levels, i.e. the spread of metabolically active PVY (Forterre 2010). The rate of spread of viral RNA might be different. Nonetheless, to our knowledge this is the first study where an estimation of cell-to-cell spread was done.

The use of PVY N605-GFP in other *Nicotiana* and *Solanum* species and cultivars was investigated to assess the potential of fluorescently labelled PVY for other applications (Table 2).

The well established protocols for transient expression of fluorescent fusion proteins by agroinfiltration in *N. benthamiana* (Du et al. 2014) with the use of PVY N605-GFP (systemic infection already at 6 DPI) could enable detailed studies of plant protein expression and/or localisation in relation to PVY localisation with high spatial resolution.

We have detected the GFP signal on inoculated leaves of three cultivars of *S. tuberosum* i.e. Bintje, Bea and Desiree. The preliminary data on *S. tuberosum* suggests that the PVY N605-GFP cell-to-cell movement is slower than in Nt cv. Xanthii. At 6-8 DPI only small fluorescent foci were detected in comparison to Nt cv. Xanthii where majority of the veins and surrounding lamina are infected by 7

V procesu objavljanja. Rupar in sod., 2014

DPI. The pattern of spread in systemic leaves seemed different as well. The GFP signal on systemic leaves was detected as discrete spots and not continuously throughout the veins and lamina as in Xanthii. The tested cultivars have different susceptibility to the PVY; hence the use of PVY N605a-GFP on these cultivars would enable to study the effect of plant susceptibility on the rate of cell-to-cell and long distance movement of the PVY.

Other *Solanum* species (i.e. wild potato relatives) are used in our laboratory as a tool for functional analysis of genes involved in potato PVY interaction. Until now, the experiments involved the use of non-labelled viruses, laborious RNA isolations followed by RT-qPCR experiments to evaluate the extent of virus spread. Since the GFP signal was detected systemically in many wild potato relatives (Table 2), those species will enable the use of PVY N605-GFP together with functional genomics approaches (such as VIGS) to study plant–virus interactions. Studies of involvement of individual plant genes in the resistance to PVY infection would now be much faster, and non-invasive observations under the microscope would enable the use of less plant material in time course studies.

The PVY N605-GFP will serve to improve the study of plant-PVY interactions with transcriptomics and other "omics" approaches as well. It will enable accurate sampling of the fluorescent and non-fluorescent parts on same inoculated and systemic leaves to unveil the plant response/affect of the PVY on plant metabolism in greater detail.

As in the case of *Wheat streak mosaic virus* (Tatineni and French 2014), the PVY N605-GFP will also enable the search for molecular determinants in the PVY genome responsible for cell-to-cell and long distance movement, host specificity and possibly PTNRD symptom induction.

V procesu objavljanja. Rupar in sod., 2014

Materials and methods

Construction of the GFP labelled PVY N605

The GFP tagged PVY used in this study was derived from a bi-partite PVY N605 infectious clone system developed by Jakab et al. (1997). The system consists of two overlapping PVY sub-clones (5' end and 3' PVY N605 sub-clone) which are ligated into a full length PVY N605 clone before biolistic inoculation on *Nicotiana tabacum* cv. Clevelandii (3-4 leaf stage). The ligation of the bipartite system, biolistic inoculation and ELISA testing of the inoculated plants were done according to Tribodet et al. (2005).

The gfp gene (sGFP S65T) was inserted by fusion PCR and ligation between Nib and CP coding sequences (Chen et al. 2007) in the 3' PVY N605 sub-clone applying three different cloning strategies. Two of them, involved introduction of unique restriction sites between Nib and Cp genes, where the gfp gene would be i) linked to CP or, ii) excised out from polyprotein after translation. In the third attempt the gfp gene was inserted between Nib and CP using fusion PCR directly. The latter, which was the only one resulting in an infective GFP labelled PVY clone, is described in detail below and illustrated in the Appendix A.

The 5' end of the *gfp* gene was flanked by viral NIa protease recognition site (PRS), while 3' end was flanked by a modified PRS, where alternative codons were used to prevent recombination events due to sequence similarity. Upon translation, the GFP is excised from the polyprotein by NIa protease and released into the cytoplasm (adopted from Guo et al. 1998).

All PCR steps were carried out in an ABI GeneAmp PCR cycler using PfuUltra High-Fidelity DNA Polymerase (Agilent Technologies, Inc.). Fragments of appropriate length were excised and cleaned with GeneJETTM gel extraction kit (Thermo Scientific) with minor modifications.

Three PCR fragments were amplified in order to insert the *gfp* gene between NIb and CP. The schematic representation of cloning steps and primers used are listed Appendix A. PCR fragments 1 and 3 were amplified with primers pairs F1_F/R and F3_F/R, respectively, using 3' PVY N605 sub-clone as a matrix. PCR fragment 2 was amplified with F2_F/R primer pair from *gfp* containing plasmid pICPPV-GFP. NIa protease recognition site and fusion PCR hybridisation sites were added on the primer tails. Fragments were amplified in 50 µl reactions and the following cycling conditions: 3min 94°C; first 5 cycles: 1 min 94°C, 1 min 49°C, 1min 72°C; 35 cycles: 1 min 94°C, 1 min 52°C, 1min 72°C; 10 min 72°C. Fusion PCR was done in two steps. First, purified PCR fragments 1 and 2 were fused using F1_F an F2_R primer pair, fusion product (F1-2) was excised from the agarose gel and cleaned as described above. In the second step, the PCR fragment 3 was fused with fusion PCR product F1-2 using F1_F and F3_R primer pair. PCR conditions for the first fusion step were as described above except for annealing and elongation times were prolonged to 2 min. The second step of fusion PCR was done with single annealing step 52°C, 2 min, 15 cycles only and the primers were added to the reaction after cycle 5. Full fusion PCR product (2236 bp in length) was restricted by PflFI (NEB, UK) and BglII (Invitrogen, UK) restriction endonucleases sequentially, in 50 µl reactions according to manufacturer's protocol. After each restriction step the restriction reaction was cleaned with MinElute gel extraction kit (Qiagen); 150 µl of QG buffer and 50 µl of isopropanol were added to 50 µl reactions, the mixtures were loaded to MinElute columns. Cleaning was finalised following manufacturer's instructions. The 3' PVY N605 sub-clone was restricted in the same manner but with an additional phosphatase treatment step. One µl of Calf intestinal phosphatase (NEB, UK) was added to the reactions at the beginning and after 30 min of incubation. The fusion PCR product was ligated into opened, phosphatase treated 3' PVY N605 sub-clone with T4-DNA ligase (NEB, UK), insert to plasmid molecular ratio 3:1. Ligation mixture was composed according to manufacturer's instructions and incubated at 16°C overnight. Afterwards the ligase was inactivated (10 min, 65°C) and the ligation mixture dialysed on MF-Millipore disc (0.22 µm pore size, mixed cellulose esters) against ddH_2O for 60 min, before electroporation to electrocompetent *E.coli* DH5α. Transformed bacteria were plated on LB media supplemented with 200 µg/ml of ampiciline. Colonies were screened with colony PCR using F1_F/F3_R primer pair. The colonies with expected fragment size

(cca. 2,2 kb) were transferred to 5 ml liquid LB with 200 µg/ml of ampicilin and incubated overnight at 37°C on a shaker. The next day the plasmids were isolated with NucleoSpin® Plasmid (Macherey-Nagel), following instruction manual and sequenced with all 6 primers to check for PCR induced errors in the sequence. Only error free 3' PVY N605 sub-clone with inserted *gfp* gene was used to construct the full length PVY N605-GFP infective clone.

Plant material and Inoculation

Nicotiana tabacum cv. Xanthii and *Nicotiana benthamiana* were grown from seeds and inoculated at 3 to 4 leaf stage. All *Solanum* species and cultivars (Table 2) were grown in stem node tissue culture. Two weeks after node segmentation they were transferred to soil in growth chambers and grown as previously described by Baebler et al. (2009). Four weeks old plants were used for inoculation. Plants were grown at 24°C with a long-day (16-h) photoperiod of light (light intensity 4000 lm/m^2 and 60 - 70% relative humidity

Source of inoculums for PVY N605-GFP and PVY N605-wt were systemically infected leaves of *Nicotiana tabacum* cv. Xanthii at 12-16 DPI. Three lower leaves (L1-L3) were inoculated on all tested species and cultivars except for Nt cv. Xanthii where only one leaf (L1) was inoculated. Inoculums for all plant species except for *Solanum tuberosum* cultivars were prepared in 1:5 plant material to buffer ratio with 20mM phosphate buffer supplemented with 2% (w/w) of polyvinylpyrrolidone (average mol wt 10,000) (Sigma). For inoculation of *Solanum tuberosum* cultivars 1:3 plant to buffer ratio was used with 20 mM phosphate buffer supplemented with 12.5 mM sodium diethyldithiocarbamate (DIECA) (Sigma). Classical mechanical inoculation was done with carburundum (0.65 µm) (Sigma), cca. 200 µl of inoculums per leaf and washing with tap water after 10 min incubation.

RNA extraction

Samples (0.01-0.1 g fresh weight) were ground with TissueLyser (Qiagen) (3 min at 30/s) in 2 ml eppendorf tubes supplemented with 1x 5 mm stainless steel beads, 90 µl of Lysis Binding Solution concentrate and 10 µl of Plant RNA Isolation Aid RNA (MagMAX™-96 Total RNA Isolation Kit, Life technologies). After homogenisation, samples were centrifuged at 14,000 g, 5 min. RNA was extracted from 50 µl of clear homogenate with MagMAX™-96 Total RNA Isolation Kit and MagMAX™ Express Magnetic Particle Processor according to manufacturer's instructions specific for plant samples. A DNAse step was included in the isolation procedure.

qPCR quantification

To assess the stability of the *gfp* insert a combination of different real-time qPCR assays was used (schematically represented in Appendix A). To quantify total PVY gRNA content in host plants a universal PVY assay developed by Kogovsek et al. (2008) was used, in this manuscript denoted as "UNI". Quantification of *gfp* gene was done with the assay by Joshi et al. (2008), here denoted as "PVY-*gfp*". The forward primer sequence was modified to hybridise perfectly on the *gfp* sequence used in this study (5'-CCACATGAAGCAGCACGACTT –3' the modified nucleotide is underlined). To detect the PVY genomes that have lost the *gfp* gene we have developed an assay specifically detecting only the PVY N605-wt genome, denoted "PVY-wt". The forward primer is located in the Nib gene, reverse primer in CP gene and the TaqMan MGB probe is located partly on the natural PRS and partly on CP sequence. Probe was designed manually while primers were designed by Primer Express 3 software (ABI). The assay's specificity was tested *in silico* using BLAST algorithm and *in vitro* on healthy plant RNA extracts and PVY-GFP infected plant RNA extracts. The range of quantification and amplification efficiency of PVY-wt assay were evaluated according to Kogovsek et al. (2008) (Appendix F). A stable plant gene coding cytochrome oxidase (COX) was used for normalisation of different total RNA input in PCR reactions (Baebler et al. 2009; Weller et al. 2000).

All samples were analysed with all 4 assays; UNI, PVY-gfp, PVY-wt and COX (Appendix I, Appendix J). Theoretical copy number (TCN) of targets detected with each assay was determined using dilution series approach (Kogovsek et al. 2008), assuming that the Cq values at the end of linear range of each assay correspond 10 copies in the RT-qPCT reaction. Dilution series curve is shown in (Appendix F). To normalise for different total RNA imput in the qPCR reaction all determined copy numbers of UNI, PVY-gfp and PVY-wt were expressed as copies per 10^6 copies of COX gene in the same sample. The reason of choosing specifically 10^6 copies of COX for normalization is due to the presence of higher concentrations (at least 6 orders of magnitude higher) of COX in contrast to the other targets in majority of the samples.
To assure the quality of the qPCR analysis, appropriate controls were used (non template control, negative control of isolation and positive control of amplification).
Real time PCR quantifications were done in an ABI 7900 HT Fast instrument with AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems®). Cycling conditions were: reverse transcription 48°C, 10 min; denaturation 95°C, 10 min, 45 cycles: 95°C, 15 s; 60°C 1 min. The amplification curves for every sample were checked manually in SDS 2.3 software (ABI), manual threshold (0.1 for PVY-*gfp* and PVY-wt, 0.25 for COX and 0.15 for UNI) and automatic baseline were used to determine the Cq values.

Fluorescent Microscopy and Image Analysis

The samples of Nt cv. Xanthii and S. *tuberosum* were examined with a fully motorised Nikon Eclipse Ti inverted microscope equipped with an ANDOR Clara E high sensitivity black and white camera (ANDOR Technologies). Two fluorescent channels were used in image acquisitions; green (excitation 465-495 nm, emission 515-550 nm) and red (excitation 540/25 nm, emission 605/55 nm), both at 700 ms exposition time. Imaging was done with 40x magnification. Large leaf samples were imaged by taking individual images sequentially (5% overlap between images) in both fluorescent channels across the whole sample. The objective was focused in one focal plane defined manually for each sample. Up to 600 high resolution images were acquired per sample and stitched together in NIS Elements software (Nikon) to form a detailed image of the entire sample.
For visualisation purposes the "Look Up Table" (LUT) was adjusted in the NIS elements software for each channel separately, however the same adjustment was used for all images (Appendix G).
In wild *Solanum* relatives the GFP was visualized with a Leica TCS SP5 laser-scanning microscope mounted on a Leica DMI 6000 CS inverted microscope (Leica Microsystems, Germany) with a HC PL FLUOTAR 10.0x0.30 DRY objective. For excitation, the 488 nm line of an Argon laser was used. Fluorescence emissions with wavelengths of 505–520 nm and 590–680 nm were collected simultaneously through two channels. Images were processed and assembled using Leica LAS AF Lite software (Leica Microsystems, Germany).
To distinguish GFP signal from auto fluorescence artefacts, only the signals specific to green channel were considered as a genuine GFP signal. To exclude artefacts from the images taken on the PVY N605-GFP infected leaves, a range of control samples taken from healthy plants, mock inoculated plants (healthy Nt leaves as a source of inoculums) and PVY N605-wt inoculated plants were examined and imaged (Appendix H). The GFP specific fluorescent signal was present in the cytoplasm of the infected epidermal cells resulting in a "puzzle" pattern seen only in the green channel (Figure 4, i, ii and iii).
To correlate the RNA concentration with fluorescence, the average intensity was measured in separate sections of the imaged leaf. Measurements were done with FIJI software (Schindelin et al. 2012), using raw integrated density of a selected area.

In vivo tracking *of PVY N605-GFP*

Modification of the plant growing technique to enabled tracking the movement of PVY N605-GFP in the same plant at different DPI. Nt cv. Xanthii plants were grown in plastic falcon tubes as shown in Appendix D. Each day one half of inoculated (L1) and systemic leaves (L2, L3, L4) was carefully placed

between two objective glasses and the whole bottom side of the leaves was scanned to produce high resolution composite image as described above.
The images from *in vivo* tracking were used to estimate the rate of movement of PVY N605-GFP on the inoculated leaf. All measurements were done in NIS Element software (Nikon). Nine fluorescent foci were chosen, their diameter measured with "circular measurement tool" at 3, 4 and 5 DPI (exactly 27 h interval) and their area calculated. The area of the "average epidermal cell" was measured each day separately, to account for the growth rate of the cells. Three groups of 10 cells were chosen on three locations across the leaf (near the fluorescent foci) and their area was measured using a "freehand area measurement tool" (Figure 8, B). To estimate the number of cells in the fluorescent foci at each day, the total area of the foci was divided by the calculated average cell area in that particular day. The estimated no. of infected cells in each fluorescent foci at each day was used to calculate the rate of the movement of PVY expressed as no. of newly infected cells per hour.

Acknowledgements
We would like to thank to Prof. Dr. Richard G. F. Visser (Wageningen University) for providing collection of wild potato relatives and Dr. XYZ (INRA, Montpellier) for providing the pICPPV-GFP plasmid used for sub cloning the *gfp* gene. We would like to acknowledge Maja Križnik for her assistance with RNA isolation of the samples used in this study. The work has been supported by a Slovenian Research Agency through a PhD research to Matevž Rupar (Contract No. 1000-10-310196) and through bilateral SLO-FR bilateral project PROTEUS (BI-FR/10-11-Proteus).

583 *Literature cited*

584 Avesani, L., Marconi, G., Morandini, F., Albertini, E., Bruschetta, M., Bortesi, L., Pezzotti,
585 M., and Porceddu, A. 2007. Stability of Potato virus X expression vectors is related to
586 insert size: implications for replication models and risk assessment. Transgenic Res.
587 16:587–97

588 Baebler, S., Krecic-Stres, H., Rotter, A., Kogovsek, P., Cankar, K., Kok, E. J., Gruden, K.,
589 Kovac, M., Zel, J., Pompe-Novak, M., and Ravnikar, M. 2009. PVY(NTN) elicits a
590 diverse gene expression response in different potato genotypes in the first 12 h after
591 inoculation. Mol. Plant Pathol. 10:263–275

592 Baulcombe, D. C., Chapman, S., and Cruz, S. 1995. Jellyfish green fluorescent protein as a
593 reporter for virus infections. Plant J. 7:1045–1053

594 Bedoya, L. C., Martínez, F., Orzáez, D., and Daròs, J.-A. 2012. Visual Tracking of Plant
595 Virus Infection and Movement Using a Reporter MYB Transcription Factor That
596 Activates Anthocyanin Biosynthesis. Plant Physiol. 158:1130–8

597 Blanchard, A., Rolland, M., Lacroix, C., Kerlan, C., and Jacquot, E. 2008. Potato virus Y: a
598 century of evolution. Curr. Top. Virol. Vol. 7. :21–32

599 Chare, E. R., and Holmes, E. C. 2006. A phylogenetic survey of recombination frequency in
600 plant RNA viruses. Arch. Virol. 151:933–946

601 Chen, C.-C., Chen, T.-C., Raja, J. A. J., Chang, C.-A., Chen, L.-W., Lin, S.-S., and Yeh, S.-D.
602 2007. Effectiveness and stability of heterologous proteins expressed in plants by Turnip
603 mosaic virus vector at five different insertion sites. Virus Res. 130:210–27

604 Cohen, Y., Gisel, a, and Zambryski, P. C. 2000. Cell-to-cell and systemic movement of
605 recombinant green fluorescent protein-tagged turnip crinkle viruses. Virology. 273:258–
606 66

607 Cruz, S. S., Chapman, S., Roberts, A. G., Roberts, I. M., Prior, D. A. M., and Oparka, K. J.
608 1996. Assembly and movement of a plant virus carrying a green fluorescent protein
609 overcoat. Proc. Natl. Acad. Sci. U. S. A. 93:6286–90

610 Dietrich, C., and Maiss, E. 2003. Fluorescent labelling reveals spatial separation of potyvirus
611 populations in mixed infected Nicotiana benthamiana plants. J. Gen. Virol. 84:2871–
612 2876

613 Du, J., Rietman, H., and Vleeshouwers, V. G. A. A. 2014. Agroinfiltration and PVX
614 Agroinfection in Potato and Nicotiana benthamiana. J. Vis. Exp.

615 Forterre, P. 2010. Defining life: the virus viewpoint. Orig. Life Evol. Biosph. 40:151–60

616 Gao, R., Tian, Y.-P., Wang, J., Yin, X., Li, X.-D., and Valkonen, J. P. T. 2012. Construction
617 of an infectious cDNA clone and gene expression vector of Tobacco vein banding
618 mosaic virus (genus Potyvirus). Virus Res. 169:276–81

619 Ghanim, M., Brumin, M., and Popovski, S. 2009. A simple, rapid and inexpensive method for
620 localization of Tomato yellow leaf curl virus and Potato leafroll virus in plant and insect
621 vectors. J. Virol. Methods. 159:311–4

622 Gray, S. M., DeBoer, S. H., Lorenzen, J., Karasev, A. V., Whitworth, J., Nolte, P., Singh, R.
623 P., Boucher, A., and Xu, H. 2010. Potato virus Y: A significant and evolving threat to
624 potato crops in the United States and Canada. Plant Dis. 94:1384–1397

625 Guo, H. S., López-Moya, J. J., and García, J. a. 1998. Susceptibility to recombination
626 rearrangements of a chimeric plum pox potyvirus genome after insertion of a foreign
627 gene. Virus Res. 57:183–95

628 Halim, V. A., Hunger, A., Macioszek, V., Landgraf, P., Nürnberger, T., Scheel, D., and
629 Rosahl, S. 2004. The oligopeptide elicitor Pep-13 induces salicylic acid-dependent and -
630 independent defense reactions in potato. Physiol. Mol. Plant Pathol. 64:311–318

631 Hofius, D., Maier, A. T., Dietrich, C., Jungkunz, I., Börnke, F., Maiss, E., and Sonnewald, U.
632 2007. Capsid protein-mediated recruitment of host DnaJ-like proteins is required for
633 Potato virus Y infection in tobacco plants. J. Virol. 81:11870–80

634 Hren, M., Nikolić, P., Rotter, A., Blejec, A., Terrier, N., Ravnikar, M., Dermastia, M., and
635 Gruden, K. 2009. "Bois noir" phytoplasma induces significant reprogramming of the leaf
636 transcriptome in the field grown grapevine. BMC Genomics. 10:460

637 Jakab, G., Droz, E., Brigneti, G., Baulcombe, D., and Malnoë, P. 1997. Infectious in vivo and
638 in vitro transcripts from a full-length cDNA clone of PVY-N605, a Swiss necrotic isolate
639 of potato virus Y. J. Gen. Virol. 78 (Pt 12:3141–5

640 Joshi, M., Keith Pittman, H., Haisch, C., and Verbanac, K. 2008. Real-time PCR to determine
641 transgene copy number and to quantitate the biolocalization of adoptively transferred
642 cells from EGFP-transgenic mice. Biotechniques. 45:247–58

643 Kerlan, C. 2006. Potato virus Y. C. Descr. Plant Viruses. 414

644 King, A., Adams, M. J., Lefkowitz, E., and Carstens, E. B. 2011. *Virus Taxonomy: Ninth*
645 *Report of the International Committee on Taxonomy of Viruses*. Elsevier.

646 Kogovsek, P., Gow, L., Pompe-Novak, M., Gruden, K., Foster, G. D., Boonham, N., and
647 Ravnikar, M. 2008. Single-step RT real-time PCR for sensitive detection and
648 discrimination of Potato virus Y isolates. J. Virol. Methods. 149:1–11

649 Kogovšek, P., Kladnik, A., Mlakar, J., Tušek-Žnidarič, M., Dermastia, M., Ravnikar, M., and
650 Pompe-Novak, M. 2011. Distribution of Potato virus Y in Potato Plant Organs, Tissues
651 and Cells. Phytopathology. 101:1292–1300

652 Lansac, M., Eyquard, J. P., Salvador, B., Garcia, J. A., Le Gall, O., Decroocq, V., and
653 Schurdi-Levraud Escalettes, V. 2005. Application of GFP-tagged Plum pox virus to
654 study Prunus-PPV interactions at the whole plant and cellular levels. J. Virol. Methods.
655 129:125–33

V procesu objavljanja. Rupar in sod., 2014

656 Lee, M. Y., Song, Y. S., and Ryu, K. H. 2011. Development of infectious transcripts from
657 full-length and GFP-tagged cDNA clones of Pepper mottle virus and stable systemic
658 expression of GFP in tobacco and pepper. Virus Res. 155:487–94

659 Leisner, S. M., Turgeon, R., and Howell, S. H. 1992. Long Distance Movement of
660 Cauliflower Mosaic Virus in Infected Turnip plants. Mol Plant-Microbe. 5:41–47

661 Lucas, W. J. 2006. Plant viral movement proteins: agents for cell-to-cell trafficking of viral
662 genomes. Virology. 344:169–84

663 Mas, P., and Pallas, V. 1996. Long-distance movement of cherry leaf roll virus in infected
664 tobacco plants. J. Gen. Virol. 77:531–540

665 Masuta, C., Yamana, T., Tacahashi, Y., Uyeda, I., Sato, M., Ueda, S., and Matsumura, T.
666 2000. Development of clover yellow vein virus as an efficient, stable gene-expression
667 system for legume species. Plant J. 23:539–46

668 Mehle, N., Kovač, M., Petrovič, N., Novak, M. P., Baebler, Š., Stres, H. K., Gruden, K., and
669 Ravnikar, M. 2004. Spread of potato virus YNTN in potato cultivars (Solanum
670 tuberosum L.) with different levels of sensitivity. Physiol. Mol. Plant Pathol. 64:293–300

671 Naderpour, M., and Johansen, I. E. 2011. Visualization of resistance responses in Phaseolus
672 vulgaris using reporter tagged clones of Bean common mosaic virus. Virus Res. 159:1–8

673 Nass, P. H., Domier, L. L., Jakstys, B. P., and D'Arcy, C. J. 1998. In Situ Localization of
674 Barley Yellow Dwarf Virus-PAV 17-kDa Protein and Nucleic Acids in Oats.
675 Phytopathology. 88:1031–9

676 Oparka, K. J., Boevink, P., and Santa Cruz, S. 1996. Studying the movement of plant viruses
677 using green fluorescent protein. Trends Plant Sci. 1:412–418

678 Rajamäki, M.-L., Kelloniemi, J., Alminaite, A., Kekarainen, T., Rabenstein, F., and
679 Valkonen, J. P. T. 2005. A novel insertion site inside the potyvirus P1 cistron allows
680 expression of heterologous proteins and suggests some P1 functions. Virology. 342:88–
681 101

682 Rajamäki, M.-L., and Valkonen, J. P. T. 2003. Localization of a potyvirus and the viral
683 genome-linked protein in wild potato leaves at an early stage of systemic infection. Mol.
684 Plant. Microbe. Interact. 16:25–34

685 Rasheed, M. S., Selth, L. A., Koltunow, A. M. G., Randles, J. W., and Rezaian, M. A. 2006.
686 Single-stranded DNA of Tomato leaf curl virus accumulates in the cytoplasm of phloem
687 cells. Virology. 348:120–32

688 Roberts, A. G., Cruz, S. S., Roberts, I. M., Prior, D. A. M., Turgeon, R., and Oparka, K. J.
689 1997. Phloem Unloading in Sink Leaves of Nicotiana benthamiana : Comparison of a
690 Fluorescent Solute with a Fluorescent Virus. Plant Cell. 9:1381–1396

3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

Za uspešno omejevanje širjenja je potrebno hitro in občutljivo določanje virusa predvsem v semenskem materialu krompirja. Odstotek okušenih gomoljev namreč prispeva največji delež k širjenju virusa in k zmanjšanju obsega in kakovosti pridelka (Karasev in Gray, 2013). V študiji vpliva PVY na pridelek krompirja so namreč izračunali, da se za vsak odstotek okuženega semenskega materiala obseg pridelka zmanjša v povprečju za cca 130 kg/ha polj ne glede na občutljivost kultivarja (Nolte, 2013, osebna komunikacija). Kar pomeni, da če posejemo 10 % okuženih gomoljev, izgubimo cca 1,3 t/ha pridelka, pri tem pa je treba upoštevati še dodatno zmanjšane kakovosti preostanka pridelka v primeru okužbe z PVY^{NTN} različki. V primeru okužbe s PVY je problematično tudi določanje bolezenskih znamenj na polju in posledično odstranjevanje okuženih rastlin, saj nekateri kultivarji ne kažejo močnih bolezenskih znamenj, čeprav je koncentracija virusa v le teh enaka ali celo višjo kot v simptomatičnih kultivarjih (Hamm in sod., 2009). Prav zaradi različnega vpliva različnih PVY različkov na različne kultivarje je potrebna natančna, hitra in robustna diagnostična metoda za določanja in karakterizacijo različkov.

3.1.1 Primerjava metod za določanje PVY

V prvem delu te naloge smo najprej naredili pregled razvoja obstoječih načinov določanja in klasifikacije izolatov PVY, od bioloških testov in seroloških metod določanja do širokega nabora molekularnih metod na podlagi PCR in drugih. Klasifikacija PVY je precej kompleksna zaradi hitrega spreminjanja virusov in neenotnega pristopa k analizi različnih lastnosti (genetskih, seroloških, molekulskih) (Singh in sod., 2008). Največji problem predstavlja združitev ali sinhronizacija bioloških in molekularnih skupin, saj so znane le molekularne determinante bolezenskih znamenj na tobaku (Faurez in sod., 2012) ne pa tudi na krompirju, na katerem temelji genetska klasifikacija na različnih kultivarjih. Na podlagi do sedaj znanih dejstev je bilo predlagano dvojno poimenovanje različkov, ki obsega tako biološko skupino kot tudi molekularni značaj, npr. $PVY^{Z/NTN}$ izzove preobčutljivostjo reakcijo na kultivarjih krompirja, ki imajo hipotetični gen *Nz*, molekularno pa so rekombinantni med PVY^{O} in PVY^{N} s tremi ali štirimi mesti rekombinacije (Kehoe in Jones, 2011). Zadnji poskus združitve vseh različic karakterizacije je prikazan na sliki 2 in opisan v objavi Karasev in Gray (2013).

Uporaba testnih rastlin za klasifikacijo je edina metoda, ki lahko razvrsti vse izolate glede na njihov biološki vpliv na gostitelja (Kerlan in sod., 2011), ni pa primerna za testiranje velikega števila vzorcev, saj gojenje rastlin in opazovanje bolezenskih znamenj potrebuje ogromno prostora in časa. Še večji problem pa predstavlja dejstvo,

da je odziv rastlin odvisen od razmer, v katerih rastline rastejo. Metoda bi morala biti dobro medlaboratorijsko standardizirana (temperatura, svetloba, vlaga in uporabljeni kultivarji), da bi omogočala primerljive rezultate. Določanje proteina plašča PVY z monoklonskimi protitelesi (serološko določanje npr. z ELISA metodo), omogoča procesiranje večjega števila vzorcev ob znatno nižji ceni kot biološki testi. Največja slabost metode je pomanjkanje dobre korelacije med serološkimi in biološkimi lastnostmi, kar je najbolj očitno v primeru različkov PVY^{N-Wi} (Chrzanowska, 1991) $PVY^{N:O}$ (Gray in sod., 2010), ki serološko spadajo v skupino PVY^{O}, biološko pa v PVY^{N} ali PVY^{Z}, saj povzročajo nekroze žil pri tobaku, nekateri pa tudi PTNRD (slika 2) (Piche in sod., 2004; Schubert in sod., 2007). Problem pa se je poglobil z odkritjem novih izolatov z nekonvencionalnim serotipom, kot je npr. PVY^{NTN-NW} (O serotip, povzroča PTNRD in nekroze žil). Tovrstne izolate bi z ELISA testom razvrstili v skupino PVY^{O} in napačno označili kot manj agresivne (Chikh Ali in sod., 2010b). Najden je bil tudi izolat PVY^{O-05}, ki reagira tudi z nekaterimi monoklonskimi protitelesi proti PVY^{N} (Karasev in sod., 2011). Serološke metode tudi ne omogočajo ločevanja PVY^{N} in PVY^{NTN}.

Ker molekularne metode nudijo največjo občutljivost v smislu določanja prisotnosti nizkih koncentracij PVY (Kogovšek in sod., 2008) ter hkrati nudijo največjo ločljivost določanja različkov PVY, smo se osredotočili na natančnejšo analizo le teh. Molekularne metode le izjemoma zaznavajo biološko signifikantna zaporedja/mesta v genomu PVY. Večina jih bazira na metodi PCR z nukleotidnimi začetniki, ki se hibridizirajo v odsekih, značilnih za PVY^{O}, PVY^{N} ali njune rekombinante (Boonham in sod., 2002b; Chikh Ali in sod., 2010a; Nie in Singh, 2002; Rigotti in Gugerli, 2007). Tehnika PCR v realnem času (qPCR) je še dodatno izboljšala občutljivost ter omogočila kvantifikacijo posameznih PVY različkov (Agindotan in sod., 2007; Balme-Sinibaldi in sod., 2006; Kogovšek in sod., 2008). Odkritje molekularnih determinant PVY, direktno povezanih s povzročanjem nekroz žil na tobaku (Tribodet in sod., 2005), je omogočilo razvoj metode, ki poleg genetskih označevalcev specifično zaznava tudi polimorfizme posameznih nukleotidov, odgovorne za povzročanje nekroz žil, SNaPshot (Rolland in sod., 2008). Znanje o molekulskih determinantah nekroz žil na tobaku je bilo pred kratkim razširjeno z dodatnimi determinantami (Faurez in sod., 2012).

V raziskavi smo izbrali dve molekularni metodi določanja in klasifikacije PVY, enokoračni RT-qPCR (Kogovšek in sod., 2008) in SNaPshot (Rolland in sod., 2008) ter analizirali njuno delovanje s poudarkom na učinkovitem in natančnem zaznavanju PVY^{NTN} različkov (Rupar in sod., 2013a). V študiji smo uporabili slovenske izolate PVY, ki smo jih najprej okarakterizirali z biološkim testom na rastlinah krompirja (*Solanum tuberosum* cv. igor) in tobaka (*Nicotiana tabacum* cv. xanthii). Kultivar krompirja igor je služil za določanje, ali izolati povzročajo PTNRD, saj je zelo občutljiva sorta. Spričo tega dejstva je tudi popolnoma izginil iz pridelave po prihodu PVY^{NTN} različkov na območje Slovenije, čeprav je igor slovenska avtohtona sorta

krompirja (Kus, 1995). Vsi izolati, testirani v tej nalogi, so povzročali tako PTNRD kot tudi nekroze žil na tobaku. Izolate smo nato testirali še s serološkim ELISA testom z obema izbranima molekularnima metodama; nekatere izolate pa smo še dodatno preverili in analizirali z določevanjem nukleotidnega zaporedja. Obe izbrani metodi sta detektirali vse izolate, vendar pa sta se razlikovali prav v zanesljivem določanju PVY^{NTN} različkov. Enokoračni RT-qPCR je pravilno razvrstil vse PVY^{NTN} različke, medtem ko je SNaPshot nekatere razvrstil med PVYN namesto PVY^{NTN}. Vzrok smo natančneje preučili s pomočjo določanja nukleotidnih zaporedij in ugotovili, da metodi zaznavata različne molekulske označevalce (polimorfizme posameznih nukleotidov (SNP), značilne za PVY^{N} in PVY^{O}. Tako enokoračni RT-qPCR zaznava SNP mesta, ki so bolj značilna za rekombinacijo v genomu PVY in s tem za PTNRD, kot tista, ki jih zaznava SNaPshot. Dejstvo, da metoda SNaPshot zaznava le po eno nukleotidno mesto v določeni regiji genoma PVY naenkrat, kar pomeni, da je tudi bolj občutljiva na točkovne mutacije kot RT-qPCR, katerega sonda naenkrat pokrije več značilnih SNP mest. SNaPshot pa ima tudi svoje prednosti, saj poleg zaznavanja bioloških determinant tudi bolj zanesljivo zazna mešane okužbe, zazna pa tudi različke tipa PVY^{N-Wi}, katerih RT-qPCR ne loči od različkov PVY^{O}. Po drugi strani pa RT-qPCR omogoča poleg določanja in karakterizacije tudi natančno kvantifikacijo ter s tem uporabo tako v diagnostiki kot tudi za raziskave. Po natančni analizi obeh metod smo nato predlagali tudi izboljšave v smeri bolj zanesljivega določanja in karakterizacije (Rupar in sod., 2013a).

Do sedaj je bilo znano le eno zaporedje slovenskega izolata PVY (Barker in sod., 2009), in sicer iz skupine PVY^{NTN}, zato smo določili delna nukleotidna zaporedja šestim novim izolatom (cca 60-75 % celotnega genoma). Štirje izolati so bili iz skupine PVY^{NTN}, ki v Sloveniji prevladuje od 90ih let prejšnjega stoletja dalje (Kus, 1995), določili pa smo tudi zaporedja slovenskih izolatov iz skupin PVY^{O} in PVY^{N-Wi}. Zbrana zaporedja smo uporabili v širši študiji evolucije gena CP PVY (Cuevas in sod., 2012a). Z molekulskimi, biološkimi in serološkimi metodami smo analizirali skupno 24 izolatov (Rupar in sod., 2013a). S tem smo ocenili prisotnost različnih skupin različkov v Sloveniji. Čeprav bi za dobro oceno bilo potrebno testirati večje število vzorcev, lahko že na manjšem številu vzorcev opazimo, da v Sloveniji močno prevladujejo različki iz skupine PVY^{NTN}, kar sovpada s podatki iz Nemčije in Francije (Blanchard in sod., 2008) ter najnovejšimi podatki iz Združenih držav Amerike (Karasev in Gray, 2013).

3.1.2 Virus se spreminja in adaptira: evolucija gena za protein plašča PVY (CP)

Zaradi velike pomembnosti CP proteina smo preučili njegovo evolucijo. Z uporabo bayesianskih statističnih orodij smo analizirali 212 nerekominantnih CP zaporedij in skušali poiskati aminokislinska mesta (kodone), izpostavljenim pozitivnim in negativnim evolucijskim pritiskom, ter mesta, ki se hkrati spreminjajo (kovarianca). Obliko filogenetskega drevesa vseh zbranih zaporedij gena CP smo pojasniti tudi preko

i) geografskega izvora izolatov, ii) gostiteljske rastline, na kateri so bil najdeni in iii) klasifikacijske skupine PVY, kateri pripadajo (Cuevas in sod., 2012a). Vsi izolati so se na filogenetskem drevesu razporedili v tri najbolj tipične skupine (PVY^{O}, PVY^{N}, $PVY^{C)}$, čeprav razmejitev med PVY^{O} in PVY^{C} ni bila dobro podprta. Največ izolatov je pripadalo PVY^{O} skupini. Podpora razmejitvam znotraj PVY^{O} skupine je bila globalno slaba, kar nakazuje na genetsko homogeno skupino z nedavnim nastankom in minimalnim vplivom selekcije. Edine dobro podprte veje v podskupini PVY^{O} so bile tiste, v katerih so bili izolati s skupnim geografskim izvorom. Podoben trend je bil opažen tudi v skupini PVY^{N}, kjer so se izolati razvrstili v dve glavni podskupini evropskih in severnoameriških izolatov. Signifikanten vpliv na obliko filogenetskega drevesa smo lahko pripisali tudi gostiteljski rastlini, na kateri je bil izolat najden. Opazili smo tri podskupine v našem drevesu, ki so pripadale izolatom iz krompirja, tobaka in paprike. Drugim gostiteljem nismo mogli pripisati večjega vpliva zaradi premajhnega števila vzorcev. Večina vzorcev, ki niso bili izolati iz krompirja, je hkrati pripadalo tudi PVY^{C} podskupini, kar nakazuje vpliv gostitelja na adaptacijo virusa. Podobno so poročali tudi že Schubert in sod. (2007). Možno je tudi, da so agresivnejši rekombinantni različki, kot npr. PVY^{NTN}, izpodrinili PVY^{C} iz krompirja. Obstajajo namreč poročila o tem, da se PVY^{NTN} bolje prenaša z listnimi ušmi, s PVY^{NTN} okužene rastline imajo manj simptomov na listih in več okuženih gomoljev ter se tako hitreje širijo v naravi (Karasev in Gray, 2013). Kljub temu nismo našli aminokislinskega mesta, ki bi bilo značilno za določenega gostitelja. Za večino kodonov v CP se je izkazalo, da so evolucijsko nevtralni ali pa izpostavljeni vplivom negativnega pritiska, t.i. purifikacijske selekcije v vseh gostiteljskih rastlinah. To nakazuje na dobro prilagojenost PVY na trenutne gostitelje in daljšo skupno evolucijo. Uporabili smo več različnih metod določanja kodonov, izpostavljenim pozitivnim selekcijskim pritiskom (FEL, IFEL, MEME) (Cuevas in sod., 2012b), in našli le eno mesto, ki je bilo skupno vsem trem metodam, kodon 1. Našli smo tudi nekaj mest, na katerih je v določeni skupini (PVY^{N}, PVY^{O}, PVY^{C}) prevladovala specifična a.k. Za posamezen različek značilna dominantna aminokislina pa je bila vedno najdena tudi pri drugih skupinah različkov, vendar s precej manjšo frekvenco. Še najbolj primerni sta a.k. 187, na podlagi katere lahko dokaj dobro ločimo PVY^{N} od PVY^{O} in PVY^{C}, ne pa tudi med PVY^{O} in PVY^{C} ter a.k. 193, ki zanesljivo ločuje med PVY^{N} in PVY^{O}, vendar se a.k., značilne za omenjene skupine, pojavljajo tudi v PVY^{C} skupini.

S to študijo smo želeli ugotoviti tudi, ali v proteinu CP obstajajo mesta, ki so značilna za i) geografsko območje, od koder izolat prihaja, ii) gostitelja, iz katerega prihaja. Na podlagi teh podatkov bi lahko sledili izvoru izolatov in širjenju na nova področja. Te informacije lahko uporabimo za epidemiološke študije in pri načrtovanju ukrepov za omejevanje širjenja PVY. Študija je pokazala, da v genu CP ni posameznih popolnoma zanesljivih molekulskih determinant niti za klasifikacijo različkov niti značilnih za posameznega gostitelja ali geografsko poreklo. To lahko pomeni, da evolucijske sile, ki oblikujejo CP, delujejo neodvisno od gostitelja in geografskega porekla, kar precej oteži

sledenje širjenja različkov. Druga razlaga, zakaj nismo našli zanesljivih determinant, je lahko tudi, da ne poznamo natančno, koliko časa je bil nek izolat na področju/na gostiteljski rastlini, na kateri je bil najden. Obstaja možnost, da je bil izolat prenesen na novo območje/gostitelja pred kratkim, kar bi pomenilo, da ima še vedno determinante, značilne za prejšnje geografsko področje/gostitelja. Edini najdeni kodon, izpostavljen pozitivni selekciji, pojasnjuje tudi rezultate prejšnje študije, kjer smo ugotovili, da metoda SNaPshot manj natančno zaznava PVY^{NTN} različke. SNaPshot namreč cilja prav prvi nukleotid v prvem kodonu CP gena, za katerega sedaj vemo, da se v populaciji diverzificira in je zato manj primeren za zanesljivo uporabo v diagnostiki.

3.1.3 Kako virusne delce osamiti iz kompleksnega vzorca?

Specifična in občutljiva monoklonska protitelesa so ključna za dobro serološko določanje ter klasifikacijo PVY. Za pripravo monoklonskih protiteles (Mab) proti novonastajajočim izolatom PVY je najprej priprava raztopine čistih virusnih delcev, ki jo uporabimo v koraku imunizacije (Ohshima in sod., 1990; Sanz in sod., 1990). Raztopino virusnih delcev lahko nato uporabimo za evalvacijo protiteles. Z metodo površinske plazmonske resonance (SPR) lahko namreč opazujemo specifičnost interakcij med virusnimi delci različnih izolatov PVY in Mab. Merimo lahko afiniteto vezave Mab na virusne delce, opazujemo pa lahko tudi, ali različna Mab zaznavajo iste, prekrivajoče ali popolnoma neodvisne epitope (Gutiérrez-Aguirre in sod., 2014). S tovrstnimi informacijami lahko nato pripravimo mešanico Mab, ki z visoko afiniteto zaznajo različne epitope. V primeru, da se en izmed tarčnih epitopov spremeni zaradi hitre virusne evolucije lahko virus zaznamo z ostalimi monoklonskimi protitelesi v mešanici. Tako lahko bolj zanesljivo zaznamo vse različke PVY.

Zaradi njihove uspešne in enostavne uporabe za čiščenje bioloških makromolekul smo v tretjem delu te doktorske naloge CIM monolitne nosilce implementirali v razvoju hitre in enostavne metode za pridobivanje čistih delcev PVY (Rupar in sod., 2013b). Preizkusili smo anionske kolone z različno kemijsko sestavo vezavne površine (QA in DEAE), različne puferske sisteme za optimalno vezavo virusnih delcev na kolono. Nato smo optimizirali še postopke čiščenja pred uporabo kolone in postopke spiranja virusnih delcev iz kolone. Vse korake optimizacije smo analizirali z več različnimi metodami, na podlagi katerih smo nato vodili postopke optimizacije. Z metodo enokoračnega RT-qPCR smo spremljali tako koncentracijo virusne RNA kot tudi prisotnost rastlinskih nukleinskih kislin. S tem smo lahko spremljali izkoristke kromatografske ločbe ter postopkov predčiščenja glede na vsebnost PVY v začetnem materialu. Opazovali pa smo lahko tudi prisotnost neželenih rastlinskih nukleinskih kislin v frakcijah očiščenega virusa PVY. Čistost virusa v posameznih korakih smo spremljali z metodo SDS-PAGE, kjer smo prisotnost PVY potrjevali s prenosom na membrano in določanjem s poliklonskimi protitelesi proti PVY i.e. metoda »Western Blot«. Zaradi filamentozne oblike in velike fleksibilnosti delcev bi se le ti lahko v postopku čiščenja poškodovali,

česar ne moremo opaziti niti z RT-qPCR niti z SDS-PAGE metodo. V ta namen smo obliko in integriteto delcev v posameznih korakih opazovali z metodo presevne elektronske mikroskopije. Določili smo tudi vpliv čiščenja na infektivnost PVY. Inokulirali smo rastline *Nicotiana tabacum* cv. xanthii in opazovali razvoj bolezenskih znamenj na rastlinah. Kombinacija kvantitativnih in kvalitativnih metod za opazovanje prisotnosti PVY v posameznih korakih je ključna, saj je v rastlini poleg virusnih delcev prisotno tudi veliko proste virusne RNA in proteinov CP, ki niso del virusnega delca. Z uporabo npr. RT-qPCR metode ali SDS PAGE metode posamično ne bi mogli zanesljivo spremljati prisotnosti virusnih delcev skozi korake izolacije. Spremljanje infektivnosti in posledično optimizacije čiščenja k večji infektivnosti je v primeru rastlinskih virusov zelo težavno in dolgotrajno, saj nimamo na voljo metod, kot je npr. analiza pojavljanja plakov pri živalskih virusih (Gerster in sod., 2013) ali bakteriofagih (Monjezi in sod., 2010; Smrekar in sod., 2008). Zato je bilo potrebno kombinirati presevno elektronsko mikroskopijo, s katero smo potrdili, da so delci dovolj veliki in večinoma nepoškodovani, z inokulacijo na rastlinah.

Izkoristki CIM čiščenja so bili v tej študiji nižji kot v primeru čiščenja virusa ToMV (Kramberger in sod., 2007), koncentriranja rotavirusov (Gutiérrez-Aguirre in sod., 2009) ali čiščenja bakteriofagov (Smrekar in sod., 2008). To dejstvo lahko deloma pojasnimo z načinom kvantifikacije virusa, kompleksnostjo izhodnega materiala in obliko virusa. V tej študiji smo za to uporabili za kvantifikacijo metodo RT-qPCR, ki zazna poleg RNA v delcih tudi prosto virusno RNA v začetnem vzorcu. V postopku čiščenja na CIM koloni eluiramo le virusne delce in kvantificiramo RNA, ki je v njih. Prosta RNA se namreč veže na kolono mnogo močneje od delcev in se eluira kasneje. S tem podcenimo dejanski izkoristek izolacije delcev. V primeru izolacije ToMV (Kramberger in sod., 2007) je bil izkoristek določen preko semikvantitativnega ELISA testa, ki je veliko manj natančen kot RT-qPCR. Pri koncentriranju rotavirusov iz pitne in rečne vode (Gutiérrez-Aguirre in sod., 2009) so izkoristke določali enako kot v tej študiji, vendar pa je bil začetni vzorec veliko manj kompleksen, virusni delci pa veliko manjši in bolj primerne oblike (kroglasti) za čiščenje na kromatografski koloni, posledično tudi izkoristki okoli 100 %. Dolg, gibljiv filamentozni delec PVY se lažje veže na druge delce, prisotne v homogenatu, se hitreje poškoduje na koloni, zato je njegova izolacija težavnejša in z nižjim izkoristkom. Kljub vsem oviram je postopek izolacije PVY s CIM monoliti primerljiv s klasičnim postopkom izolacije PVY (Leiser in Richter, 1978), tako v smislu čistosti kot tudi izkoristka.

Čiščenje s CIM monoliti skrajša postopek iz štirih na manj kot dva dni brez uporabe ultracentrifugiranja v gradientu saharoze in cezijevega klorida. V primeru, da potrebujemo večjo količino čiste virusne suspenzije, lastnosti CIM monolitov omogočajo enostavno povečanje obsega čiščenja v enakem času brez spreminjanja pogojev čiščenja. V postopku čiščenja s CIM monoliti odstranimo tudi 99,99 % vseh prostih nukleinskih kislin, o čemer so poročali tudi v primeru izolacije ToMV

(Kramberger in sod., 2007) in virusom podobnih delcev (VLP) (Urbas in sod., 2011). Odstranjevanje prostih nukleinskih kislin s pridom uporabimo v pripravi knjižnic virusnih nukleinskih kislin virusov za sekvenciranje nove generacije (NGS). Nukleinske kisline v enkapsidiranih virusnih delcih lahko predstavljajo le nekaj odstotkov vseh nukleinskih kislin, ki jih izoliramo iz rastline, zato večina odčitkov NGS pripada gostiteljski rastlini. Z odstranitvijo nukleinskih kislin gostitelja izboljšamo verjetnost detektiranja celotnih genomov enkapsidiranih virusov, poenostavimo pa tudi obdelavo podatkov (Steyer in sod., 2013).

3.1.4 Kako slediti virusu v gostiteljski rastlini?

Spremljanje premikanja virusa v gostiteljskih rastlinah nam omogoča raziskovanje interakcij med gostiteljem in virusom. Omogoča nam funkcionalno analizo tako virusnih (Fedorkin in sod., 2000; Lansac in sod., 2005; Lucas, 2006; Oparka in sod., 1996) kot tudi rastlinskih proteinov (Hofius in sod., 2007; Vijayapalani in sod., 2012; Wei in sod., 2010), udeleženih v procesu širjenja virusne infekcije v gostitelju. Prav tako lahko pomaga osvetliti mehanizme prepoznave, signalizacije in rastlinske odpornosti na viruse (Carr in sod., 2010)

Lokalizacijo in premikanje virusa PVY v gostiteljskih rastlinah smo do sedaj lahko raziskovali s kombiniranjem naslednjih metod: i) opazovanje pojavljanja bolezenskih znamenj (Mehle in sod., 2004), ii) določanje prisotnosti virusnih proteinov s tehnikami imunolokalizacije s svetlobno in elektronsko mikroskopijo ter z ELISA testom (Kogovšek in sod., 2011; Mehle in sod., 2004; Rajamäki in Valkonen, 2003) iii) ter z določanjem in kvantifikacijo virusnih nukleinskih kislin z metodo RT-qPCR (Kogovšek in sod., 2011). Metode zaznajo le posamezne virusne elemente (proteine, nukleinske kisline), ne pa metabolno aktivnega virusa ali z drugo besedo celic, kjer se virus aktivno namnožuje. Dodatna pomanjkljivost večine naštetih metod (razen opazovanja bol. znamenj) je tudi ta, da omogočajo le *ex-vivo* opazovanje s predhodnim vzorčenjem. Ker z vzorčenjem rastline poškodujemo, zahtevajo te metode uporabo večjega števila rastlin pri poskusih, kjer spremljamo širjenje virusa po rastlini skozi čas. Za izboljšanje študij širjenja PVY v gostiteljskih rastlinah in interakcij med virusom in rastlino zato potrebujemo orodje za *in vivo* opazovanje širjenja PVY.

V zadnjem delu doktorske naloge smo s pomočjo infektivnih klonov PVY N605 izolata (Jakab in sod., 1997) skonstruirali z zelenim fluorescentnim proteinom označen PVY (PVY N605-GFP). Natančno smo analizirali njegove biološke parametre in stabilnost ter ga uporabili za *in vivo* sledenje širjenju virusa in za prvo oceno hitrosti širjenja med celicami.

Gen za protein GFP smo vstavili v genom PVY med gena, ki kodirata proteina NIb in CP. Na N- in C-terminalnem koncu smo ga obdali s prepoznavnima mestoma za proteinazo NIa, ki GFP po translaciji odcepi iz poliproteina. Fluorescenco GFP zato

zaznamo v citoplazmi celic, kjer se virusni proteini aktivno sintetizirajo. Da lahko PVY N605-GFP uporabljamo kot orodje za opazovanje širjenja PVY med celicami, moramo najprej potrditi njegovo biološko primerljivost z neoznačenim PVY N605 izolatom. Potrdili smo, da PVY N605-GFP ob enakem času po inokulaciji povzroča enako močna bolezenska znamenja kot neoznačen PVY N605. S tehniko RT-qPCR (Kogovšek in sod., 2008) smo potrdili, da oba dosegata primerljivo koncentracijo virusnih nukleinskih kislin v sistemsko okuženih listih. Fluorescentni označevalec torej ne vpliva na fitnes PVY N605. Tekom pomnoževanja virusnega genoma v gostitelju nastaja veliko število tako mutacij kot tudi rekombinacij (Chare in Holmes, 2006; Guo in sod., 1998; Regoes in sod., 2012; Routh in sod., 2012). Vsak neesencialen gen, ki predstavlja za PVY dodatno genetsko breme, se zato lahko hitro odstrani iz genoma (Seaberg in sod., 2012). Za uspešno sledenje fluorescentno označenega virusa je stabilnost vstavljenega fluorescentnega gena ključna. V primeru, da se gen izreže ali poškoduje, namreč ne moremo več zanesljivo zaznati lokalizacije virusa, saj ne vemo, ali deli rastline brez GFP signala označujejo mesta, kjer se virus ne namnožuje, ali mesta, kjer je izgubil gen za GFP. Stabilnost gena za GFP protein v genomu PVY N605 smo določili s kombinacijo treh specifičnih RTq-PCR testov: i) kvantificirali smo količino gena za GFP (Wang in sod., 2004), ii) količino vseh genomov PVY (Kogovšek in sod., 2008) ter iii) specifično kvantificirali količino genomov z izrezanim genom za GFP. Ugotovili smo, da sta koncentracija vseh virusnih genomov in koncentracij gena za GFP skoraj enaki v vseh vzorcih. Manjša odstopanja od razmerja 1:1 so bila opažena v obeh smereh (več GFP kot vseh virusnih genomov in obratno), kar lahko pripišemo napaki meritve. Rezultati kažejo na to, da se GFP pomnožuje stabilno in ekvimolarno skupaj z virusnim genomom. V skoraj polovici od 164 analiziranih vzorcev nismo našli genomov brez gena za GFP. V vzorcih, kjer pa smo take genome zaznali, le ti niso presegli 0,05 % celotne populacije PVY genomov. Še več, njihova koncentracija se ni povečevala tekom trajanja okužbe, temveč so se vzorci, kjer smo zaznali PVY N605 brez GFP gena, pojavljali naključno v vseh testiranih dnevih po inokulaciji. Spričo hitre virusne evolucije torej nastane PVY N605 brez gena za GFP, vendar pa le ti ne izpodrinejo PVY N605-GFP v *Nicotiana tabacum* cv. xanthii. Posebej smo namreč analizirali tudi mesta brez fluorescence GFP, ki so se nahajala v bližini flourescentnih območij. Tudi v teh delih smo dobili razmerje med genom za protein GFP in vsemi virusnimi genomi 1:1, zato domnevamo, da se gen za GFP ni izrezal, temveč protein še ni prisoten v zadostni koncentraciji za detekcijo z mikroskopsko metodo, medtem ko se je RNA že namnožila do stopnje, ko jo lahko zaznamo z RT-qPCR. Tovrstna natančna analiza biološke primerljivosti in stabilnosti fluorescentno označenega virusa do sedaj še ni bila izvedena.

Dobro okarakteriziran PVY N605-GFP smo nato uporabili za sledenje PVY v rastlinah *Nicotiana tabacum* cv. xanthii. Fluorescenco smo opazovali s pomočjo avtomatiziranega fluorescentnega mikroskopa, s katerim smo zaporedoma zajeli več sto slik visoke resolucije pri 40x povečavi in jih sestavili v detajlne slike fluorescence velikih

delov lista ali celo celotnega lista naenkrat. Tako smo najprej opazovali potek širjenja PVY med celicami na inokuliranih listih, kjer smo drobna območja (nekaj 10 celic) z GFP fluorescenco označenega virusa zaznali že po dveh do treh dneh po inokuolaciji. Kot smo predpostavljali, smo prva bolezenska znamenja zaznali šele tri do štiri dni potem, ko smo zaznali širjenje PVY preko fluorescence. Za sledenje virusa *in vivo* smo prilagodili tehniko gojenja rastlin tako, da smo lahko inokulirane in sistemsko okužene liste brez poškodb vsak dan pregledali. Tako smo lahko spremljali širjenje virusa med celicami inokuliranega lista ter sistemsko širjenje po rastlini. Pri inokulaciji enega lista na rastlino smo opazili trend, ki kaže na to, da se virus hitreje razširi v višje liste nad inokuliranim kot v list točno nad inokuliranim. Fluorescenco smo zaznali v višjih sistemsko okuženih listih že po 4 dneh po inokulaciji, najprej v žilah in njihovi okolici, nato pa po celem listu. V listu točno nad inokuliranim smo fluorescenco zaznali le v nekaj primerih in še to pri bazi lista. Ta opažanja lahko razložimo s tokom fotosintetskih asimilatov iz razvitih v razvijajoče se liste (»source to sink flow«), katerim se virus sistemsko širi po rastlini (Roberts in sod., 1997). Tok asimilatov in s tem virusa je najmočnejši iz razvitega inokuliranega lista proti mladim višjim listom, ki se zato najhitreje okužijo. Listi se razvijejo iz porabnikov asimilantov v proizvajalce v bazipetalni smeri, najprej vrh, nato sredina in baza lista (Meng in sod., 2001). Ob času inokulacije je bil prvi list nad inokuliranim deloma ali pa že v celoti razvit, zato smo zaznali fluorescenco GFP označenega virusa le takrat, ko je bila baza lista še v fazi porabnika fotosintetskih asimilatov, s katerimi se je virus tja razširil. Zajeli smo slike istih mest okužbe na inokuliranih listih ob različnih dnevih po okužbi. Tako smo lahko določili hitrost širjenja PVY med celicami v tobaku. Opazili smo, da se virus širi pospešeno, saj je drugi dan okužil več novih celic (v povprečju 9 celic na uro) kot prvi dan (v povprečju 6,5 celic na uro) opazovanja. Tovrsten trend smo opazili pri vseh opazovanih mestih okužbe. V literaturi do sedaj še ni bilo natančnega podatka o tem, kako hitro se PVY širi med celicami.

Poleg tobaka smo s PVY N605-GFP uspešno okužili tudi štiri kultivarje krompirja (*Solanum tuberosum* L.), désireé, NagG-désireé, bea in bintje. Preliminarni podatki kažejo na to, da se PVY širi v krompirju počasneje in tudi z drugačnim vzorcem kot v tobaku. V sistemsko okuženih listih smo fluorescenco GFP zaznali med 18-22 DPI, medtem ko smo jo pri tobaku že pri 5 DPI. V tobaku se je fluorescenca GFP enakomerno širila iz vseh žil preko celotnega lista, medtem ko smo na sistemsko okuženih listih kultivarjev krompirja zaznali le manjša med seboj ločena območja z GFP fluorescenco. Testirali smo tudi dovzetnost divjih sorodnikov krompirja na okužbo s PVY N605-GFP. Fluorescenco GFP smo zaznali na inokuliranih listih vseh desetih testiranih vrst, v vrstah *Solanum mochiquense, Solanum papit*a in *Solanum venturii* pa tudi sistemsko. Divji sorodniki omogočajo lažjo prehodno transformacijo z *Agrobacterium tumefaciens* ter so genetsko sorodni kultiviranemu krompirju, zato jih uporabljamo kot nadomestni model za študij interakcij med PVY in krompirjem.

Zanesljivo in natančno orodje za *in vivo* lokalizacijo PVY bo pripomoglo k študijam funkcionalne analize rastlinskih in virusnih genov, udeleženih pri okužbi in premikanju znotraj rastline. Fluorescentno označen virus je uporaben tudi pri študijah transkriptoma okuženih rastlin. Brez poznavanja lokacije PVY lahko slepo vzorčimo dele rastlin, ki so okuženi, skupaj s tistimi, ki niso, in tako nevede »razredčimo« okužen material. S tem zabrišemo razlike v izraženju genov med zdravimi in okuženimi rastlinami. PVY N605-GFP omogoča ločeno vzorčenje delov rastline, kjer se virus aktivno pomnožuje, od tistih, kjer metabolno aktiven virus še ni prisoten. S tem povečamo občutljivost zaznavanja razlik v izražanju genov, ki so udeleženi pri obrambi rastline proti PVY.

3.2 SKLEPI

V nalogi smo prikazali različne načine, s katerimi lahko določamo in razvrščamo izolate PVY v skupine različkov. Natančneje, primerjali smo zanesljivo določevanje različkov, ki povzročajo PTNRD t.i. PVY^{NTN} z dvema najmodernejšima molekularnima metodama, enokoračni RT-qPCR (Kogovšek in sod., 2008) in SNaPshot (Rolland in sod., 2008). RT-qPCR se je izkazal za bolj zanesljivo metodo pri določanju PVY^{NTN} različkov, vendar pa z njo ni mogoče razločevati nekaterih drugih skupin. SNaPshot je razlikoval med vsemi skupinami različkov, kljub vsemu pa je nekatere izolate pripisal PVY^{N} namesto PVY^{NTN} skupini. Za obe metodi smo predlagali nekaj izboljšav. Dokler metode ne bodo ciljale molekulskih determinant, zaradi katerih PVY povzroča PTNRD, ostajata testirani metodi najbolj zanesljivi orodji za klasifikacijo PVY.

S sekvenciranjem nukleotidnih zaporedij slovenskih izolatov PVY smo opazovali pestrost PVY v Sloveniji. Ugotovili smo, da so v Sloveniji prisotni večinoma zelo sorodni PVY^{NTN} različki, našli pa smo tudi izolata iz skupin PVY^{O} in PVY^{N-Wi}.

Zbrana zaporedja virusnega proteina CP smo združili z več kot 200 drugimi zaporedji iz celega sveta ter preko bayesianske analize in filogenetskih primerjav opazovali vpliv geografskega porekla in gostitelja na evolucijo CP. Razporeditev vej filogenetskega drevesa je najbolj sovpadalo s skupinami različkov. Znotraj veje PVY^{N} različkov sta bili jasno ločeni veji evropskih in severnoameriških izolatov. Jasno so bile tudi ločene tudi skupine izolatov iz Azije in južne Afrike. Ločene veje na filogenetskem drevesu smo zaznali tudi zaradi evolucijske prilagoditve na gostiteljsko rastlino. Formirale so se ločene veje izolatov iz krompirja, tobaka in paprike. Raziskali smo tudi, kateri kodoni v proteinu CP so pod pozitivnim in negativnim evolucijskim pritiskom. Našli smo en sam kodon pod signifikantno pozitivnim evolucijskim pritiskom ter nekaj aminokislinskih mest, značilnih za določeno skupino različkov. Virusni protein CP je večinoma pod negativnim evolucijskim pritiskom. Ugotovili smo, da geografski izvor in prilagoditev

na gostitelja pomembno vplivata na evolucijo proteina CP, vendar pa s tem ne moremo pojasniti celotne pestrosti.

Namesto dolgotrajnih in zamudnih klasičnih postopkov čiščenja virusnih delcev PVY smo razvili hitro in učinkovito metodo na podlagi ionsko izmenjevalne kromatografije z monolitnimi CIM nosilci. Metoda skrajša postopek iz štirih na manj kot dva dni, dosega primerljivo čistost in izkoristek čiščenja kot klasična metoda, vendar brez uporabe dragih naprav za ultracentrifugiranje. Po čiščenju so virusni delci nepoškodovani in infektivni, metoda pa omogoča tudi hitro in enostavno povečanje kapacitete čiščenja.

S pomočjo infektivnih klonov PVY smo skonstruirali z GFP označen PVY. Konstrukt je stabilen in biološko primerljiv z neoznačenim virusom. Omogoča *in vivo* sledenje metabolno aktivnega virusa na inokuliranih listih in sistemsko. Uspešno okužuje rastline tobaka *Nicotiana tabacum, Nicotiana benthamiana*, različne kultivarje krompirja *Solanum tuberosum* L. ter divje sorodnike krompirja. S fluorescentno označenim virusom smo opazovali širjenje virusa med celicami *in vivo* ter ocenili hitrost napredovanja okužbe. Z GFP označen PVY lahko uporabimo za funkcionalno analizo virusnih in rastlinskih genov, udeleženih v širjenju virusa in v rastlinski obrambi proti PVY.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Izziv predloženega doktorskega dela je razumevanje širjenja najpomembnejšega virusnega povzročitelja bolezni na krompirju, krompirjevega virusa Y (Potato virus Y, v nadaljevanju PVY). Krompir (*Solanum tuberosum* L.) je v prehrani ljudi tretja najpomembnejša poljščina, ima široko podnebno območje pridelave. Uspeva namreč od najjužnejših področij Južne Amerike do Grenlandije, od regij tik nad morsko gladino pa vse do 4700 m nadmorske višine. Pridelavo krompirja poleg PVY ogroža še širok spekter bolezni in škodljivcev: koloradski hrošč, ogorčice, glivne in bakterijske gnilobe ter druge virusne okužbe. Krompirjev virus Y povzroča številna bolezenska znamenja, katerih tip in jakost sta močno odvisna od kultivarja krompirja, različka virusa in tipa okužbe (primarna ali sekundarna). Pojavnost znamenj je odvisna tudi on klimatskih pogojev. Najpogostejša bolezenska znamenja na listih so: mozaik, pegavost, nagubanost in nepravilna rast listov, lokalne in sistemske nekroze ter rumenenje in odpadanje listov. Nekateri različki povzročajo tudi nekroze na površini gomoljev, ki so značilno obročkaste oblike. Bolezen imenujemo obročkasta nekroza gomoljev krompirja (Potato Tuber Necrotic Ringspot Disease ali PTNRD), zaradi katere so gomolji neuporabni za prodajo. Rast rastlin je lahko zaradi PVY zmanjšana in zakrnela.

PVY je predstavnik rodu Potyvirus družine Potyviridae. PVY je RNA virus, katerega filamentozni virusni delec (dolg 740 nm in 11 nm v preseku) je sestavljen iz približno 10 kb dolge enoverižne pozitivno usmerjene RNA. Virusna RNA kodira velik poli-protein, ki se po translaciji razreže v 10 manjših proteinov. V 2+ odprtem bralnem okviru se nahaja enajsti protein. PVY ima širok krog gostiteljskih rastlin, večinoma iz družine razhudnikovk (*Solanaceae*), med katerimi sta ekonomsko najpomembnejši vrsti krompir (*Solanum tuberosum*) in tobak (*Nicotiana tabaccum*). PVY je razširjen po celem svetu, v naravi ga prenaša vsaj 40 vrst listnih uši (primarna okužba), prenaša se iz materinske na hčerinsko rastlino preko gomoljev (sekundarna okužba) in mehansko z rastlinskim sokom.

Poznamo veliko različkov virusa PVY, ki jih ločimo na podlagi molekulskih in genetskih kriterijev. Glede na zmožnost različkov izzvati izražanje obrambnih genov *N* v različnih kultivarjih krompirja jih delimo v pet skupin (genetska klasifikacija): PVY^{O}, PVY^{N},PVY^{C}, PVY^{E} in PVY^{Z}. Genetska karakterizacija omogoča najbolj neposredno in s tem relevantno klasifikacijo različkov v skupine s podobnim biološkim, vendar pa je genetska metoda klasifikacije dolgotrajna (priprava rastlin, inokulacija, opazovanje bolezenskih znamenj), zahteva veliko prostora in je težavna za dobro medlaboratorijsko standardiziranje. Mnogo hitrejše in enostavnejše so metode serološkega določanja in klasifikacije PVY z monoklonskimi protitelesi. S pomočjo protiteles proti proteinu plašča (CP) lahko izolate razvrstimo v dve serološki skupini, serotip O in N. Izolatov

PVY s serološkimi tehnikami ne moremo natančno ločevati, saj je znotraj obeh seroloških skupin več podskupin z različnimi lastnostmi, ki jih lahko ločimo le z molekulskimi metodami. Velika večina izolatov, ki povzroča PTNRD, pripada serološki N skupini, kar pomeni, da je serološka lastnost še vedno precej dober pokazatelj za uporabo v diagnostiki.. Problem seroloških testov je tudi njihova občutljivost, saj ne omogočajo zanesljive določanje nizkih koncentracij virusa. Določitev nukleotidnih zaporedij velikega števila različkov PVY je omogočil razvoj molekularnih metod določanja in karakterizacije PVY. Glede na sorodnost nukleotidnih zaporedij razvrščamo različke v devet skupin različkov. Štiri skupine, katerih genom ni rekombiniran: PVY^{O}, PVY^{N}, PVY^{NA-N} in PVY^{C}; in pet rekombiniranh skupin različkov: PVY^{Z}, $PVY^{N:O}$, PVY^{N-Wi}, PVY^{E} in PVY^{NE-11}. V Evropi prevladujejo različki PVY^{Z} podskupine PVY^{NTN}, ki so posledica rekombinacij genomov iz skupine PVY^{O} in PVY^{N}. V pridelavi krompirja so najpomembnejši prav različki PVY^{NTN}, saj vsi povzročajo PTNRD. Hitra evolucija in adaptacija je ena izmed značilnosti PVY, zaradi česar je razvoj zanesljivih metod za določanje zelo otežen. Za natančnejše razlikovanje in boljše poznavanje pestrosti izolatov PVY potrebujemo vedno nova nukleotidna zaporedja. S pomočjo informacije o nukleotidnih zaporedjih so bila razvita številna molekulska orodja, ki zanesljivo ločijo med različki PVY.

V tej nalogi smo primerjali dve najnovejši metodi za razlikovanje med različki PVY, SNaPshot (Rolland in sod., 2008), ki različke ločuje na podlagi točkovnih polimorfizmov preko celotnega genoma, in metodo verižnega pomnoževanja z reverzno transkripcijo v realnem času (RT-qPCR) (Kogovšek in sod., 2008), ki poleg ločevanja med različki omogoča tudi kvantifikacijo števila kopij virusnega genoma. Raziskavo smo usmerili predvsem v zanesljivo določanje PVY^{NTN} različkov. Sklepali smo, da bomo z metodo RT-qPCR zanesljiveje ločili med PVY^{N} in PVY^{NTN} različki in da metoda ne bo tako občutljiva za točkovne mutacije kot SNaPshot metoda, za katero pričakujemo, da bo določila vse različke tudi v mešanih okužbah. Obe metodi smo uporabili za karakterizacijo več kot 20 slovenskih izolatov PVY. Rezultate smo potrdili z biološkimi testi, ELISA testom in določanjem nukleotidnih zaporedij. Snapshot metoda je razlikovala med več različnimi skupinami različkov, vendar pa je veliko izolatov napačno razvrstila v skupino PVYN namesto v PVY^{NTN}, medtem ko smo z RT-qPCR pravilno določili vse PVY^{NTN} različke. Metoda pa ne more razlikovati med PVY^{O} in PVY^{N-Wi}. Z analizo nukleotidnih zaporedij smo ugotovili, da gre razlike v določanju pripisati različnim molekulskim označevalcem, ki jih metodi zaznavata. RT-qPCR cilja za PVY^{NTN} bolj značilne molekulske označevalce ter je tudi manj podvržen napačni identifikaciji zaradi hitre virusne evolucije, ker cilja s sondo več označevalcev hkrati. Za obe metodi smo predlagali izboljšave v smislu zanesljivejšega določanja PVY različkov.

Do sedaj je bilo določeno le eno zaporedje genoma virusa PVY izoliranega v Sloveniji, in sicer iz skupine PVY^{NTN}. Sklepali smo, da bomo z določitvijo nukleotidnih zaporedij

izbranih genov virusa PVY ugotovili prisotnost več različkov PVY v Sloveniji. Določili smo delna nukleotidna zaporedja šestim novim izolatom (cca 60-75% celotnega genoma), z molekulskimi metodami pa analizirali še dodatnih 18 izolatov. Večina slovenskih izolatov (22/24) so pripadali skupini PVY^{NTN}, ki v Sloveniji prevladuje od 90ih let prejšnjega stoletja dalje, določili pa smo tudi zaporedja slovenskih izolatov iz skupin PVY^{O} in PVY^{N-Wi}. Za boljšo sliko sestave različkov v Sloveniji bi potrebovali večje število vzorcev, vendar pa rezultati sovpadajo s podatki iz Nemčije, Francije kjer prav tako prevladujejo različki PVY^{NTN}.

Z naborom večjega števila nukleotidnih zaporedij iz različnih gostiteljev in geografskih področij lahko nato raziskujemo, kateri deli genoma so vključeni v procese adaptacije na gostiteljsko rastlino, katere aminokisline so podvržene pozitivnim in negativnim evolucijskim pritiskom in tako ocenimo, kateri molekulski označevali so bolj ali manj primerni za uporabo v molekulski diagnostiki. Zaporedje gena za CP je eno izmed najpogosteje študiranih delov genoma, saj se pogosto uporablja za filogenetske študije in klasifikacijo PVY različkov, direktno pa je povezan tudi s serološkim določanjem PVY. Potivirusni CP je primer večfunkcionalnega virusnega proteina, ki sodeluje pri izražanju bolezenskih znamenj, pri prenosu virusa z vektorji, sodeluje pri interakcijah s potivirusnim proteinom Hc-Pro in z veliko podenoto encima RubisCO. Zaradi velike pomembnosti proteina CP smo preučili njegovo evolucijo. Z uporabo bayesianskih statističnih orodij smo analizirali CP zaporedja in skušali poiskati aminokislinska mesta (kodone) izpostavljenim pozitivnim in negativnim evolucijskim pritiskom ter mesta. ki se hkrati spreminjajo, (ko-varianca). Izolati so se na filogenetskem drevesu razporedili v tri najbolj tipične skupine, PVY^{O}, PVY^{N}, PVY^{C}. Dobro so bile podprte tudi veje v skupinah PVY^{O} PVY^{N}, kjer so bili izolati s skupnim geografskim izvorom. V skupini PVY^{N} so se izolati razvrstili v dve glavni podskupini evropskih in severnoameriških izolatov. Na obliko filogenetskega drevesa je vplivala tudi gostiteljska rastlina, opazili smo namreč podskupine, ki so pripadale izolatom iz krompirja, tobaka in paprike. Zanesljivega aminokislinskega mesta, ki bi bilo značilno za določenega gostitelja, nismo našli. Za večino kodonov v CP se je izkazalo, da so evolucijsko nevtralni ali pa izpostavljeni vplivom negativne selekcije. To nakazuje na dobro prilagojenost PVY na trenutne gostitelje in daljšo skupno evolucijo. Uporabili smo več različnih metod (FEL, IFEL, MEME) določanja kodonov, izpostavljenim pozitivnim selekcijskim pritiskom Prvi kodon v CP genu je bil edino mesto, ki je bilo skupno vsem trem metodam. S tem lahko pojasnimo tudi rezultate prejšnje študije. SNaPshot namreč cilja prav prvi nukleotid v prvem kodonu CP gena, za katerega sedaj vemo, da se v populaciji diverzificira in je zato manj primeren za zanesljivo uporabo v diagnositki. S to študijo smo želeli ugotoviti, ali v proteinu PC obstajajo mesta, ki so značilna za i) geografsko območje, od koder izolat pridaja, ii) gostitelja, iz katerega prihaja. Na podlagi teh podatkov bi lahko sledili izvoru izolatov in širjenju na nova področja. Ugotovili smo, da geografski izvor in prilagoditev na gostitelja pomembno vplivata na evolucijo virusnega CP proteina, vendar pa s tem ne moremo pojasniti celotne pestrosti. Pokazali smo, da v

genu CP ni posameznih popolnoma zanesljivih molekulskih determinant niti za klasifikacijo različkov niti značilnih za posameznega gostitelja ali geografsko poreklo.

Hitra evolucija virusnega genoma zahteva potrebno sprotno proizvajanje protitelesa proti novo nastajajočim različkom PVY. Za pridobivanju novih monoklonskih protiteles potrebujemo suspenzijo očiščenih virusnih delcev, ki pa je uporabna tudi za študije interakcij virusnih delcev s protitelesi ter za študije in-vitro prenosa z vektorji. Klasična izolacija čistih virusnih delcev PVY iz rastlinskega materiala je navadno dolg (4-5 dni) in težaven proces, ki vključuje homogenacijo, bistrenje homogenata, čiščenje z ultracentrifugiranjem skozi gradient saharoze in cezijevega klorida, frakcioniranje gradienta in dializo. Proces zahteva izurjeno osebje ter drago opremo, kot so ultarcentrifuge, zato je mnogim laboratorijem nedostopen. Metakrilatni monolitni kromatografski nosilci imajo zelo veliko skupno površino za vezavo in hkrati relativno velike pore/kanalčke (800-1200 μm), ki so med seboj povezani, kar pomeni, da je vsa površina kolone dostopna za vezavo, ne da bi bila za to potrebna difuzija delcev. Tako je ločljivost kromatografske ločbe neodvisna od velikosti kolone in pretoka Kromatografski nosilci pot tržnim imenom CIM® (BiaSeparations, Slovenia) so bili že uspešno uporabljeni za koncentriranje in čiščenje paličastih in kroglastih rastlinskih in živalskih virusov in viroidov, nukleinskih kislin in celo ribosomov, ne pa tudi filamentoznih virusnih delcev. Izolacija s CIM monoliti močno skrajša čas izolacije, ne potrebuje korakov ultracentrifugiranja, odlikujejo pa jo tudi visoki izkoristki, visoka resolucija ločbe in enostavno povečevanje kapacitete čiščenja (scale up).

Na podlagi dosedanjih izkušenj smo predpostavljali, da je CIM monolitno tehnologijo možno uporabiti za izboljšano čiščenje infektivnih virusnih delcev filamentoznega virusa PVY. V postopkih optimizacije kromatografskega čiščenja smo preizkusili anionske kolone (QA in DEAE) in različne puferske sisteme. Nato smo optimizirali še postopke čiščenja pred uporabo kolone in postopke spiranja virusnih delcev iz kolone. Izkoristke kromatografske ločbe ter postopkov predčiščenja smo analizirali z metodo enokoračnega RT-qPCR (spremljali smo koncentracijo virusne RNA in rastlinskih nukleinskih kislin). Čistost virusa v posameznih korakih smo spremljali z metodo SDS-PAGE, kjer smo prisotnost PVY potrdili z metodo»Western Blot«. Obliko in integriteto delcev v posameznih korakih smo opazovali z metodo presevne elektronske mikroskopije. Določili smo tudi infektivnost očiščenih delcev PVY z inokulacijo rastlin *Nicotiana tabacum* cv. xanthii.. Pri izolaciji intaktnih filamentoznih delcev PVY (RNA in proteini) iz zelo kompleksnega rastlinskega homogenata je kombinacija kvantitativnih in kvalitativnih metod za opazovanje prisotnosti PVY ključna, saj s posamičnimi metodami ne dobimo dovolj dobrih podatkov o kvaliteti ločbe. Čiščenje s CIM monoliti skrajša postopek iz štirih na manj kot dva dni brez uporabe ultracentrifugiranja v gradientu saharoze in cezijevega klorida. Metoda dosega primerljiv izkoristek in čistost kot klasična metoda izolacije, odstrani 99,99 % vseh prostih nukleinskih kislin, očiščeni delci so intaktni in infektivni, metoda pa omogoča

tudi enostavno povečanje obsega čiščenja v enakem času brez spreminjanja pogojev čiščenja. S CIM očiščene delce lahko uporabimo za opazovanje interakcij med delci in protitelesi ter za sekvenciranje nove generacije, kjer z odstranitvijo nukleinskih kislin gostitelja poenostavimo obdelavo podatkov.

Poleg poznavanja nukleotidnih zaporedij, je za uspešno določanje in preučevanje interakcij potrebno tudi poznati, kje se virus v rastlini nahaja in kako se po rastlini širi. V ta namen smo pripravili z zelenim fluorescentnim proteinom (GFP) označen PVY infektivni klon. Predvidevali smo, da bomo lahko z GFP označenimi infektivnimi kloni virusa PVY opazovali razporeditev PVY v gostiteljskih rastlinah ter da metabolna aktivnost virusa ne bo korelirala s pojavom vidnih bolezenskih znamenj.

Infektivni kloni omogočajo tarčno spreminjanje virusnega genoma in opazovanje sprememb virusnega fitnesa (patogenost, hitrost pomnoževanja in širjenja po rastlini, prenos z vektorji) zaradi sprememb genoma. Problem priprave infektivnih klonov PVY je predvsem v tem, da lahko pride do izražanja toksičnih proteinov v *E. coli*, kar onemogoča postopke kloniranja. Prvi infektivni klon PVY so razvili Jakab in sod. (1997). Genom PVY-N605 (Švicarski nekrotični izolat) so prepolovili v proteinu CI tako, da so dobili dva manjša klona, ki nista toksična za *E. coli*. Z omenjenim infektivnim klonom so ugotovili, kateri del genoma PVY je odgovoren za bolezensko znamenje nekroz žil na tobaku.

Uporaba infektivnih klonov je tudi edini način, s katerim lahko viruse označimo s fluorescentnimi in drugimi označevalci, kar omogoča sledenje premikanju virusa med celicami in sistemsko po rastlini. Fluorescentno ter z drugimi označevalci je bilo označenih že nekaj virusov iz rodu *Potivirus*, npr.: krompirjev virus A (PVA), virus združevanja žil in mozaika tobaka (TVBMV), virus šarke (PPV) in drugi. Obstaja tudi z GFP označen PVY in je bil omenjen v študiji proteinsko-proteinskih interakcij med PVY in *N. tabacum*, vendar pa ni bil okarakteriziran niti biološko niti s stališča stabilnosti.

V nalogi smo gen za protein GFP vstavili v genom PVY med gena, ki kodirata proteina NIb in CP tako, da se po translaciji odcepi iz poliproteina. Fluorescenco GFP zato zaznamo v citoplazmi celic, kjer se virusni proteini aktivno sintetizirajo. Najprej smo potrditi njegovo biološko primerljivost z neoznačenim PVY N605 izolatom. Potrdili smo, da PVY N605-GFP ob enakem času po inokulaciji povzroča enako močna bolezenska znamenja kot neoznačen PVY N605. S tehniko RT-qPCR (Kogovšek in sod., 2008) smo potrdili, da dosegata primerljivo koncentracijo virusnih nukleinskih kislin v sistemsko okuženih listih. Protein GFP torej ne vpliva na fitnes PVY N605. Tekom pomnoževanja virusnega genoma v gostitelju nastaja veliko število tako mutacij kot tudi rekombinacij, preko katerih se gen za GFP lahko odstrani iz genoma. Za uspešno sledenje fluorescentno označenega virusa je stabilnost vstavljenega fluorescentnega gena ključna. Stabilnost gena za protein GFP v genomu PVY N605 smo določili s kombinacijo treh specifičnih RT q-PCR testov: i) kvantificirali smo

količino gena za GFP, ii) količino vseh genomov PVY ter iii) specifično kvantificirali količino genomov z izrezanim genom za GFP. Koncentracija vseh virusnih genomov in koncentracija gena za GFP sta bili skoraj enaki v vseh vzorcih. Rezultati kažejo na to, da se GFP pomnožuje stabilno in ekvimolarno skupaj z virusnim genomom. Genomi PVY z izrezanim genom za GFP smo našli v nekaj vzorcih, vendar pa je bil njihov delež večinoma nižji od 0,05 % in se po času od okužbe ni povečeval. Spričo hitre virusne evolucije torej nastanejo PVY N605 brez gena za GFP, vendar pa le ti ne izpodrinejo PVY N605-GFP v *Nicotiana tabacum* cv. xanthii. Tovrstna natančna analiza biološke primerljivosti in stabilnosti fluorescentno označenega virusa do sedaj še ni bila izvedena. Okarakteriziran PVY N605-GFP smo nato uporabili za sledenje PVY v rastlinah *Nicotiana tabacum* cv. xanthii. Detajlne slike fluorescence velikih delov lista ali celo celotnega lista naenkrat smo opazovali s pomočjo avtomatiziranega fluorescentnega mikroskopa. Najprej smo opazovali potek širjenja PVY med celicami na inokuliranih listih, kjer smo drobna območja (nekaj 10 celic) z GFP fluorescenco označenega virusa zaznali že po dveh do treh dneh po inokuolaciji. Kot smo predpostavljali, smo prva bolezenska znamenja zaznali šele tri do štiri dni potem, ko smo zaznali širjenje PVY preko fluorescence. Za sledenje virusa *in vivo* smo prilagodili tehniko gojenja rastlin tako, da smo lahko inokulirane in sistemsko okužene liste brez poškodb vsak dan pregledali. Fluorescenco smo zaznali v višjih sistemsko okuženih listih že po 4 dneh po inokulaciji, najprej v žilah in njihovi okolici, nato pa po celem listu. V listu točno nad inokuliranim smo fluorescenco zaznali le v nekaj primerih in še to pri bazi lista. Zajeli smo slike istih mest okužbe na inokuliranih listih ob različnih dnevih po okužbi. Tako smo lahko določili hitrost širjenja PVY med celicami v tobaku. Opazili smo, da se virus širi pospešeno, saj je drugi dan okužil več novih celic (v povprečju 9 celic na uro) kot prvi dan (v povprečju 6,5 celic na uro) opazovanja. Tovrsten trend smo opazili pri vseh opazovanih mestih okužbe. V literaturi do sedaj še ni bilo natančnega podatka o tem, kako hitro se PVY širi med celicami. Poleg tobaka lahko PVY N605-GFP uspešno sistemsko okuži tudi štiri kultivarje krompirja (*Solanum tuberosum* L.): désireé, NagG-désireé, bea in bintje; ter divje sorodnike krompirja (*Solanum mochiquense*, *Solanum papita* in *Solanum venturii*). Divji sorodniki omogočajo lažjo prehodno transformacijo z *Agrobacterium tumefaciens* ter so genetsko sorodni kultiviranemu krompirju, zato jih uporabljamo kot nadomestni model za študij interakcij med PVY in krompirjem. Zanesljivo in natančno orodje za *in vivo* lokalizacijo PVY bo pripomoglo k študijam funkcionalne analize rastlinskih in virusnih genov udeleženih pri okužbi in premikanju PVY po rastlini.

4.2 SUMMARY

The aim of this doctoral thesis was to understand the spread of the most important viral pathogen infecting potato, Potato virus Y (PVY). Potato (*Solanum tuberosum* L.) is the third most important crop according to human consumption; it has a wide climate range of production. Potato thrives from the southernmost areas of South America to Greenland, from the regions just above the sea level and all the way to 4700 m above the sea. In addition to PVY, potato production is damaged by a wide range of diseases and pests: the Colorado potato beetle, nematodes, fungal and bacterial rots and other viral infections. Potato virus Y causes a number of symptoms of which type and strength are strongly dependent on the potato cultivar, variant of the virus infecting and type of infection (primary or secondary). The incidence of symptoms is dependent on the climatic conditions as well. The most common symptoms on leaves are: mosaic, rugose, mottling, irregular growth of leaves, local and systemic necrosis, yellowing and leaf drop. Some variants of PVY are causing necrosis on the surface of the tubers, which are characteristic ring shaped. The disease is called Potato Tuber Necrotic Ringspot Disease (PTNRD), due to which the tubers are unfit for sale. Plant growth may be reduced and stunned due to PVY as well.

PVY is representative of the genus *Potyvirus* family *Potyviridae*. It is an RNA virus with a filamentous virus particle (740 nm in length and 11 nm in cross-section) is composed of approximately 10 kb long single-stranded positive-sense RNA molecule. The viral RNA encodes a large poly-protein which, is cut into 10 smaller proteins after translation. The 11^{th} protein is located in the +2 open reading frame. PVY has a wide host range, mostly from the *Solanaceae* family. Economically most important are potato (*Solanum tuberosum* L.) and tobacco (*Nicotiana tabaccum*). PVY is present worldwide. It is transmitted from plant to plant by at least 40 species of aphids (primary infection), from a mother to daughter plant through the tubers (secondary infection) and mechanically with the plant sap.

There are many variants of the PVY, which are classified on the basis of the molecular and genetic criteria. Depending on their ability to induce defence *N* genes in different potato cultivars we classify PVY isolates into five strain groups (genetic classification): PVY^{O}, PVY^{N}, PVY^{C}, PVY^{E} and PVY^{Z}. Genetic characterization enabled the most direct biologically relevant classification of isolates into strain groups with similar biological properties. However the methodology is time-consuming (preparation of plants, inoculation, observation of symptoms), requires a lot of space and it is difficult to standardize between the laboratories. Serological methods of detection and classification of PVY with monoclonal antibodies are faster and easier to use. We can distinguish between two serotypes using antibodies against coat protein (CP), serotype O and N. All strain groups of PVY cannot be distinguished with serological techniques, since more sub-groups with different characteristics are present within the two

serotypes. Those can be distinguished only by molecular methods. The vast majority of isolates causing PTNRD belongs to serological group N. Therefore this serological property is still a valuable indicator for the use in diagnostic tests. The problem of serological tools is also their low sensitivity which does not allow reliable detection and characterisation of PVY in low concentrations. Availability of the nucleotide sequences of a large number of PVY isolates has enabled the development of molecular methods for detection and characterization of PVY. On the base of the similarity of the nucleotide sequences strains are classified into nine groups: four groups with non-recombinant genome: PVY^{O}, PVY^{N}, PVY^{NA-N} and PVY^{C}; and five groups with recombinant genome: PVY^{Z}, $PVY^{N:O}$, PVY^{N-Wi}, PVY^{N} , PVY^{E} and PVY^{NE-11}. The prevalent strain group in Europe is PVY^{Z} more specifically sub-group PVY^{NTN} which genome is a result of recombination between genomes from the PVY^{O} and PVY^{N} groups. The isolates from PVY^{NTN} sub-group are also the most important in potato production since they all cause PTNRD. The rapid evolution and adaptation is one of the characteristics of PVY, which makes the development of reliable molecular methods for detection and classification very difficult. For a more precise distinction and a better understanding of the diversity of isolates of PVY, their recent nucleotide sequences are needed. On the base of nucleotide sequences many molecular tools that reliably distinguish between versions of the PVY have been developed.

In this study we compared two most recent methods for detection and classification of PVY, SNaPshot (Rolland in sod., 2008), which separates variants based on single nucleotide polymorphisms (SNP) present across the PVY genome, and the method based on the real-time polymerase chain reaction with reverse transcription (RT-qPCR) (Kogovšek in sod., 2008), which in addition to the classification of the isolates into the sub-groups enables also the quantification of PVY. We focused our research on accurate detection of isolates from the PVY^{NTN} sub-group. Our hypothesis was that RT-qPCR would reliably distinguish between PVY^{N} and PVY^{NTN} strain groups, and that the method will not be less sensitive to point mutations than SNaPshot method, for which we expect to distinguish between all strain groups even in mixed infections. Both methods were used to characterize more than 20 Slovenian isolates of PVY. The results were confirmed by biological tests, ELISA and by analysis of nucleotide sequences. Snapshot method indeed distinguished betted between all different PVY sub-groups, but many isolates were incorrectly classified in the group PVY^{N} instead of PVY^{NTN}. The RT-qPCR method on the other side correctly classified all PVY^{NTN} variants, but is not able to distinguish between PVY^{O} and PVY^{N-Wi} sub-groups. The analysis of the nucleotide sequences revealed that difference in the classification accuracy between the methods lied in the different molecular markers targeted by the two methods. The RT-qPCR targeted more stable and reliable molecular markers for PVY^{NTN} than SNaPshot. It is also less prone to false identification due to the SNP mutations induced by rapid viral evolution, because the qPCR probe targeted several markers at the same time. For

both methods we proposed improvements for more reliable discrimination between of PVY sub-groups.

Only one sequence of the PVY genome isolated from Slovenia was available till now, the sequence of one Slovenian PVY^{NTN} isolate. We proposed that with sequencing of more Slovenian PVY isolates will reveal the presence of several PVY sub-groups in Slovenia.

We determined the partial nucleotide sequences of six new isolates (appx. 60-75 % of the total genome) and analyzed additional 18 isolates with molecular methods. Majority of the isolates were from the PVY^{NTN} sub-group which is the dominant subgroup in Slovenia since the 1990s. We have also detected and sequenced Slovenian isolates from sub-groups PVY^{O} and PVY^{N-Wi}. To elucidate the variability of Slovenian isolates more accurately we would need to test more samples, however the results correspond to the ones from Germany, France, where PVY^{NTN} is the prevalent sub-group as well.

With a wide range of PVY sequences from different plant hosts and geographical regions we are able to investigate which parts of the genome are involved in the processes of adaptation to the host plant, which amino acids are subjected to positive and negative evolutionary pressure and thus assess which molecular markers are more or less suitable for targeting with molecular methods. The sequence of the CP gene is one of the most studied parts of the PVY genome since it is often used for phylogenetic studies and classification of PVY subgroups and directly related to the serological determination of PVY. Potyviral CP is an example of a multi-functional viral protein that is involved in the expression of the symptoms, transmission of the PVY by vectors, it interacts with potyviral HC- Pro protein and also with the large subunit of RubisCO. Due to the great importance of the CP protein, we examined its evolution. By using Bayesian statistical tools we have analyzed the CP sequence and tried to find amino acid sites (codons) under positive and negative evolutionary pressure and the positions exhibiting co-variance. We constructed phylogenetic tree where isolates clustered in the three most typical groups PVY^{O}, PVY^{N} and PVY^{C}. Well were supported branches within the PVY^{O} and PVY^{N} groups contained isolates with a common geographical origin. The isolates in the group PVY^{N} were classified into two major subgroups, European and North American. The branching of the phylogenetic tree was also influenced by the host plant. We have observed subgroup on the tree with isolates from potato, tobacco and pepper. Amino acid sites characteristic for a particular host were not found in the PVY genome.

The majority of the codons in the CP were under neutral or purifying evolutionarily selection, which indicates good adaptation of PVY on the current hosts and extended common evolution. We have used several methods (FEL, IFEL, MEME) to determine the codons under positive selection pressure. The first codon of the CP gene was the only position that was detected by all methods. This may explain the why SNaPshot method was less accurately detecting PVY^{NTN} strains (first part of this study). The

method detects the recombinant isolates with the SNP of the first position in the first codon of the CP gene, for which we know that is diversifying in the population and is therefore less suitable for reliable use in diagnostics. The aim was also to determine if there are any positions in CP gene characteristic for i) the geographical area in which the isolate was found, ii) the host from which it was isolated from. These data could enable tracing the origin of the isolates and their spread to new areas. We haven't found any individual reliable molecular determinants nor for the classification of sub-groups, neither specific to a particular host or geographic origin We found that geographical origin and adaptation to the host have a major impact on the evolution of the CP protein, but the two parameters do not explain the overall diversity observed.

Due to the rapid evolution of the virus genome continuous production of antibodies against newly emerging variants of PVY is required. Production of new monoclonal antibodies requires suspensions of purified viral particles for immunisation procedures. Pure viral particle solutions are also used for the study of interactions between viral particles and antibodies and for studies *in vitro* transmission of PVY by vectors. Classical isolation of pure PVY particles from plant material is usually long (4-5 days) and laborious process that involves homogenisation, clarification of the homogenate, cleaning by ultracentrifugation through a sucrose gradient and caesium chloride gradient, fractionation and dialysis. The process requires trained personnel and expensive equipment, such as ultracentrifuge, thus inaccessible to many laboratories. Methacrylate monolithic chromatographic supports have a very large total binding surface area and relatively large pores / channels (800-1200 μm) at the same time. The pores are all connected to each other therefore the whole binding area of the column is accessible to the solutes in the flow without the diffusion of solutes as in classical columns. Thus, the resolution of the chromatographic separation is independent of column size and flow rate. Chromatographic supports with the trade name Convective interaction media® (CIM) (BiaSeparations, Slovenia) have already been successfully used for the concentration and purification of the rod-like and spherical plant and animal viruses, viroids, nucleic acids and even ribosomes, but not filamentous virus particles. Isolation of the CIM monoliths significantly shorten the time of isolation, isolation does not require ultracentrifugation steps, enables high recovery and resolution of separations and simple scale up of the method.

Based on previous experience, we assumed that the CIM monolithic technology can be used for improving purification of infective filamentous viral particles of PVY. In the optimization procedure different anion columns (QA and DEAE) and various buffer systems were tested. Then we optimized the sample preparation steps preceding the chromatography and the elution of the virus particles from the column. Recovery of the PVY in the chromatographic separation was analyzed with single step RT- qPCR (we tracked the concentration of viral RNA and plant nucleic acids as well). The purity of the virus in the individual steps was monitored by SDS-PAGE, wherein the presence of

PVY was confirmed with "Western Blot". The shape and the integrity of the particles in the individual purification were observed by transmission electron microscopy. We determined the infectivity of purified PVY particles by inoculating test plants of *Nicotiana tabacum* cv. Xanthii. A combination of quantitative and qualitative methods monitoring the filamentous PVY particles (composed of RNA and proteins) is required during the optimisation of purification from a complex plant homogenate since individual methods do not provide sufficient data regarding the quality of separation of the particles. Cleaning with CIM monoliths shorten the process from four to less than two days, without the use of gradient ultracentrifugation in sucrose and caesium chloride. The method achieves comparable yield and purity as a conventional isolation, method removes 99.99% of all free nucleic acids and purified particles remain intact and infectious as well. Method also allows simple scale up of the purification without changing the conditions of the purification or time required. The CIM purified particles can be used for observing the interactions between the particles and the antibodies, and for the next generation sequencing, where the removal of the host nucleic acids of simplifies the data processing.

In addition to the PVY sequence information, the knowledge on localisation and movement of PVY in the plant is necessary for a successful detection and study of interactions. To enable the study of localisation we have prepared PVY infective clone labelled with a green fluorescent protein (GFP). We assumed that we can use the GFP labelled PVY infective clones to observe the distribution the virus in host plants and that of the metabolic activity of the virus will not correlate with the appearance of visible symptoms.

With the infectious clone strategy we are able to modify the viral genome and observe the changes in viral fitness (pathogenicity, rate of amplification and spread in the plant, efficiency of the vector transmission) due to changes in the genome. The main difficulty of the PVY infectious clone development is the fact that some proteins are being expressed in *E. coli*, which impairs the cloning procedures. The first infectious clone of PVY was developed by Jakab in sod. (1997). The genome of PVY-N605 (Swiss necrotic isolate) was split in two halves in the CI protein, resulting in get two smaller clones, which are not toxic for *E. coli*. This infective clone was used to elucidate the exact positions in the PVY genome responsible for the vein necrosis symptom on tobacco.

The use of infectious clones is the only technique by which viruses can be labelled with fluorescent and other markers, for tracking the movement between cells and systemically through the plant. Fluorescent and other markers have used to label for several potyviruses eg.: *Potato virus A* (PVA), *Tobacco vein banding mosaic virus* (TVBMV), *Plum pox virus* (PPV) and others. The GFP labelled PVY was already mentioned in the study of protein-protein interactions between PVY and *N. tabacum*,

however the construct was not characterised in terms of stability and biological suitability for localisation studies.

In this study the GFP protein was inserted into the PVY genome between the genes for NIb and CP proteins. The GFP protein is cut out of the polyprotein after translation. Fluorescence of GFP is detected in the cytoplasm of the cells, where PVY proteins are being actively synthesized. First, we confirm the biological comparability of the construct in comparison to non-labelled PVY N605 isolate. We confirmed that PVY N605-GFP causes the same symptoms, with similar severity and at the same time pos inoculation as non-labelled virus. With the RT-qPCR technique (Kogovšek in sod., 2008), we confirmed that the PVY N605-GFP reached comparable levels of viral nucleic acids in systemically infected leaves. The addition of the GFP protein did not affect the fitness of PVY N605. Stability of the inserted fluorescent gene is crucial in order to reliably follow fluorescently labelled virus. During the amplification of the viral genome in the host, high frequency of mutation and recombination can occur, resulting in the removal of the GFP gene from the PVY genome. The stability of the gene GFP in the PVY N605 genome was determined by a combination of three specific RT-qPCR tests. We quantified i)the amount of the gene for GFP, ii) the amount of all PVY genomes, and iii) the amount of the genomes that have lost the *gfp* gene. The concentration of the gene for GFP was nearly identical to the concentration of all viral genomes in all samples. The results indicate that the *gfp* gene replicates stably and and in equimolar concentrations with the viral genome. PVY genomes with the *gfp* gene cut out of the genome were detected in some samples, but their amount was lower than 0.05% and it did not increase with the time after infection. Rapid viral evolution resulted in the lost of th*gfp* gene in some cases, however the variants without the *gfp* gene did not displace the labelled PVY N605-GFP in *Nicotiana tabacum* cv. Xanthii. The detailed biological and stability analysis of the fluorescently labelled virus was not carried out before.

The characterized PVY N605-GFP was then used for tracking the PVY in *Nicotiana tabacum* cv. Xanthii . Detailed images of GFP fluorescence in the large sections of the leaves or even the whole leaves were taken with automated fluorescence microscope. First, we observe the movement of PVY between the cells in the inoculated leaves, where small areas of infected GFP fluorescent cells (cca 10 cells) were observed already at two to three days after the inoculation. As we assumed, the first symptoms appeared three to four days after we detected the spread of PVY with fluorescence. We adapted the plant growing technique to follow the movement of PVY in inoculated and systemically infected leaves *in vivo* without damaging the plant. The fluorescence was detected in higher systemic infected leaves as early as 4 days post inoculation. PVY was first detected in the veins and their surroundings, then across the whole leaf lamina. In the leaf directly above the inoculated leaf the fluorescence was detected only in a few cases and mostly at the base of the leaf. Images of the same infection sits taken at

different days post inoculation, enabled the estimation of the rate of movement of PVY between the cells in tobacco. It was observed that the virus movement accelerates during the initial steps of infection. The movement rate was on average 6.5 cells per hour in the first day and on average of 9 cells per hour in the second day. Similar trend was observed in all analysed sites of infection. This is the first estimation of cell-to-cell movement rate for PVY. In addition to tobacco, PVY N605-GFP could successfully systemically infect four cultivars of potato (*Solanum tuberosum* L.): Désireé, NagG-Désireé, Bea and Bintje, as well as wild potato relatives (*Solanum mochiquense*, *Solanum papita* and *Solanum venturii*). Wild potato relatives facilitate transient transformation with *Agrobacterium tumefaciens* and are genetically related to cultured potatoes thus, we can use them as a surrogate model to study the interactions between PVY and potato. Reliable and accurate tool for the *in vivo* localization of PVY will contribute to the study of functional analysis of the plant and viral genes involved in PVY infection and movement through the plant.

5 VIRI

Agindotan B. O., Shiel P. J., Berger P. H. 2007. Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan real-time RT-PCR. Journal of Virological Methods, 142, 1–9

Andrejeva J., Puurand U., Merits a, Rabenstein F., Järvekülg L., Valkonen J. P. 1999. Potyvirus helper component-proteinase and coat protein (CP) have coordinated functions in virus-host interactions and the same CP motif affects virus transmission and accumulation. Journal of Virology, 80, 5: 1133–1139

Atreya P. L., Atreya C. D., Pirone T. P. 1991. Amino acid substitutions in the coat protein result in loss of insect transmissibility of a plant virus. The Proceedings of the National Academy of Sciences of the United States of America, 88, 17: 7887–7891

Atreya P. L., Lopez-Moya J. J., Chu M., Atreya C. D., Pirone T. P. 1995. Mutational analysis of the coat protein N-terminal amino acids involved in potyvirus transmission by aphids. Journal of General Virology, 76, 2: 265–270

Balme-Sinibaldi V., Tribodet M., Croizat F., Lefeuvre P., Kerlan C., Jacquot E. 2006. Improvement of Potato virus Y (PVY) detection and quantitation using PVY(N)- and PVY(O)-specific real-time RT-PCR assays. Journal of Virological Methods, 134, 1-2: 261–266

Barker H., Mcgeachy K. D., Toplak N., Gruden K., Žel J., Browning I. 2009. Comparison of Genome Sequence of PVY Isolates with Biological Properties. American Journal of Potato Research, 86, 3: 227–238

Baulcombe D. C. D. C., Chapman S., Santa Cruz S., Cruz S. 1995. Jellyfish green fluorescent protein as a reporter for virus infections. Plant Journal, 7, 6: 1045–1053

Beczner L., Horváth J., Romhányi I., Förster H., Box P. O. 1984. Studies on the etiology of tuber necrotic ringspot disease in potato. Potato Research, 27, 3: 339–352

Bedoya L. C., Martínez F., Orzáez D., Daròs J.-A. 2012. Visual Tracking of Plant Virus Infection and Movement Using a Reporter MYB Transcription Factor That Activates Anthocyanin Biosynthesis. Plant Physiology, 158, 3: 1130–1138

Blanchard A., Rolland M., Lacroix C., Kerlan C., Jacquot E. 2008. Potato virus Y: a century of evolution. Current Topics in Virology, 7: 21–32

Boonham N., Walsh K., Hims M., Preston S., North J., Barker I. 2002a. Biological and sequence comparisons of Potato virus Y isolates associated with potato tuber necrotic ringspot disease. Plant Pathology, 51, 2: 117–126

Boonham N., Walsh K., Preston S., North J., Smith P., Barker I. 2002b. The detection of tuber necrotic isolates of Potato virus Y, and the accurate discrimination of PVY(O), PVY(N) and PVY(C) strains using RT-PCR. Journal of Virological Methods, 102, 1-2: 103–112

Bukovinszki Á., Götz R., Johansen E., Maiss E., Balázs E. 2007. The role of the coat protein region in symptom formation on Physalis floridana varies between PVY strains. Virus Research, 127, 1:122–125

Carr J. P., Lewsey M. G., Palukaitis P. 2010. Signaling in induced resistance. Advances in Virus Research, 76, 10: 57–121

Centro Internacional de la Papa 2010. Facts and figures about potato; Retrieved from http://cipotato.org/potato/publications/pdf/005449.pdf (18.2.2014)

Ceřovská N. 1998. Production of monoclonal antibodies to potato virus Y NTN strain and their use for strain differentiation. Plant Pathology, 47, 4: 505–509

Chare E. R., Holmes E. C. 2006. A phylogenetic survey of recombination frequency in plant RNA viruses. Archives in Virology, 151, 5: 933–946

Cheng N. H., Su C. L., Carter S. A., Nelson R. S. 2000. Vascular invasion routes and systemic accumulation patterns of tobacco mosaic virus in Nicotiana benthamiana. Plant Journal, 23, 3: 349–362

Chikh Ali M., Maoka T., Natsuaki K. T. 2007. The Occurrence and Characterization of New Recombinant Isolates of PVY Displaying Shared Properties of PVY N W and PVY NTN. Journal of Phytopathology, 155, 7-8: 409–415

Chikh Ali M., Maoka T., Natsuaki K. T. 2007. A point mutation changes the serotype of a potato virus Y isolate; genomic determination of the serotype of PVY strains. Virus Genes, 35, 2: 359–367

Chikh Ali M., Maoka T., Natsuaki K. T., Natsuaki T. 2010a. The simultaneous differentiation of Potato virus Y strains including the newly described strain PVY(NTN-NW) by multiplex PCR assay. Journal of Virological Methods, 165, 1: 15–20

Chikh Ali M., Maoka T., Natsuaki T., Natsuaki K. T. 2010b. PVY NTN-NW , a novel recombinant strain of Potato virus Y predominating in potato fields in Syria. Plant Pathology, 59, 1: 31–41

Chikh Ali M., Said Omar a, Natsuaki T. 2011. An infectious full-length cDNA clone of potato virus Y (NTN-NW), a recently reported strain of PVY that causes potato tuber necrotic ringspot disease. Archives in Virology, 8: 6–10

Chrzanowska M. 1991. New isolates of the necrotic strain of potato virus Y (PVYN) found recently in Poland Potato Research, 34, 2: 179–182

Chung B. Y.-W., Miller W. A., Atkins J. F., Firth A. E. 2008. An overlapping essential gene in the Potyviridae. The Proceedings of the National Academy of Sciences of the United States of America, 105,15: 5897–5902

Cuevas J. M., Delaunay A., Rupar M., Jacquot E., Elena S. F. 2012a. Molecular evolution and phylogeography of potato virus Y based on the CP gene. Journal of General Virology, 93, 11: 2496–501

Cuevas J. M., Delaunay A., Visser J. C., Bellstedt D. U., Jacquot E., Elena S. F. 2012b. Phylogeography and Molecular Evolution of Potato virus Y. PLoS One, 7, 5: e37853

Desbiez C., Chandeysson C., Lecoq H., Moury B. 2012. A simple, rapid and efficient way to obtain infectious clones of potyviruses. Journal of Virological Methods, 183,1: 94–97

Dietrich C., Maiss E. 2003. Fluorescent labelling reveals spatial separation of potyvirus populations in mixed infected Nicotiana benthamiana plants. Journal of General Virology, 84, 10: 2871–2876

Faurez F., Baldwin T., Tribodet M., Jacquot E. 2012. Identification of new Potato virus Y (PVY) molecular determinants for the induction of vein necrosis in tobacco. Molecular Plant Pathology, 13, 8: 948–959

Fedorkin O. N., Merits a, Lucchesi J., Solovyev a G., Saarma M., Morozov S. Y., Mäkinen K. 2000. Complementation of the movement-deficient mutations in potato virus X: potyvirus coat protein mediates cell-to-cell trafficking of C-terminal truncation but not deletion mutant of potexvirus coat protein. Virology, 270,1: 31–42

Feki S., Loukili M. J., Triki-Marrakchi R., Karimova G., Old I., Ounouna H., Nato A., Nato F., Guesdon J.-L., Lafaye P., Elgaaied a. B. A. 2005. Interaction between tobacco Ribulose-l,5-biphosphate Carboxylase/Oxygenase large subunit (RubisCO-LSU) and the PVY Coat Protein (PVY-CP). European Journal of Plant Pathology, 112, 3: 221–234

Food and Agriculture Organisation 2014. FAOSTAT: Potato (116); Retrieved from http://faostat3.fao.org/faostat-gateway/go/to/search/potato/E (15.2.2014)

Forcic D., Branovic-Cakanic K., Ivancic J., Jug R., Barut M., Strancar A. 2005. Purification of genomic DNA by short monolithic columns. Journal of Chromatography A, 1065,1: 115–120

Gao R., Tian Y.-P., Wang J., Yin X., Li X.-D., Valkonen J. P. T. 2012. Construction of an infectious cDNA clone and gene expression vector of Tobacco vein banding mosaic virus (genus Potyvirus). Virus Research, 169, 1: 276–281

Gerster P., Kopecky E.-M., Hammerschmidt N., Klausberger M., Krammer F., Grabherr R., Mersich C., Urbas L., Kramberger P., Paril T., Schreiner M., Nöbauer K., Razzazi-Fazeli E., Jungbauer A. 2013. Purification of infective baculoviruses by monoliths. Journal of Chromatography A, 1290, 36–45

Glais L., Kerlan C., Tribodet M., Tordo V. M.-J., Robaglia C., Astier-Manifacier S. 1996. Molecular characterization of potato virus YN isolates by PCR-RFLP. European Journal of Plant Pathology, 102 7, 655–662

Glais L., Tribodet M., Kerlan C. 2002. Genomic variability in Potato potyvirus Y (PVY): evidence that PVY(N)W and PVY(NTN) variants are single to multiple recombinants between PVY(O) and PVY(N) isolates. Archives of Virology, 147, 2: 363–78

Glais L., Tribodet M., Kerlan C. 2005. Specific detection of the PVY(N)-W variant of Potato virus Y. Journal of Virological Methods, 125, 2: 131–136

Grangeon R., Jiang J., Wan J., Agbeci M., Zheng H., Laliberté J.-F. 2013. 6K2-induced vesicles can move cell to cell during turnip mosaic virus infection. Frontiers in Microbiology, 4, 1-10

Gray S. M., DeBoer S. H., Lorenzen J., Karasev A. V., Whitworth J., Nolte P., Singh R. P., Boucher A., Xu H. 2010. Potato virus Y: A significant and evolving threat to potato crops in the United States and Canada. Plant Disease, 94, 12: 1384–1397

Guo H. S., López-Moya J. J., García J. A. 1998. Susceptibility to recombination rearrangements of a chimeric plum pox potyvirus genome after insertion of a foreign gene. Virus Research, 57, 2: 183–195

Gutiérrez-Aguirre I., Banjac M., Steyer A., Poljsak-Prijatelj M., Peterka M., Strancar A., Ravnikar M. 2009. Concentrating rotaviruses from water samples using monolithic chromatographic supports. Journal of Chromatography A, 1216, 2700–2704

Gutiérrez-Aguirre I., Hodnik V., Glais L., Rupar M., Jacquot E., Anderluh G., Ravnikar M. 2014. Surface plasmon resonance for monitoring the interaction of Potato virus Y with monoclonal antibodies. Analytical Biochemistry, 447, 74–81

Gutiérrez-Aguirre I., Steyer A., Banjac M., Kramberger P., Poljšak-Prijatelj M., Ravnikar M. 2011. On-site reverse transcription-quantitative polymerase chain reaction detection of rotaviruses concentrated from environmental water samples using methacrylate monolithic supports. Journal of Chromatography A, 1218, 17: 2368–2373

Hamm P. B., Hane D. C., Pavek M. J., Leroux L. D., Gieck S. L., David N. L. 2009. Potato Cultivars Differ in Current Season Potato Virus Y (PVY) Infection. American Journal of Potato Research, 87, 1: 19–26

Hofius D., Maier A. T., Dietrich C., Jungkunz I., Börnke F., Maiss E., Sonnewald U. 2007. Capsid protein-mediated recruitment of host DnaJ-like proteins is required for Potato virus Y infection in tobacco plants. Journal of Virology, 81, 21: 11870–11880

Hu X., Nie X., He C., Xiong X. 2011. Differential pathogenicity of two different recombinant PVY(NTN) isolates in Physalis floridana is likely determined by the coat protein gene. Virology Journal, 8, 1: 207

Huet H., Gal-On a, Meir E., Lecoq H., Raccah B. 1994. Mutations in the helper component protease gene of zucchini yellow mosaic virus affect its ability to mediate aphid transmissibility. Journal of General Virology, 75, 6: 1407–1414

Jakab G., Droz E., Brigneti G., Baulcombe D., Malnoë P. 1997. Infectious in vivo and in vitro transcripts from a full-length cDNA clone of PVY-N605, a Swiss necrotic isolate of potato virus Y. Journal of General Virology, 78, 12, 3141–3145

Karasev A. V, Hu X., Brown C. J., Kerlan C., Nikolaeva O. V, Crosslin J. M., Gray S. M. 2011. Genetic diversity of the ordinary strain of Potato virus Y (PVY) and origin of recombinant PVY strains. Phytopathology, 101, 7: 778–785

Karasev A. V., Gray S. M. 2013. Continuous and emerging challenges of Potato virus Y in potato. Annual Reviews of Phytopathol., 51, 571–586

Karasev A. V., Nikolaeva O. V., Hu X., Sielaff Z., Whitworth J., Lorenzen J. H., Gray S. M. 2009. Serological Properties of Ordinary and Necrotic Isolates of Potato virus Y: A Case Study of PVYN Misidentification. American Journal of Potato Research, 87, 1: 1–9

Kehoe M. A., Jones R. A. C. 2011. A proposal to help resolve the disagreement between naming of potato virus Y strain groups defined by resistance phenotypes and those defined by sequencing. Archives of Virology, 156, 12: 2273–2278

Kerlan C. 2006. Potato virus Y. Description of Plant Viruses 414; Retrieved from http://www.dpvweb.net/dpv/showdpv.php?dpvno=414 (12.3.2014)

Kerlan C., Nikolaeva O. V, Hu X., Meacham T., Gray S. M., Karasev A. V 2011. Identification of the Molecular Make-Up of the Potato virus Y Strain PVY(Z): Genetic Typing of PVY(Z)-NTN. Phytopathology, 101, 9: 1052–1060

Kogovšek P., Gow L., Pompe-Novak M., Gruden K., Foster G. D., Boonham N., Ravnikar M. 2008. Single-step RT real-time PCR for sensitive detection and discrimination of Potato virus Y isolates. Journal of Virological Methods, 149, 1: 1–11

Kogovšek P., Kladnik A., Mlakar J., Tušek-Žnidarič M., Dermastia M., Ravnikar M., Pompe-Novak M. 2011. Distribution of Potato virus Y in Potato Plant Organs, Tissues and Cells. Phytopathology, 101, 11: 1292–1300

Kovac K., Gutiérrez-Aguirre I., Banjac M., Peterka M., Poljsak-Prijatelj M., Ravnikar M., Mijovski J. Z., Schultz A. C., Raspor P. 2009. A novel method for concentrating hepatitis A virus and caliciviruses from bottled water. Journal of Virological Methods, 162, 1-2: 272–5

Krajacić M., Ivancic-Jelecki J., Forcic D., Vrdoljak A., Skorić D. 2007. Purification of plant viral and satellite double-stranded RNAs on DEAE monoliths. Journal of Chromatogr. A, 1144, 1: 111–9

Krajnc N. L., Smrekar F., Cerne J., Raspor P., Modic M., Krgovic D., Strancar A., Podgornik A. 2009. Purification of large plasmids with methacrylate monolithic columns. Journal of Separation Science, 32, 15-16: 2682–2690

Kramberger P., Honour R. C., Herman R. E., Smrekar F., Peterka M. 2010. Purification of the Staphylococcus aureus bacteriophages VDX-10 on methacrylate monoliths. Journal of Virological Methods, 166, 1-2: 60–64

Kramberger P., Peterka M., Boben J., Ravnikar M., Strancar A. 2007. Short monolithic columns--a breakthrough in purification and fast quantification of tomato mosaic virus. Journal of Chromatogr. A, 1144, 1: 143–149

Kramberger P., Petrovic N., Strancar A., Ravnikar M. 2004. Concentration of plant viruses using monolithic chromatographic supports. Journal of Virological Methods, 120, 1: 51–57

Kus M. 1995. The epidemic of the tuber necrotic ringspot strain of potato virus Y PVY NTN and its effect on potato crops in Slovenia. Proc. 9th Eur. Assoc. Potato Research Virology Sect. Bled, Slov., 159–160

Lansac M., Eyquard J. P., Salvador B., Garcia J. A., Gall O. Le, Decroocq V., Schurdi-Levraud Escalettes V. 2005. Application of GFP-tagged Plum pox virus to study Prunus-PPV interactions at the whole plant and cellular levels. Journal of Virological Methods, 129, 2: 125–133

Lee M. Y., Song Y. S., Ryu K. H. 2011. Development of infectious transcripts from full-length and GFP-tagged cDNA clones of Pepper mottle virus and stable systemic expression of GFP in tobacco and pepper. Virus Research, 155, 2: 487–494

Leiser R.-M., Richter J. 1978. Reinigung und einige Eigenschaften des Kartoffel-Y-Virus. Archives Of Phytopathology And Plant Protection, 14, 6: 337–350

López-Moya J. J., García J. a 2000. Construction of a stable and highly infectious intron-containing cDNA clone of plum pox potyvirus and its use to infect plants by particle bombardment. Virus Research, 68, 2: 99–107

Lorenzen J. H., Piche L. M., Gudmestad N. C., Meacham T., Shiel P. 2006. A Multiplex PCR Assay to Characterize Potato virus Y Isolates and Identify Strain Mixtures. Plant Disease, 90, 7: 935–940

Lucas W. J. 2006. Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. Virology, 344, 1: 169–184

MacFarlane S. A., Popovich A. H. 2000. Efficient expression of foreign proteins in roots from tobravirus vectors. Virology, 267, 1: 29–35

Mehle N., Kovač M., Petrovič N., Novak M. P., Baebler Š., Stres H. K., Gruden K., Ravnikar M. 2004. Spread of potato virus YNTN in potato cultivars (Solanum tuberosum L.) with different levels of sensitivity. Physiological Molecular Plant Pathology, 64, 6: 293–300

Meng Q., Siebke K., Lippert P., Baur B., Mukherjee U., Weis E. 2001. Sink-source transition in tobacco leaves visualized using chlorophyll fluorescence imaging. New Phytologist, 151, 3: 585–595

Monjezi R., Tey B. T., Sieo C. C., Tan W. S. 2010. Purification of bacteriophage M13 by anion exchange chromatography. Journal of Chromatogr. B, 878, 21: 1855–1859

Moravec T., Cerovska N., Boonham N. 2003. The detection of recombinant, tuber necrosing isolates of Potato virus Y (PVYNTN) using a three-primer PCR based in the coat protein gene. Journal of Virological Methods, 109, 1: 63–68

Moury B., Simon V. 2011. dN/dS-Based Methods Detect Positive Selection Linked to Trade-Offs between Different Fitness Traits in the Coat Protein of Potato virus Y. Molecular Biology of Evolution, 28, 9: 2707–2717

Nie X., Singh R. P. 2002. A new approach for the simultaneous differentiation of biological and geographical strains of Potato virus Y by uniplex and multiplex RT-PCR. Journal of Virological Methods, 104, 1: 41–54

Nie X., Singh R. P. 2003. Evolution of North American PVY(NTN) strain Tu 660 from local PVY(N) by mutation rather than recombination. Virus Genes, 26, 1: 39–47

Nikolaeva O., Roop D., Galvino-Costa S., Figueira A., Gray S., Karasev A. 2012. Epitope mapping for monoclonal antibodies recognizing tuber necrotic isolates of Potato virus Y. American Journal Potato Research, 89, 121– 128

Nolte P., Whitworth J., Thornton M. K., McIntosh C. 2004. Effect of seedborne Potato virus Y on performance of Russet Burbank, Russet Norkotah, and Shepody potato. Plant Disease, 88, 248–252

Ohshima K., Inoue A. K., Ishikawa Y., Shikata E., Hagita T. 1990. Production and application of monoclonal antibodies specific to ordinary strain and necrotic strain of potato virus Y. Japanese Journal Phytopathology, 56, 4: 508–514

Oparka K. J., Roberts A. G., Roberts I. M., Prior D. A. M., Cruz S. 1996. Viral coat protein is targeted to, but does not gate, plasmodesmata during cell-to-cell movement of potato virus X. Plant Journal, 10, 5: 805–813

Peng Y. H., Kadoury D., Gal-On a, Huet H., Wang Y., Raccah B. 1998. Mutations in the HC-Pro gene of zucchini yellow mosaic potyvirus: effects on aphid transmission and binding to purified virions. Journal General Virology, 79, 4: 897–904

Perica M. C., Sola I., Urbas L., Smrekar F., Krajacić M. 2009. Separation of hypoviral double-stranded RNA on monolithic chromatographic supports. Journal Chromatogr. A, 1216, 13: 2712–2716

Peterka M., Strancar A., Banajc M., Kramberger P., Maurer E., Muster T. 2008. Method for influenza virus purification. World Patent application. WO 2008/006780 A1, 44 str.

Piche L. M., Singh R. P., Nie X., Gudmestad N. C. 2004. Diversity Among Potato virus Y Isolates Obtained from Potatoes Grown in the United States. Phytopathology, 94, 12: 1368–1375

Podgornik A., Strancar A. 2005. Convective Interaction Media (CIM)-short layer monolithic chromatographic stationary phases. Biotechnology Annual Reviews, 11: 281–333

Quenouille J., Vassilakos N., Moury B. 2013. Potato virus Y: a major crop pathogen that has provided major insights into the evolution of viral pathogenicity. Molecular Plant Pathology, 14, 5: 439–452

Rajamäki M.-L., Kelloniemi J., Alminaite A., Kekarainen T., Rabenstein F., Valkonen J. P. T. 2005. A novel insertion site inside the potyvirus P1 cistron allows expression of heterologous proteins and suggests some P1 functions. Virology, 342, 1: 88–101

Rajamäki M.-L., Valkonen J. P. T. 2003. Localization of a potyvirus and the viral genome-linked protein in wild potato leaves at an early stage of systemic infection. Molecular Plant Microbe Interactions, 16, 1: 25–34

Regoes R. R., Hamblin S., Tanaka M. M. 2012. Viral mutation rates: modelling the roles of within-host viral dynamics and the trade-off between replication fidelity and speed. Proceedings of the Royal society B: Biological Science, 280, 1750: 1-9

Rigotti S., Gugerli P. 2007. Rapid identification of potato virus Y strains by one-step triplex RT-PCR. Journal of Virological Methods, 140, 1-2: 90–94

Roberts A. G., Cruz S. S., Roberts I. M., Prior D. A. M., Turgeon R., Oparka K. J. 1997. Phloem Unloading in Sink Leaves of Nicotiana benthamíana : Comparison of a Fluorescent Solute with a Fluorescent Virus. The Plant Cell, 9: 1381–1396

Rolland M., Glais L., Kerlan C., Jacquot E. 2008. A multiple single nucleotide polymorphisms interrogation assay for reliable Potato virus Y group and variant characterization. Journal of Virological Methods, 147, 1: 108–117

Romancer M. Le, Kerlan C., Nedellec M. 1994. Biological characterisation of various geographical isolates of potato virus Y inducing superficial necrosis on potato tubers. Plant Pathology, 43, 1: 138–144

Rosner A., Maslenin L. 1999. Transcript conformation polymorphism : A novel approach for differentiating PVYNTN. Journal of Phytopathology, 147, 11-12: 661–664

Routh A., Ordoukhanian P., Johnson J. E. 2012. Nucleotide-resolution profiling of RNA recombination in the encapsidated genome of a eukaryotic RNA virus by next-generation sequencing. Journal of Molecular Biology, 424, 5: 257–69

Rupar M., Kogovšek P., Pompe-Novak M., Gutiérrez-Aguirre I., Delaunay A., Jacquot E., Ravnikar M. 2013a. Assessment of SNaPshot and single step RT-qPCR methods for discriminating Potato virus Y (PVY) subgroups. Journal of Virological Methods, 189, 1: 93–100

Rupar M., Ravnikar M., Tušek-Žnidarič M., Kramberger P., Glais L., Gutiérrez-Aguirre I. 2013b. Fast purification of the filamentous Potato virus Y using monolithic chromatographic supports. Journal of Chromatography A, 1272, 33–40

Ruščić J., Gutiérrez-Aguirre I., Urbas L., Kramberger P., Mehle N., Škorić D., Barut M., Ravnikar M., Krajačić M. 2013. A novel application of methacrylate based short monolithic columns: concentrating Potato spindle tuber viroid from water samples. Journal of Chromatography. A, 1274, 129–136

Sanz A., Cambra M., Roman C. P., Miguet J. G., Cortes E., Gorris M. T., Vela C. 1990. Preparation of additional monoclonal antibodies for detection and discrimination of potato virus Y isolates infecting potato. Potato Research, 33, 3: 365–375

Scholthof K. G., Adkins S., Czosnek H., Palukaitis P., Jacquot E., Hohn T., Hohn B., Saunders K., Candresse T., Ahlquist P., Hemenway C., Foster G. D. 2011. Top 10 plant viruses in molecular plant pathology. Molecular Plant Pathology, 12: 938–954

Schubert J., Fomitcheva V., Sztangret-Wiśniewska J. 2007. Differentiation of Potato virus Y strains using improved sets of diagnostic PCR-primers. Journal of Virological Methods, 140, 1-2: 66–74

Seaberg B. L., Hsieh Y.-C., Scholthof K.-B. G., Scholthof H. B. 2012. Host impact on the stability of a plant virus gene vector as measured by a new fluorescent local lesion passaging assay. Journal of Virological Methods, 179, 2: 289–294

Seo J., Kang S., Seo B. O. Y., Jung J. I. N. K. Y. O. 2010. Mutational analysis of interaction between coat protein and helper component-proteinase of Soybean mosaic virus involved in aphid transmission. Molecular Plant Pathology, 11: 265–276

Singh R. P., Valkonen J. P. T., Gray S. M., Boonham N., Jones R. A. C., Kerlan C., Schubert J. 2008. The naming of Potato virus Y strains infecting potato. Archives in Virology, 153, 1: 1–13

Smrekar F., Ciringer M., Peterka M., Podgornik A., Strancar A. 2008. Purification and concentration of bacteriophage T4 using monolithic chromatographic supports. Journal of chromatography. B, Analytical technologies in the biomedical and life sciences, 861, 2: 177–180

Steyer A., Gutiérrez-Aguire I., Kolenc M., Koren S., Kutnjak D., Pokorn M., Poljšak-Prijatelj M., Racki N., Ravnikar M., Sagadin M., Fratnik Steyer A., Toplak N. 2013. High similarity of novel orthoreovirus detected in a child hospitalized with acute gastroenteritis to mammalian orthoreoviruses found in bats in Europe. Journal Clinical Microbiology, 51, 11: 3818–3825

Szemes M., Klerks M. . M., Heuvel J. F. J. M. van den, Schoen C. D. 2002. Development of a multiplex AmpliDet RNA assay for simultaneous detection and typing of potato virus Y isolates. Journal of Virological Methods, 100, 1-2: 83–96

Trauner A., Bennett M. H., Williams H. D. 2011. Isolation of bacterial ribosomes with monolith chromatography. PLoS One, 6, 2: e16273

Tribodet M., Glais L., Kerlan C., Jacquot E. 2005. Characterization of Potato virus Y, PVY) molecular determinants involved in the vein necrosis symptom induced by PVYN isolates in infected Nicotiana tabacum cv. Xanthi. Journal General Virology, 86, 7: 2101–2105

Ullah Z., Grumet R. 2002. Localization of Zucchini yellow mosaic virus to the veinal regions and role of viral coat protein in veinal chlorosis conditioned by the zym potyvirus resistance locus in cucumber. Physiology of Molecular Plant Pathology, 60, 2: 79–89

Urbas L., Jarc B. L., Barut M., Zochowska M., Chroboczek J., Pihlar B., Szolajska E. 2011. Purification of recombinant adenovirus type 3 dodecahedric virus-like particles for biomedical applications using short monolithic columns. Journal of Chromatography A, 1218, 17: 2451–2459

Urcuqui-Inchima S., Haenni a L., Bernardi F. 2001. Potyvirus proteins: a wealth of functions. Virus Research, 74, 1-2: 157–175

Vijayapalani P., Maeshima M., Nagasaki-Takekuchi N., Miller W. A. 2012. Interaction of the Trans-Frame Potyvirus Protein P3N-PIPO with Host Protein PCaP1 Facilitates Potyvirus Movement. PLoS Pathogens, 8, 4: e1002639

Wang G., Gentry T. J., Grass G., Josephson K., Rensing C., Pepper I. L. 2004. Real-time PCR quantification of a green fluorescent protein-labeled, genetically engineered Pseudomonas putida strain during 2-chlorobenzoate degradation in soil. FEMS Microbiology Letters, 233, 2: 307–314

Wang H. L., Sudarshana M. R., Gilbertson R. L., Lucas W. J. 1999. Analysis of cell-to-cell and long-distance movement of a bean dwarf mosaic geminivirus-green fluorescent protein reporter in host and nonhost species: identification. Molecular Plant Microbe Interactions, 12, 4: 345–355

Wei T., Zhang C., Hong J., Xiong R., Kasschau K. D., Zhou X., Carrington J. C., Wang A. 2010. Formation of complexes at plasmodesmata for potyvirus intercellular movement is mediated by the viral protein P3N-PIPO. PLoS Pathogens, 6, 6: e1000962

ZAHVALA

Najprej se iskreno zahvaljujem svojima mentorjema in kolegoma prof. dr. Maji Ravnikar in dr. Ionu Gutiérrezu-Aguirreju, ki sta me usmerjala, mi svetovala in me bodrila tako na znanstveno-raziskovalnem področju, kot tudi v širših življenjskih dilemah, s katerimi sem se srečal v času doktorata. Hvala za številne konstruktivne, plodne in zabavne diskusije, za pomoč pri reševanju problemov in snovanju idej ter za odpiranje številnih novih zornih kotov. Omogočala in podpirala sta mojo svobodo znanstvenega raziskovanja ter željo po raziskovanju bližnjih in daljnih krajev. Še posebej hvala Ionu za vse poučne in zabavne ure, ki sva jih skupaj preživela v laboratoriju in izven. Pod mentorstvom Maje in Iona sem zrasel na vseh področjih, njuni nasveti in nauki me bodo vedno spremljali.

I would like to thank to Dr. Emmanuel Jacquot, Agnes Delaunay, Dr. Florence Faurez, Michel Tribodet and the rest of the "ex-VIRO" team at Institut National Recherche Agronomique, Rennes, France, where a considerable part of the research was conducted. Without their collaboration there would be no results regarding the fluorescently labelled PVY. In their pleasant company I obtained plenty of new knowledge and also international collaboration experience. Merci, merci beaucoup.

Prof. dr. Darji Žgur Bertok in prof. dr. Tatjani Avšič Zupanc se zahvaljujem za pregled doktorskega dela, dr. Sebastijanu Radišku za sodelovanje pri pripravi dispozicije doktorske naloge.

Lepa hvala študentki Maji Križnik za pomoč pri eksperimentalnem delu, Lidiji Matičič in Špeli Prijatelj Novak za skrbno vzdrževanje testnih rastlin.

Zahvala gre prav vsem zaposlenim na Oddelku za biotehnologijo in sistemsko biologijo Nacionalnega inštituta za biologijo, ki so mi tako ali drugače popestrili vsak delovni dan. Še posebej bi se zahvalil bližnjim sodelavcem in prijateljem Anni Coll Rius, Tjaši Stare, Urški Čepin, Klemnu Zupančiču, Nejcu Račkiju, Živi Ramšak, Ani Lazar, Denisu Kutnjaku, Roku Lenarčiču in Marku Petku, s katerimi sem preživel veliko nepozabnih družabno-raziskovalnih trenutkov.

Posebna zahvala gre Davidu in Riji Arko za lektoriranje doktorskega dela.

Hvala tudi družini, predvsem sestricama Barbari in Ani, ter vsem prijateljicam in prijateljem, ki morda ne vedo veliko o nukleotidnih zaporedjih in fluorescentnih proteinih, vendar pa so me kljub temu vsa leta spodbujali, podpirali in se z mano veselili uspehov.

Priloga A

Dovoljenje za objavo članka »Molecular evolution and phylogeography of potato virus Y based on the CP gene«

Cuevas J. M., Delaunay A., Rupar M., Jacquot E., Elena S. F. 2012. Molecular evolution and phylogeography of potato virus Y based on the CP gene. J. Gen. Virol., vol. 93, 2496-2501

Odgovor uredništva Journal of General Virology :

Dear Matevz,

Thank you for your permission request. SGM no longer responds to individual permission requests; these are now handled by the Copyright Clearance Center.
If you are an author (or an author's representative) of the content for which you are requesting permission:
The copyright transfer agreement/licence to publish that was signed by the authors gives express permission for authors to reuse their own content, provided that the journal is acknowledged as the original source. No further permission is required.
If another publisher requires confirmation that permission has been granted, please forward this email to them.
If you are NOT an author of the content for which you are requesting permission:
Please click on the '© Get Permissions' link that appears with the online article.
Alternatively, you can visit www.copyright.com.
Please note that the Copyright Clearance Center will charge a processing fee for each request. Additional charges may apply for reuse for "for-profit" purposes.

Kind regards,

Cath Hicklin
JGV Editorial Office

Priloga B

Dovoljenje za objavo člankov z naslovom »Assessment of SNaPshot and single step RT-qPCR methods for discriminating Potato virus Y (PVY) subgroups« in »Fast purification of the filamentous Potato virus Y using monolithic chromatographic supports«

Rupar M., Kogovšek P., Pompe-Novak M., Gutiérrez-Aguirre I., Delaunay A., Jacquot E., Ravnikar M. 2013. Assessment of SNaPshot and single step RT-qPCR methods for discriminating Potato virus Y (PVY) subgroups. J. Virol. Methods, 189 (1), 93–100

Rupar M., Ravnikar M., Tušek-Žnidarič M., Kramberger P., Glais L., Gutiérrez-Aguirre I. 2013. Fast purification of the filamentous Potato virus Y using monolithic chromatographic supports. J. Chromatogr. A, 1272, 33–40

Del korespondence z založnikom revij:

Dear Matevž:

Permission is covered by the rights you retain as an Elsevier journal author as outlined at http://www.elsevier.com/journal-authors/author-rights-and-responsibilities, which include Inclusion in a thesis or dissertation, provided that proper acknowledgement is given to the original source of publication. Permission extends to online publication of your thesis provided that the Elsevier journal article is not available for download as a standalone PDF but only embedded within the thesis itself. As this is a retained right, no written permission is necessary.

Should you require any further assistance, please let me know. Best of luck with your thesis.

Regards,
Hop

Hop Wechsler
Permissions Helpdesk Manager
Global Rights Department
Elsevier
1600 John F. Kennedy Boulevard
Suite 1800
Philadelphia, PA 19103-2899
Tel: +1-215-239-3520
Mobile: +1-215-900-5674
Fax: +1-215-239-3805
E-mail: h.wechsler@elsevier.com

Priloga C

Dodatno gradivo k članku »Rupar M., Kogovšek P., Pompe-Novak M., Gutiérrez-Aguirre I., Delaunay A., Jacquot E., Ravnikar M. 2013. Assessment of SNaPshot and single step RT-qPCR methods for discriminating Potato virus Y (PVY) subgroups. J. Virol. Methods, 189 (1), 93–100«

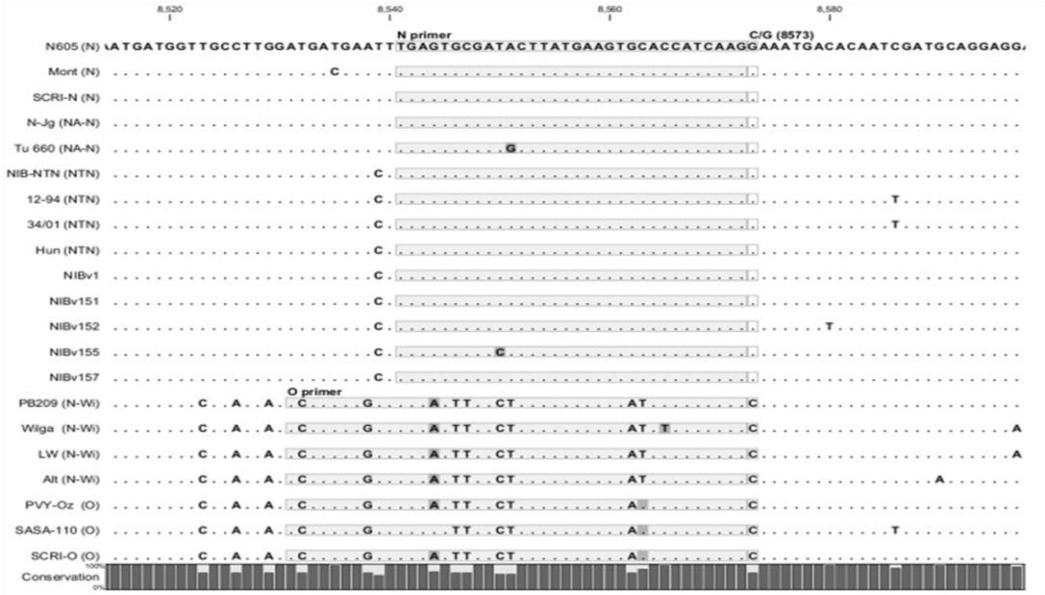


Fig. A.1 In silico analysis of the SNaPshot primer hybridizing sites and CP N terminal SNP position (C/G 8573). The PVY subgroup (listed in the NCBI Nucleotide database) is denoted in parentheses next to the strain name. Primer hybridizing positions are...*Assessment of SNaPshot and single step RT-qPCR methods for discriminating* Potato virus Y *(PVY) subgroups*

http://dx.doi.org/10.1016/j.jviromet.2013.01.013

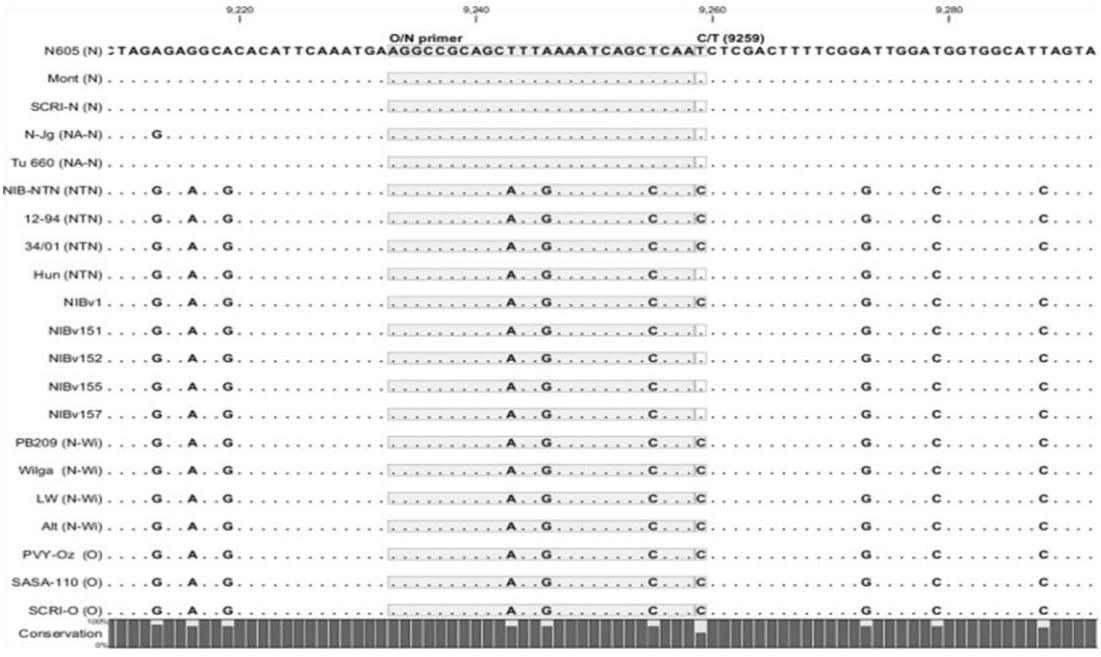


Fig. A.2 In silico analysis of the SNaPshot primer hybridizing sites and Mid CP SNP position (C/T 9259). The PVY subgroup (listed in the NCBI Nucleotide database) is denoted in parentheses next to the strain name. Primer hybridizing positions are denote...*Assessment of SNaPshot and single step RT-qPCR methods for discriminating* Potato virus Y *(PVY) subgroups*

http://dx.doi.org/10.1016/j.jviromet.2013.01.013

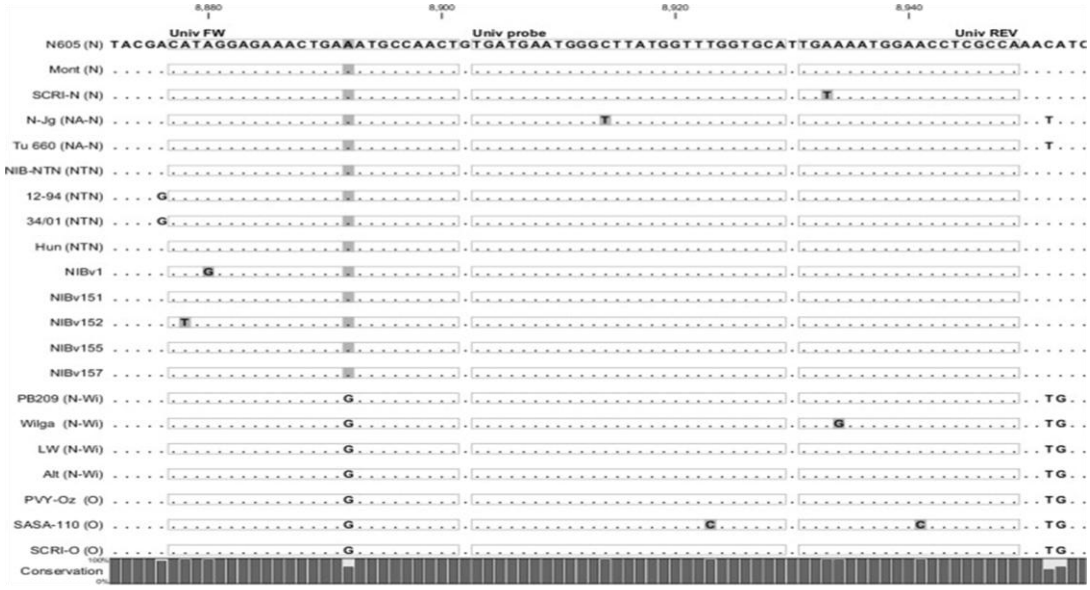


Fig. A.3 In silico analysis of the RT-qPCR primer and probe hybridizing sites of the universal PVY amplicon (Univ). The PVY subgroup (listed in the NCBI Nucleotide database) is denoted in parentheses next to the strain name. Primer and probe hybridizing ...*Assessment of SNaPshot and single step RT-qPCR methods for discriminating* Potato virus Y *(PVY) subgroups*

http://dx.doi.org/10.1016/j.jviromet.2013.01.013

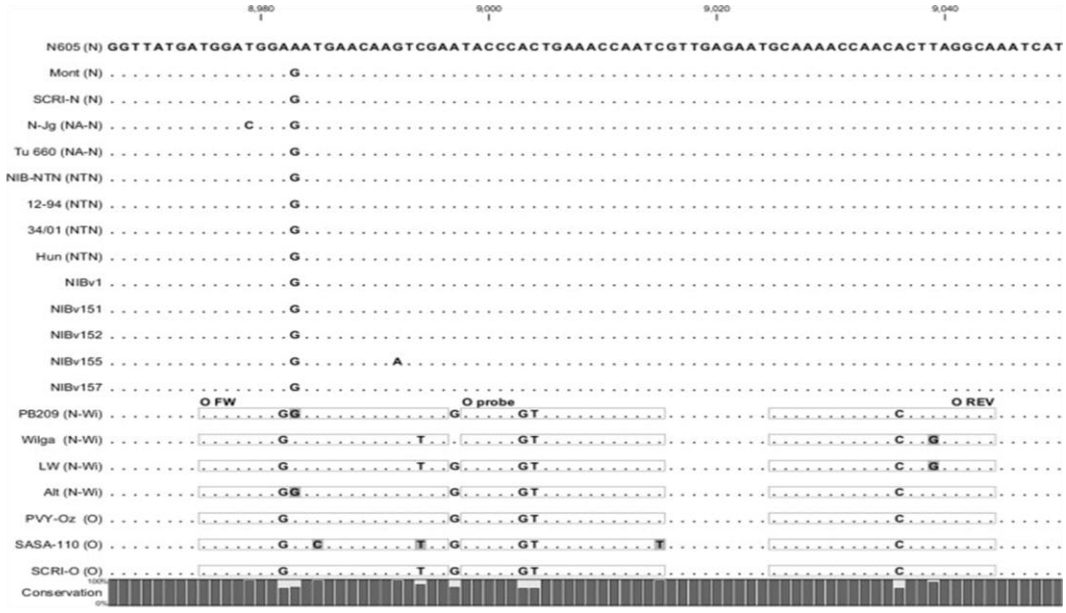


Fig. A.4 In silico analysis of the RT-qPCR primer and probe hybridizing sites of the PVY O specific amplicon. The PVY subgroup (listed in the NCBI Nucleotide database) is denoted in parentheses next to the strain name. Primer and probe hybridizing posit...*Assessment of SNaPshot and single step RT-qPCR methods for discriminating* Potato virus Y *(PVY) subgroups*

http://dx.doi.org/10.1016/j.jviromet.2013.01.013

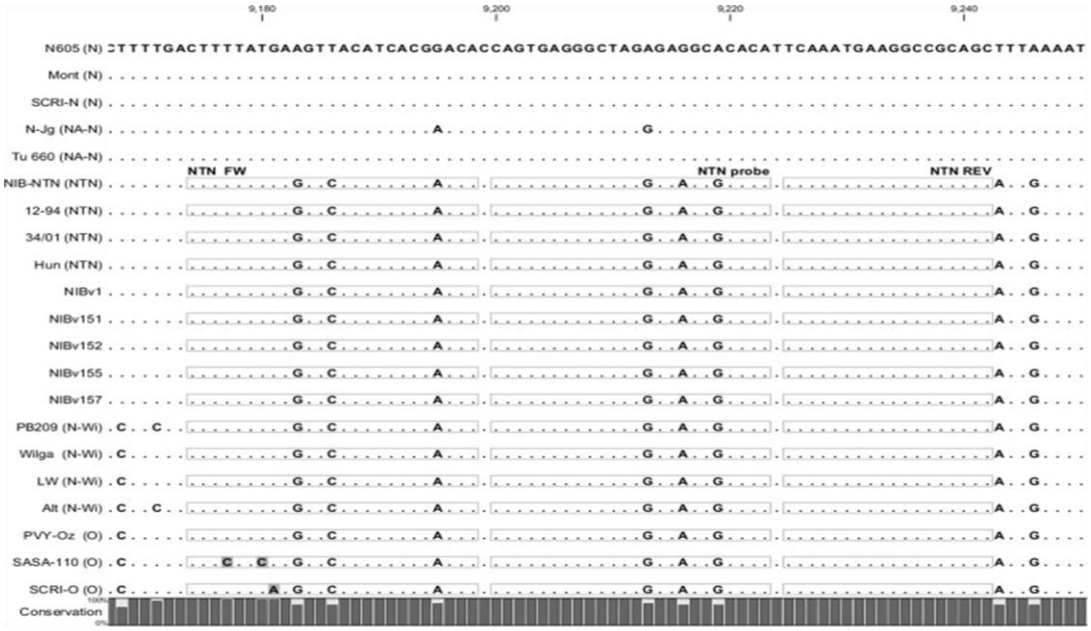


Fig. A.5 In silico analysis of the RT-qPCR primer and probe hybridizing sites of the PVY NTN specific amplicon. The PVY subgroup (listed in the NCBI Nucleotide database) is denoted in parentheses next to the strain name. Primer and probe hybridizing pos...*Assessment of SNaPshot and single step RT-qPCR methods for discriminating* Potato virus Y *(PVY) subgroups*

http://dx.doi.org/10.1016/j.jviromet.2013.01.013

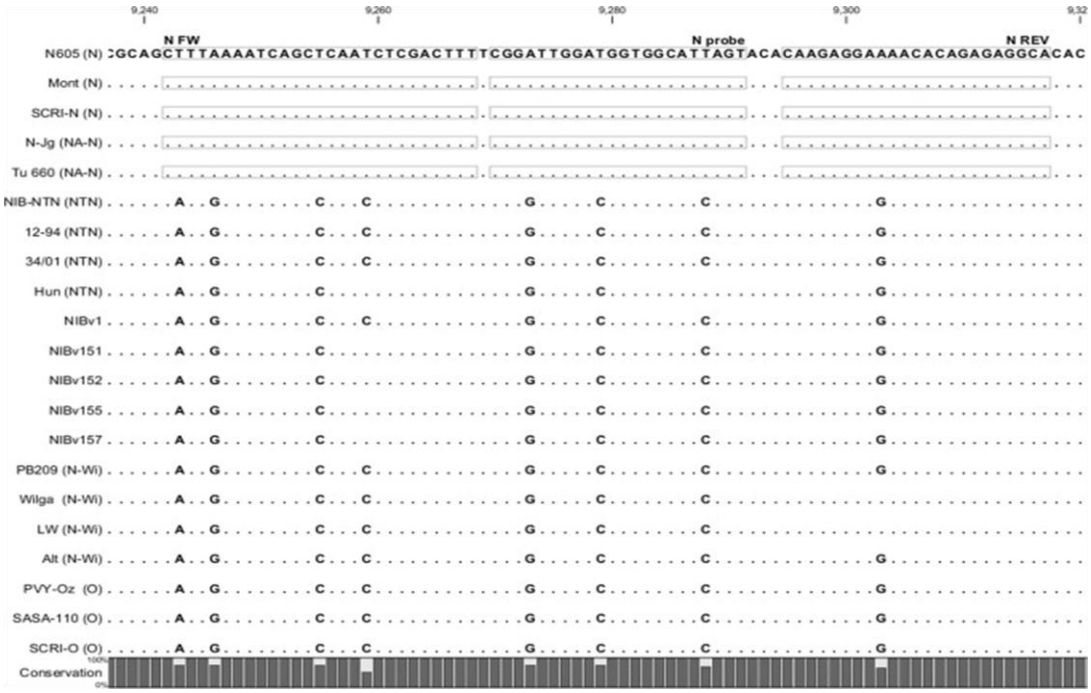


Fig. A.6 In silico analysis of the RT-qPCR primer and probe hybridizing sites of the PVY N specific amplicon. The PVY subgroup (listed in the NCBI Nucleotide database) is denoted in parentheses next to the strain name. Primer and probe hybridizing posit...*Assessment of SNaPshot and single step RT-qPCR methods for discriminating* Potato virus Y *(PVY) subgroups*

http://dx.doi.org/10.1016/j.jviromet.2013.01.013

Priloga D

Dodatno gradivo k članku »Cuevas J. M., Delaunay A., Rupar M., Jacquot E., Elena S. F. 2012. Molecular evolution and phylogeography of potato virus Y based on the CP gene. J. Gen. Virol., vol. 93, 2496-2501«

Table S1. PVY isolates used in the present study

Underlined isolates in column one, newly described in this paper.

Isolate	GenBank accession no.	Origin	Host	Collection date
156	AJ889867	Germany	*S.tuberosum*	
605	X97895	Switzerland	*S.tuberosum*	1976
12-94*	AJ889866	Poland	*S.tuberosum*	1994
156var	AJ889868	Germany	*S.tuberosum*	2004
261-4	AM113988	Germany	*S.tuberosum*	2004
34/01*	AJ890342	Poland	*S.tuberosum*	2001
423-3*	AY884982	USA	*S.tuberosum*	2002
53-29	AJ390298	Denmark	*S.tuberosum*	
53-49	AJ390299	Denmark	*S.tuberosum*	
Adgen-C	AJ890348	France	*S.tuberosum*	2005
Al-Baqa'*	EU073854	Jordan	*S.tuberosum*	
Al-Ghor*	EU073855	Jordan	*S.tuberosum*	
Al-Mafraq*	EU073857	Jordan	*S.tuberosum*	
aL-Ramtha	EU073859	Jordan	*S.tuberosum*	
Alt*	AY884985	USA	*S.tuberosum*	2002
Anqiu4	EF592517	China	*N.tabacum*	
AQ1	EF592513	China	*N.tabacum*	
Ca/H*	AJ535662	Hungary	*C.annuum*	
CAA141	JQ954317	France	*C.annuum*	1999
CAA15	JQ954318	France	*C.annuum*	2000
CAA82	JQ954315	Israel	*C.annuum*	1982
CAPA7	JQ954316	Tunisia	*C.annuum*	2006
CC24_5	GQ853667	South Africa	*S.tuberosum*	
CC55_8_146	GQ853652	South Africa	*S.tuberosum*	
CC62_20_156*	GQ853623	South Africa	*S.tuberosum*	

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

CC66_91_47	GQ853653	South Africa	*S.tuberosum*	
CC9_12_171*	GQ853621	South Africa	*S.tuberosum*	
CC9_30_175	GQ853650	South Africa	*S.tuberosum*	
CC9_47_177*	GQ853622	South Africa	*S.tuberosum*	
CC9_48_178	GQ853651	South Africa	*S.tuberosum*	
Chile3	FJ214726	Chile	*C.baccatum*	2005
DD019_141_138	GQ853661	South Africa	*S.tuberosum*	
DD020_92_30*	GQ853624	South Africa	*S.tuberosum*	
DD037F_31_186*	GQ853625	South Africa	*S.tuberosum*	
DD037F_35_188*	GQ853626	South Africa	*S.tuberosum*	
DD037F_9_154	GQ853655	South Africa	*S.tuberosum*	
DD051_14	GQ853660	South Africa	*S.tuberosum*	
DD051_7	GQ853659	South Africa	*S.tuberosum*	2007
DD103A_101_190	GQ853657	South Africa	*S.tuberosum*	
DD103A_184_191	GQ853603	South Africa	*S.tuberosum*	
DD103A_80_180	GQ853656	South Africa	*S.tuberosum*	
DD122A_25	GQ853658	South Africa	*S.tuberosum*	
DD122A_34	GQ853662	South Africa	*S.tuberosum*	
DD122A_36*	GQ853627	South Africa	*S.tuberosum*	
Ditta*	AJ890344	Austria	*S.tuberosum*	1998
Fanzhen6	EF592515	China	*S.tuberosum*	
Fanzhen8	EF592521	China	*S.tuberosum*	
Foggia	EU482153	Italy	*L.esculentum*	2007
FX24	EF592514	China	*S.tuberosum*	
German_14*	JQ954384	Germany	*S.tuberosum*	2003
German_16*	JQ954295	Germany	*S.tuberosum*	2003
German_20	JQ954387	Germany	*S.tuberosum*	2004
German_33	JQ954296	Germany	*S.tuberosum*	2004

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

German_34	JQ954297	Germany	*S.tuberosum*	2004
German_35	JQ954298	Germany	*S.tuberosum*	2004
German_37	JQ954299	Germany	*S.tuberosum*	2004
German_38	JQ954300	Germany	*S.tuberosum*	2004
German_39	JQ954301	Germany	*S.tuberosum*	2004
German_4*	JQ954302	Germany	*S.tuberosum*	2002
German_41	JQ954303	Germany	*S.tuberosum*	2004
German_42	JQ954304	Germany	*S.tuberosum*	2004
German_43	JQ954305	Germany	*S.tuberosum*	2004
German_45	JQ954306	Germany	*N.tabacum*	2006
German_47	JQ954314	Germany	*S.tuberosum*	2003
German_51	JQ954307	Germany	*S.tuberosum*	2004
German_52	JQ954308	Germany	*S.tuberosum*	2004
German_55	JQ954309	Germany	*S.tuberosum*	2004
German_56	JQ954310	Germany	*S.tuberosum*	2004
German_57	JQ954311	Germany	*S.tuberosum*	2004
German_58	JQ954312	Germany	*S.tuberosum*	2004
German_62*	JQ954342	Germany	*S.tuberosum*	2004
German_65	JQ954313	Germany	*S.tuberosum*	2004
GG517_128	GQ853635	South Africa	*S.tuberosum*	2005
GG517_170_168	GQ853636	South Africa	*S.tuberosum*	
GG517_93_160	GQ853593	South Africa	*S.tuberosum*	
Gpost*	JN936420	South Africa	*S.tuberosum*	2010
GR_PVY12	JQ954319	Greece	*L.esculentum*	1998
GR_PVY13	JQ954320	Greece	*L.esculentum*	1999
GR_PVY84*	JQ954321	Greece	*S.tuberosum*	2004
Gr99*	AJ890343	Poland	*N.tabacum*	1999
Hangzhou	AJ488834	China	*S.tuberosum*	

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

HN2	GQ200836	China	*S.tuberosum*	2007
Thole*	M95491	Hungary	*S.tuberosum*	1993
IAC†	AY840082	Brazil	*S.tuberosum*	
Irbid*	EU073856	Jordan	*S.tuberosum*	
Isol5	AJ890350	Germany	*S.tuberosum*	
IT_104	JQ954323	Italy	*S.tuberosum*	1997
IT_115*	JQ954324	Italy	*S.tuberosum*	1998
IT_117	JQ954325	Italy	*S.tuberosum*	1998
IT_101*	JQ954322	Italy	*S.tuberosum*	1998
IT_215*	JQ954326	Italy	*S.tuberosum*	1998
L26*	FJ204165	USA	*S.tuberosum*	2007
Laiwu1	EF592525	China	*S.tuberosum*	
Laiwu29	EF592527	China	*N.tabacum*	
Laiwu3	EF592516	China	*S.tuberosum*	
Laiwu9*	EF592526	China	*S.tuberosum*	
Linda*	AJ890345	Germany	*S.tuberosum*	2004
Linkou29	EF592524	China	*N.tabacum*	
LW	AJ890349	Poland	*S.tuberosum*	1970
LYE842	AJ43954	Canary Islands	*L.esculentum*	1984
ME173	FJ643479	USA	*S.tuberosum*	2006
Mengyin3	EF592518	China	*N.tabacum*	
MengyinA	EF592519	China	*N.tabacum*	
MengyinC	EF592520	China	*N.tabacum*	
Mont	AY884983	USA	*S.tuberosum*	2001
N_Nysa†	FJ666337	Poland	*S.tuberosum*	1974
N484_1	GQ853634	South Africa	*S.tuberosum*	
Naur*	EU073858	Jordan	*S.tuberosum*	
NC57	DQ309028	USA	*N.tabacum*	1973

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

NE-11*	DQ157180	USA	*S.tuberosum*	2003
N-Egypt	AF52229	Egypt	*S.tuberosum*	2001
New_Zealand	AM268435	New Zealand	*S.tuberosum*	2002
NIB-NTN*	AJ585342	Slovenia	*S.tuberosum*	
Nicola	AJ890346	Germany	*N.tabacum*	1999
N-Jg	AY166867	Canada	*S.tuberosum*	1991
NN300_155_19	GQ853597	South Africa	*S.tuberosum*	
NN300_155_22	GQ853598	South Africa	*S.tuberosum*	
NN300_41_123	GQ853595	South Africa	*S.tuberosum*	
NN300_60_23	GQ853663	South Africa	*S.tuberosum*	
NN300_76_118*	GQ853628	South Africa	*S.tuberosum*	
NN300_98_31	GQ853596	South Africa	*S.tuberosum*	
NN300_99_34	GQ853664	South Africa	*S.tuberosum*	
NN333B_28_149*	GQ853629	South Africa	*S.tuberosum*	
NN333B_87_152*	GQ853630	South Africa	*S.tuberosum*	
NN459_14	GQ853631	South Africa	*S.tuberosum*	
NN459_25	GQ853599	South Africa	*S.tuberosum*	
NN71_111	GQ853594	South Africa	*S.tuberosum*	2005
nnp*	AF237963	Italy	*C.annuum*	1992
NN-UK-N†	AJ390296	UK	*S.tuberosum*	
NN-UK-O	AJ390297	UK	*S.tuberosum*	
NTND6	AB331515	Japan	*S.tuberosum*	1997
NTNHO90	AB331517	Japan	*S.tuberosum*	1997
NTNHO92	AB331549	Japan	*S.tuberosum*	
NTNHO95	AB331550	Japan	*S.tuberosum*	
NTNK114	AB331540	Japan	*S.tuberosum*	
NTNNN99	AB331518	Japan	*S.tuberosum*	1997
NTNOK102	AB331546	Japan	*S.tuberosum*	

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

NTNOK105	AB331516	Japan	*S.tuberosum*	1997
NTNON92	AB331519	Japan	*S.tuberosum*	1997
O-Des	AJ390305	UK	*S.tuberosum*	
O-Gov	AJ390301	UK	*S.tuberosum*	
O-Tom	AJ390307	Portugal	*S.tuberosum*	
P21-82	AJ303097	Spain	*C.annuum*	
P21-82b	AJ005639	Spain	*C.annuum*	
PB_602	JQ954329	The Netherlands	*S.tuberosum*	1978
PB_702	JQ954327	The Netherlands	*S.tuberosum*	1957
PB_707	JQ954328	The Netherlands	*S.tuberosum*	1958
PB_752	JQ954330	The Netherlands	*S.tuberosum*	1995
PB312*	EF026075	USA	*S.tuberosum*	2003
PMB21	AJ390306	UK	*S.tuberosum*	
PN10A	DQ008213	USA	*S.tuberosum*	2004
PN-82	AJ303096	Spain	*C.annuum*	
PO7	U09509	Canada	*S.tuberosum*	1994
PP026B_184_111*	GQ853606	South Africa	*S.tuberosum*	
PRI-509	EU563512	The Netherlands	*S.tuberosum*	1938
PVY-12*	AB185833	Syria	*S.tuberosum*	2003
PVY-C-CM	AJ390302	UK	*S.tuberosum*	
PVY-MN	AF463399	USA	*N.tabacum*	2001
PVY-NBR	AF255660	Brazil	*S.tuberosum*	
PVY-N-RB	AJ390285	UK	*S.tuberosum*	
PVYNTN1	GQ853632	South Africa	*S.tuberosum*	2007
PVYNTN17_1*	JN936429	South Africa	*S.tuberosum*	2007
PVYNTN3_3*	GQ853607	South Africa	*S.tuberosum*	
PVY-OBR	AF255659	Brazil	*S.tuberosum*	
PVY-Sumi*	EU885418	South Korea	*S.tuberosum*	2008

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

PVY-ThaiNguyen	FM201468	Vietnam	*S.tuberosum*	
RB	HM367076	Canada	*S.tuberosum*	
RRA-1	AY884984	USA	*S.tuberosum*	2001
S25774_1*	JQ954331	Switzerland	*S.tuberosum*	2008
S25776_3*	JQ954332	Switzerland	*S.tuberosum*	2008
S25777_4*	JQ954333	Switzerland	*S.tuberosum*	2008
S25781_8*	JQ954334	Switzerland	*S.tuberosum*	2008
S25783_10*	JQ954335	Switzerland	*S.tuberosum*	2008
S25789_16*	JQ954336	Switzerland	*S.tuberosum*	2008
S25907-134	JQ954393	Switzerland	*S.tuberosum*	2008
S25972-199*	JQ954394	Switzerland	*S.tuberosum*	2008
SASA-110	AJ585195	UK	*S.tuberosum*	1997
SASA-61	AJ585198	UK	*S.tuberosum*	1997
Satina*	AJ890347	Germany	*S.tuberosum*	2002
SC143	JQ954337	Scotland	*S.tuberosum*	1996
SC190	JQ954338	Scotland	*S.tuberosum*	2000
SC61	JQ954339	Scotland	*S.tuberosum*	1986
SCRI-N	AJ585197	UK	*S.tuberosum*	1985
SCRI-O	AJ585196	UK	*S.tuberosum*	1985
Shanxi	EU719650	China	*S.tuberosum*	
Si15_Italy	AJ303093	Italy	*C.annuum*	
Si15_Turkey	AJ303094	Turkey	*C.annuum*	
SLO4	JQ954376	Slovenia	*S.tuberosum*	2009
SLO7*	JQ954377	Slovenia	*S.tuberosum*	2007
S-NTN	AJ390295	UK	*S.tuberosum*	
SON41	AJ439544	France	*S.nigrum*	1972
S-RB96†	AJ390308	UK	*S.tuberosum*	
SS082A_171_4	GQ853601	South Africa	*S.tuberosum*	

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

SS082A_194_14*	GQ853608	South Africa	*S.tuberosum*	
SS082A_88	GQ853600	South Africa	*S.tuberosum*	2005
SS121_154_10	GQ853633	South Africa	*S.tuberosum*	
SS121_166_56	GQ853637	South Africa	*S.tuberosum*	
SS121_197_16*	GQ853610	South Africa	*S.tuberosum*	
SS121_53_42*	GQ853609	South Africa	*S.tuberosum*	
SS121_82_1*	GQ853612	South Africa	*S.tuberosum*	
SS147_144_144*	GQ853611	South Africa	*S.tuberosum*	
SYR-D4*	AB295477	Syria	*S.tuberosum*	2004
SYR-D9*	AB295478	Syria	*S.tuberosum*	
SYR-II-2-8	AB461451	Syria	*S.tuberosum*	2006
SYR-II-Be1	AB461452	Syria	*S.tuberosum*	2004
SYR-NB-16	AB270705	Syria	*N.tabacum*	2006
SYR-Sn	AB295475	Syria	*S.nigrum*	2004
T50	AB331544	Japan	*S.tuberosum*	
Tamarillo	FM244834	Taiwan	*C.betacea*	2008
TC_2-186	JQ954340	Czech Republic	*S.tuberosum*	2006
TC_2-187	JQ954341	Czech Republic	*S.tuberosum*	2006
TC_2-191*	JQ954343	Czech Republic	*S.tuberosum*	2006
TC_2-196	JQ954344	Czech Republic	*S.tuberosum*	2006
TC_2-197	JQ954345	Czech Republic	*S.tuberosum*	2006
TC_2-198	JQ954346	Czech Republic	*S.tuberosum*	2006
TC_2-199	JQ954347	Czech Republic	*S.tuberosum*	2006
TC_2-200	JQ954348	Czech Republic	*S.tuberosum*	2006
TT014_184_135*	GQ853613	South Africa	*S.tuberosum*	
TT019A_107_52	GQ853665	South Africa	*S.tuberosum*	
TT026B_195_58	GQ853602	South Africa	*S.tuberosum*	
TT026B_86_128	GQ853638	South Africa	*S.tuberosum*	

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

TT026B_88_115	GQ853639	South Africa	*S.tuberosum*	
TT138D_111_79	GQ853640	South Africa	*S.tuberosum*	
TT138D_13_68*	GQ853614	South Africa	*S.tuberosum*	
TT138E_102_96	GQ853641	South Africa	*S.tuberosum*	
TT138E_111_104	GQ853666	South Africa	*S.tuberosum*	
TT138E_113_106	GQ853642	South Africa	*S.tuberosum*	
TT141A_76_73	GQ853643	South Africa	*S.tuberosum*	
Tu_660	AY166866	Canada	*S.tuberosum*	1991
Tu12.3	AJ303095	Turkey	*C.annuum*	
TU619	AJ390309	USA	*S.tuberosum*	
US04_24	JQ954392	USA	*S.tuberosum*	2004
US05_11	JQ954373	USA	*S.tuberosum*	2005
US05_12*	JQ954391	USA	*S.tuberosum*	2005
US05_13	JQ954349	USA	*S.tuberosum*	2005
US05_14	JQ954350	USA	*S.tuberosum*	2005
US05_17	JQ954385	USA	*S.tuberosum*	2005
US05_19	JQ954351	USA	*S.tuberosum*	2005
US05_20	JQ954386	USA	*S.tuberosum*	2005
US05_25	JQ954352	USA	*S.tuberosum*	2005
US05_26	JQ954353	USA	*S.tuberosum*	2005
US05_28	JQ954389	USA	*S.tuberosum*	2005
US05_3	JQ954354	USA	*S.tuberosum*	2005
US05_30	JQ954355	USA	*S.tuberosum*	2005
US05_31	JQ954390	USA	*S.tuberosum*	2005
US05_33	JQ954356	USA	*S.tuberosum*	2005
US05_36	JQ954388	USA	*S.tuberosum*	2005
US05_37	JQ954375	USA	*S.tuberosum*	2005
US05_39	JQ954357	USA	*S.tuberosum*	2005

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

US05_41	JQ954358	USA	*S.tuberosum*	2005
US05_45	JQ954359	USA	*S.tuberosum*	2005
US05_48	JQ954360	USA	*S.tuberosum*	2005
US05_49	JQ954361	USA	*S.tuberosum*	2005
US05_51	JQ954362	USA	*S.tuberosum*	2005
US05_52*	JQ954363	USA	*S.tuberosum*	2005
US05_56	JQ954364	USA	*S.tuberosum*	2005
US05_6	JQ954365	USA	*S.tuberosum*	2005
US05_64	JQ954366	USA	*S.tuberosum*	2005
US05_7	JQ954367	USA	*S.tuberosum*	2005
US05_9	JQ954368	USA	*S.tuberosum*	2005
US06_52	JQ954369	USA	*S.tuberosum*	2006
US06_55	JQ954370	USA	*S.tuberosum*	2006
US06_56	JQ954371	USA	*S.tuberosum*	2005
US06_59	JQ954372	USA	*S.tuberosum*	2005
USMN20	JQ954374	USA	*S.tuberosum*	2004
v942490*	EF016294	UK	*S.tuberosum*	1994
v951156-1	AJ390286	UK	*S.tuberosum*	
v951175	AJ390304	UK	*S.tuberosum*	
v951204	AJ390292	UK	*S.tuberosum*	
v951204 N†	AJ390291	UK	*S.tuberosum*	
v951218	AJ390287	UK	*S.tuberosum*	
v97005	AJ390303	UK	*S.tuberosum*	
Wilga	EF558545	Poland	*S.tuberosum*	1984
WW002_22_147*	GQ853615	South Africa	*S.tuberosum*	
WW002_74_150*	GQ853616	South Africa	*S.tuberosum*	
WW002_82_151*	GQ853617	South Africa	*S.tuberosum*	
WW010_146_164	GQ853645	South Africa	*S.tuberosum*	

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

WW010_147_166	GQ853646	South Africa	*S.tuberosum*	
WW010_70_158	GQ853644	South Africa	*S.tuberosum*	
WW154_175_62	GQ853647	South Africa	*S.tuberosum*	
WW154A_62_86	GQ853648	South Africa	*S.tuberosum*	
WW202B_21_172*	GQ853618	South Africa	*S.tuberosum*	
WW202B_24_184*	GQ853619	South Africa	*S.tuberosum*	
WW282E_3	GQ853649	South Africa	*S.tuberosum*	
Xinyang	EU719648	China	*S.tuberosum*	
Z14*	JN936440	South Africa	*S.tuberosum*	2009
Z16*	JN936441	South Africa	*S.tuberosum*	2010
Z26*	GQ853620	South Africa	*S.tuberosum*	2005
Zhuanglang103	EF592523	China	*N.tabacum*	
	GQ496607	Latvia	*S.tuberosum*	2007
	AM931253	China	*N.tabacum*	
	AM931254*	China	*S.tuberosum*	

*Isolates showing the common recombination point at position 9170.

†Isolates showing other recombination points at the CP cistron.

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

Table S2. Amino acid composition for potato (P) and non-potato (NP) isolates (180 and 32, respectively) at positively selected codons

The last two columns indicate those amino acids that have been detected only in P or NP isolates, respectively, for a given position. Codon positions are given as the corresponding amino acid positions in the CP cistron.

Position	P composition	NP composition	P specific	NP specific
1	113A, 60G, 5V	24A, 5G, 3V		
68	173E, 4K, 3G	31E, 1D	K, G	D
193	98V, 57G, 25M	11G, 8V, 7I, 4M, 1R, 1T		I, R, T
216	178A, 1E, 1G	32A	E, G	

Table S3. Amino acid composition for potato (P) and non-potato (NP) isolates (180 and 32, respectively) at covarying codons

The last two columns indicate those amino acids that have been detected only in potato or non-potato isolates, respectively, for a given position. Codon positions are given as the corresponding amino acid positions in the CP cistron.

Position	P composition	NP composition	P specific	NP specific
1	113A, 60G, 5V	24A, 5G, 3V		
11	113S, 45T, 21N, 1A	22S, 9N, 1T	A	
17	125P, 53Q, 1L, 1R	28P, 4Q	L, R	
24	116S, 59P, 3L, 2R	16P, 9S, 5R, 2L		
26	119P, 61L,	27P, 4L, 1S		S
29	126G, 54E	27G, 4E, 1A		A
31	126D, 53E, 1V	25D, 7E	V	
99	122M, 53L, 5V	17M, 8V, 5L, 1T, 1I		T, I
138	94D, 86N	14S, 10N, 8D		S

Table S4. Amino acid composition for PVY^C, PVY^O and PVY^N strain isolates (22, 132 and 57 isolates, respectively) at positively selected codons

The last three columns indicate those amino acids that have been detected only in PVY^C, PVY^O or PVY^N groups, respectively, for a given position. Codon positions are given as the corresponding amino acid positions in the CP cistron.

Position	PVY^C composition	PVY^O composition	PVY^N composition	PVY^C specific	PVY^O specific	PVY^N specific
1	20A, 2V	108A, 18G, 4V	47G, 8A, 2V			
68	21E, 1D	132E	50E, 4K, 3G	D		K, G
193	13G, 6I, 1M, 1T, 1V	104V, 28M	55G, 1R, 1V	I, T		R
216	22A	130A, 1E, 1G	57A		E, G	

Table S5. Amino acid composition for PVY^C, PVY^O and PVY^N strain isolates (22, 132 and 57 isolates, respectively) at covarying codons

The last three columns indicate those amino acids that have been detected only in PVY^C, PVY^O or PVY^N groups, respectively, for a given position. Codon positions are given as the corresponding amino acid positions in the CP cistron.

Position	PVY^C composition	PVY^O composition	PVY^N composition	PVY^C specific	PVY^O specific	PVY^N specific
1	20A, 2V	108A, 18G, 4V	47G, 8A, 2V			
11	19S, 3N	114S, 18N	46T, 9N, 1S, 1A			T, A
17	22P	130P, 1Q, 1L	56Q, 1R		L	R
24	11P, 7R, 3S, 1L	122S, 7P, 3L	56P, 1L	R		
26	21P, 1S	122P, 10L	55L, 2P	S		
29	21G, 1A	131G, 1E	57E	A		
31	19D, 3E	131D, 1V	57E		V	
99	12V, 7M, 1I, 1L, 1T	132M	57L	V, I, T		
138	14S, 6N, 2D	79N, 53D	47D, 10N	S		

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

Table S6. Amino acid composition for PVY^C, PVY^O and PVY^N strain isolates (22, 132 and 57 isolates, respectively) at the region showing evidence of positive selection for the internal branch leading to PVY^C clade (branch b2, codons 187–194, shown in Fig. S1)

The last three columns indicate those amino acids that have been detected only in PVY^N, PVY^O or PVY^C groups, respectively, for a given position.

Position	PVY^C composition	PVY^O composition	PVY^N composition	PVY^C specific	PVY^O specific	PVY^N specific
187	19I, 1H, 1N, 1V	130I, 2T	55V, 2I	H, N	T	
188	22R	132R	57R			
189	22N	132N	56N, 1T			T
190	22L	132L	56L, 1V			V
191	22R	132R	57R			
192	21D, 1V	132D	57D	V		
193	13G, 6I, 1M, 1T, 1V	104V, 28M	55G, 1R, 1V	I, T		R
194	17S, 5G	132G	57S			

Reference

Drummond, A. J. & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* **7**, 214. doi:10.1186/1471-2148-7-214 Medline

Jose M. Cuevas, Agnès Delaunay, Matevz Rupar, Emmanuel Jacquot, and Santiago F. Elena (2012). Molecular evolution and phylogeography of *Potato virus Y* based on the CP gene. *J Gen Virol* **93**, 2496–2501.

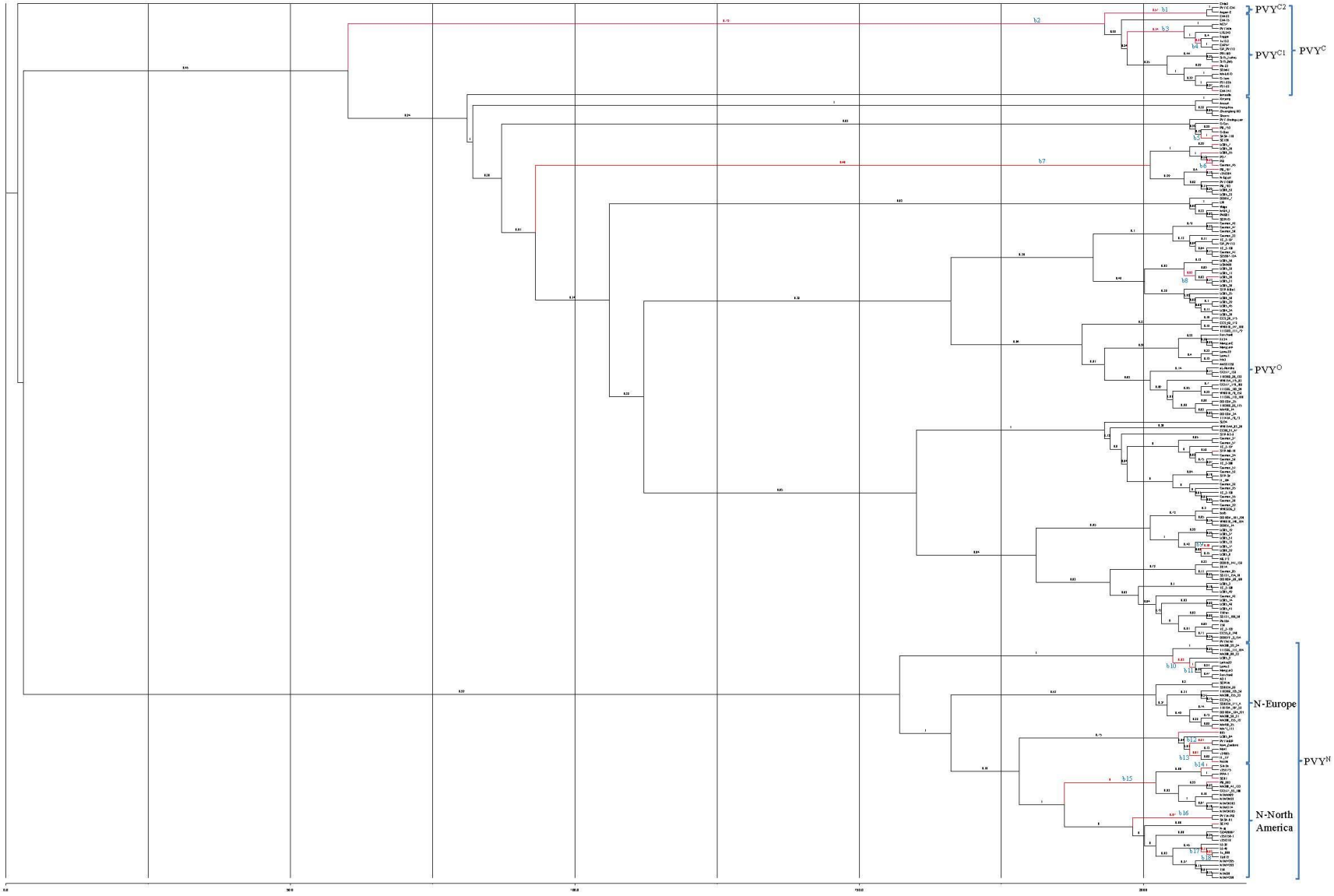

PVY^C2
PVY^C1
PVY^C
PVY^O
N-Europe
N-North America
PVY^N

Fig. S1. MCC phylogeny of the PVY isolates for the CP cistron. The tree was calculated from the posterior distribution of trees generated by Bayesian MCMC coalescent analyses with BEAST (Drummond & Rambaut, 2007). Posterior probabilities are indicated above branches. Branches detected to be under positive selection are shown in red, and internal branches are identified numbering in the range b1–b18. For clarity, branches were transformed as proportional using FigTree (www.tree.bio.ed.ac.uk).

Priloga E

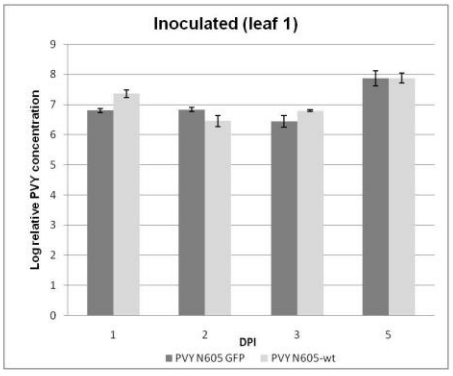
Dodatno gradivo k članku v »Fluorescentno označeni krompirjev virus Y: vsestransko orodje za funkcionalno analizo interakcij med rastlino in virusom" (v objavljanju)

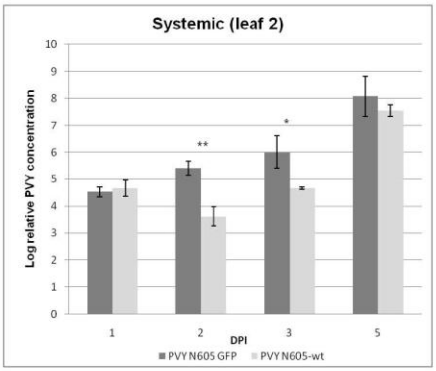
Appendix A. Table with primer pairs used in PCR; in bold are the parts of the primers hybridizing to the target, underlined are the overhangs coding modified Nla protease recognition site, in italic are the fusion PCR hybridisation sites.

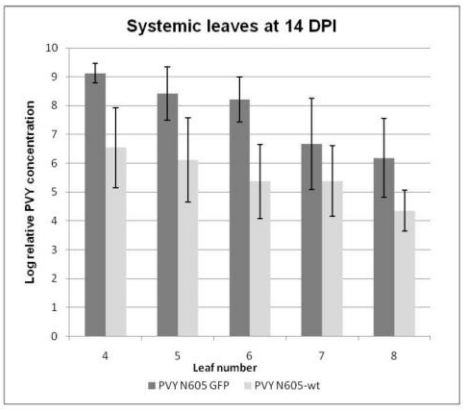
Schematic representation of cloning steps, annotated with amplicon hybridisation sites of the three qPCR assays (UNI, PVY-*gfp*, PVY-wt). Fusion PCR with fragments 1, 2 and 3 was used to insert PRS flanked *gfp* gene between Nib and CP gene sequences. In the second step restriction-ligation procedure with BglII and PflFI restriction endonucleases was used to swap the WT PVY N605 sequence with the construct bearing *gfp* gene.

Primer name	Sequence (5'-3')	Lenght (nt)	Hybridisation target
F1_F	TCACAACATTTCTCAGATCTTGG	23	middle of NIb
F1_R	*CCTGGTGATGAACCTCGTACG***TATCGCACTCAAATTCATCATCC**	44	3' NIb
F2_F	*CGTACGAGGTTCATCACCAGGG***CATGGTGAGCAAGGGCGAGG**	42	5' GFP
F2_R	TCCTTGATGGTGCACTTCATAAGT**ACCCTTGTACAGCTCGTCCAT**	45	3' GFP
F3_F	ACTTATGAAGTGCACCATCAAGGA	24	5' CP

733
734 Appendix B Comparing the concentrations of PVY N605-GFP and PVY N605-wt in inoculated and
735 systemic leaves through time. The height of the columns represents the average concentration of the
736 PVY in the leaf; the bars represent the standard errors. The lower leaves were checked at the
737 beginning of the infection (1-5 DPI) and upper systemic leaves at 14 DPI. No statistically significant
738 differences (t-test, p=0.005) were observed between PVY N065 GFP and PVY N605-wt concentrations
739 on inoculated leaf (L1) or upper systemic leaves. On systemic leaf 2 at 2 and 3 DPI the concentration
740 of PVY N605GFP and PVY N605-wt was significantly different. However both reached comparable
741 concentration at 5 DPI and the concentration of PVY N605-GFP was higher than PVY N605-wt. All
742 data combined indicated that insertion of gfp gene does not impair the virus fitness.
743



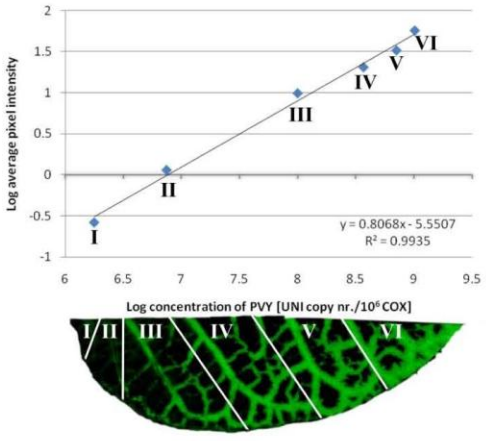




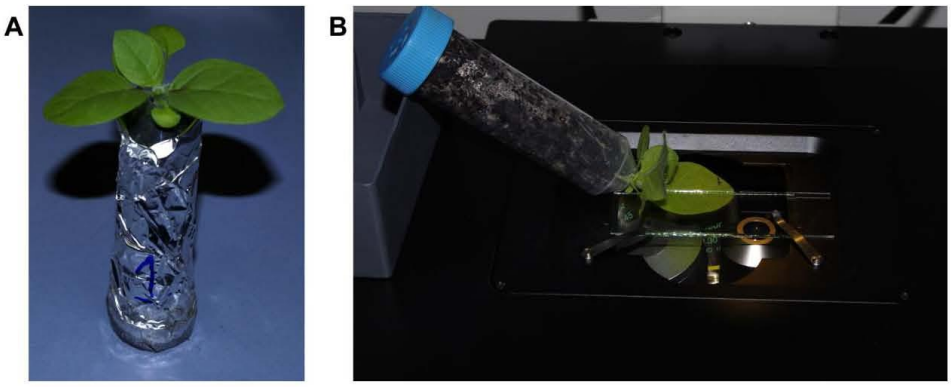
744
745 Appendix C. The qPCR data supporting Figure 5, concentrations of PVY RNA detected in each sample
746 are listed in the table. The graph is showing correlation between average pixel intensity in the green
747 channel (analysed in Image J) and PVY RNA concentration in the sections of systemically infected leaf
748 L4 (below the graph) at 7 DPI (also Figure 5, section B; underlined in the table). The analysed regions
749 of the leaf are numbered (I-VI) for visualisation purpose.The graph indicates that regions with higher
750 RNA concentrations had also higher GFP fluorescence intensity.

Figure section	Leaf type	DPI	Sample nr.	Relative PVY concentration [UNI copy nr./10^6 copies of COX]	Average Cq value		Theoretical copy nr.	
					COX	UNI	COX	UNI
A	inoculated L1	3	1	2.3E+06	18.59	22.92	3.8E+04	8.6E+04
			2	1.3E+06	18.89	24.06	3.1E+04	4.0E+04
			3	5.8E+07	18.63	18.21	3.7E+04	2.1E+06
			4	3.2E+06	18.89	22.72	3.1E+04	9.9E+04
B	systemic L4	7	5	1.0E+09	17.59	13.03	7.1E+04	7.2E+07
			6	7.0E+08	17.07	13.08	9.9E+04	6.9E+07
			7	3.7E+08	16.04	13.08	1.9E+05	6.9E+07
			8	1.0E+08	15.42	14.42	2.8E+05	2.8E+07
			9	7.5E+06	16.03	18.8	1.9E+05	1.4E+06
			10	1.8E+06	16.95	21.76	1.1E+05	1.9E+05
C	systemic L4	10	11	7.3E+08	15.64	11.69	2.4E+05	1.8E+08
			12	2.6E+08	14.92	12.55	3.8E+05	1.0E+08
			13	1.9E+08	14.22	12.36	6.0E+05	1.1E+08
D	systemic L5	7	14	1.5E+08	16.34	14.64	1.6E+05	2.4E+07
			15	6.7E+06	16.27	19.17	1.6E+05	1.1E+06
			16	2.1E+06	15.5	20.17	2.7E+05	5.6E+05
E	systemic L6	10	17	4.7E+06	14.51	18.08	5.0E+05	2.3E+06
			18	1.2E+05	13.25	22.28	1102709.2	1.3E+05

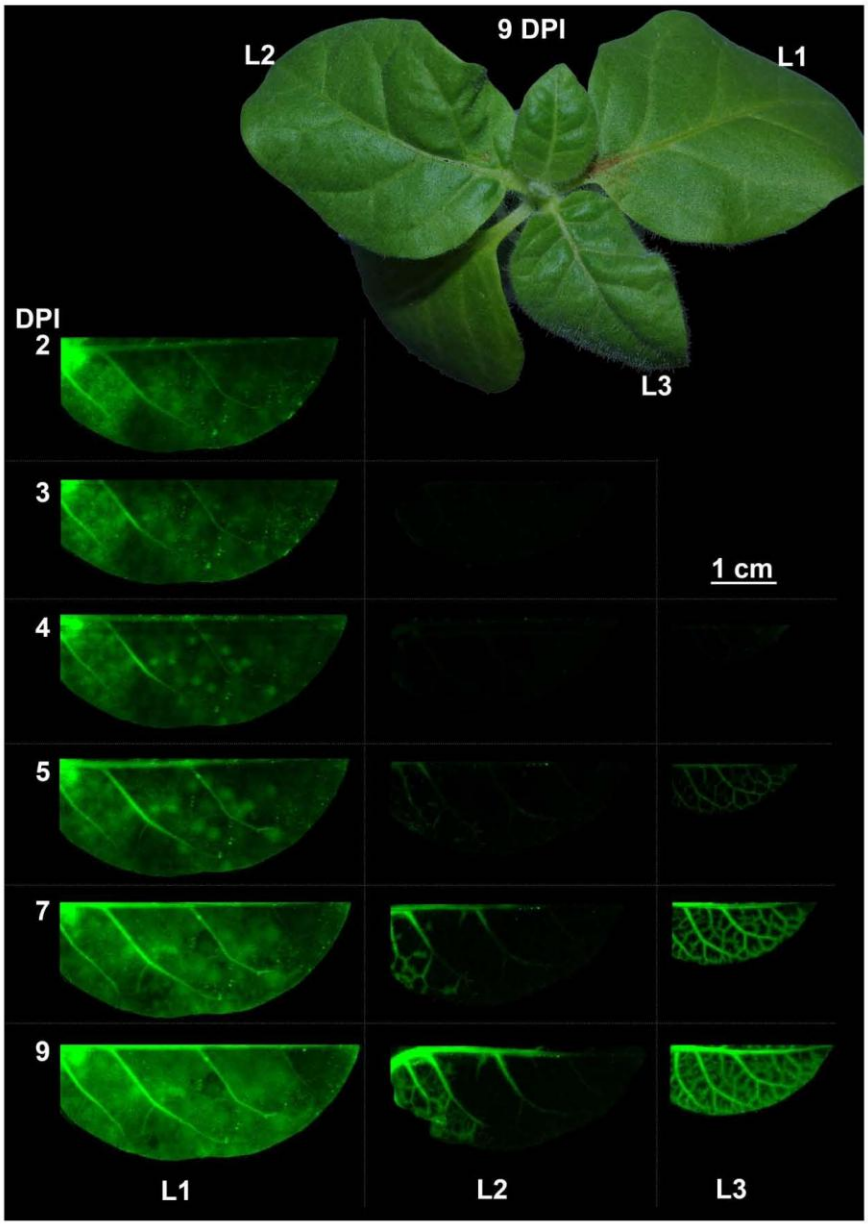
751
752
753
754



Appendix D. Technique used for *in vivo* tracking of PVY N605-GFP. Plants were grown in falcon tubes with aeration holes on the sides and bottom, aluminium foil was used to keep the roots out of light. Half of the leaf was carefully placed between two objective glasses, scanned in both green and red channel and then the plant was put back into growing chamber until next day.



763 Appendix E. Repetition of the experiment (Figure 7) of PVY N605-GFP *in vivo* tracking in *Nicotiana*
764 *tabacum* cv. Xanthii.
765
766
767



768

769 Appendix F. MIQE table for PVY-wt assay and standard dilution series for all four assays used for
770 calculating theoretical copy nr. Linear regression lines and corresponding equations used for further
771 calculations are shown.

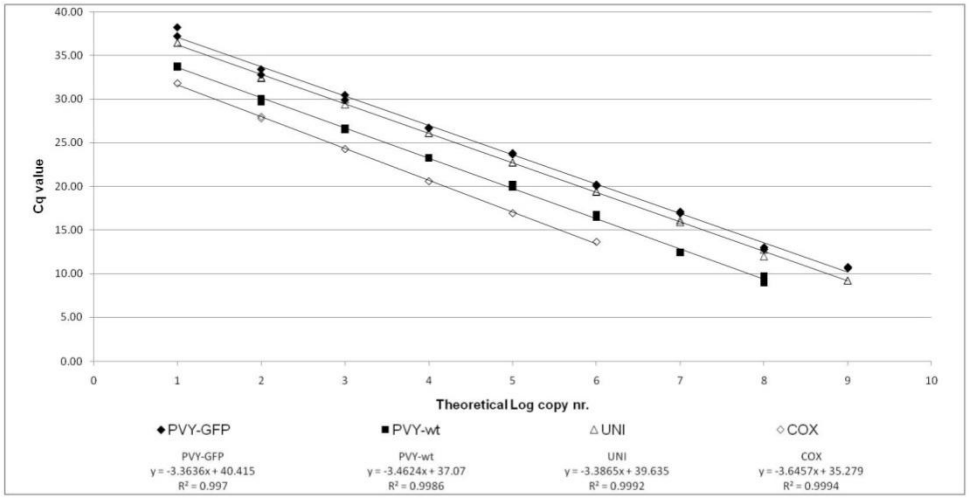
Sample/Template	details			
Source	*Nicotiana tabacum* cv. Xanthii			
Method of preservation	Frozen -20			
Storage time	< 3 months old			
Handling	frozen			
Extraction method	Magnetic based Mag Max total RNA extraction system (Ambion)			
RNA: DNA-free	DNAse reaction (turbo DNAse, Ambion)			
Concentration	Measured by Nanodrop, 0,002-2mg/ reaction			
Inhibition-free	3 point dilution series approach on representative selection of samples			
Assay optimisation/validation				
Accession number	X97895			
Amplicon details	spanning the junction of Nib and CP gene of PVY N605			
Primers and probe				
Name	Sequence	Tm [°C]	Length [bp]	Concentration in the PCR reaction [µM]
PVY-wt FW	5'- TGGTTGCCTTGGATGATGAAT -3'	61	21	0.3
PVY-wt REV	5'- TCGATTGTGTCATTTCCTTGATG -3'	60	23	0.3
PVY-wt P	5'-FAM-TGAGTGCGATACTTATGAAG-MGB-3'	55	20	0.3
In silico	BLAST/Primer-BLAST/Oligo analysed (IDT)			
Priming conditions	target-specific primers for in RT and PCR (One step)			
PCR efficiency	94.5%			
Linear dynamic range	2 – 0,00000002 mg of total RNA/reaction			

772

773

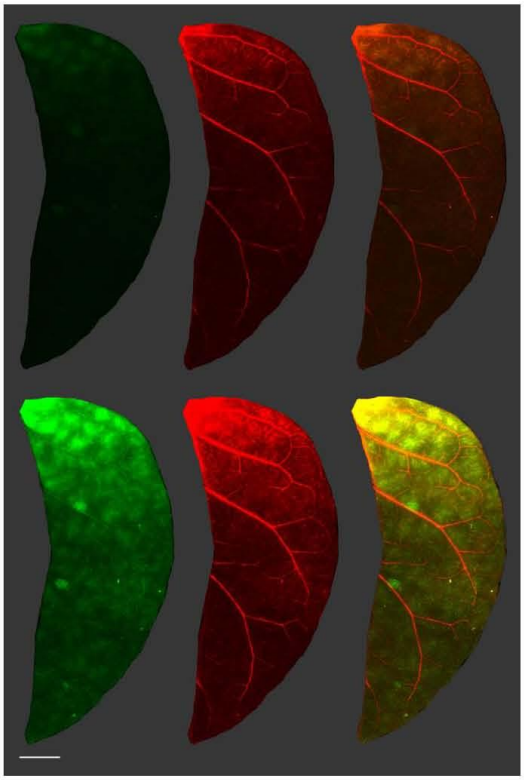
RT/PCR	
Protocol	10 µl reactions, each sample tested in duplicates Cycling conditions: reverse transcription 48°C, 10 min; denaturation 95°C, 10 min, 45 cycles: 95°C, 15 s; 60°C 1 min
Reagents	AGPATH-ID One-Step RT-PCR Reagents (Applied Biosystems®) used in concentrations according to manufacturer's protocol
Duplicate RT	Yes (dCq< 0,5)
NTC	Undetermined
NAC	Not done
Positive control	RNA extract from the symptomatic plant infected with PVY N605

774
775
776

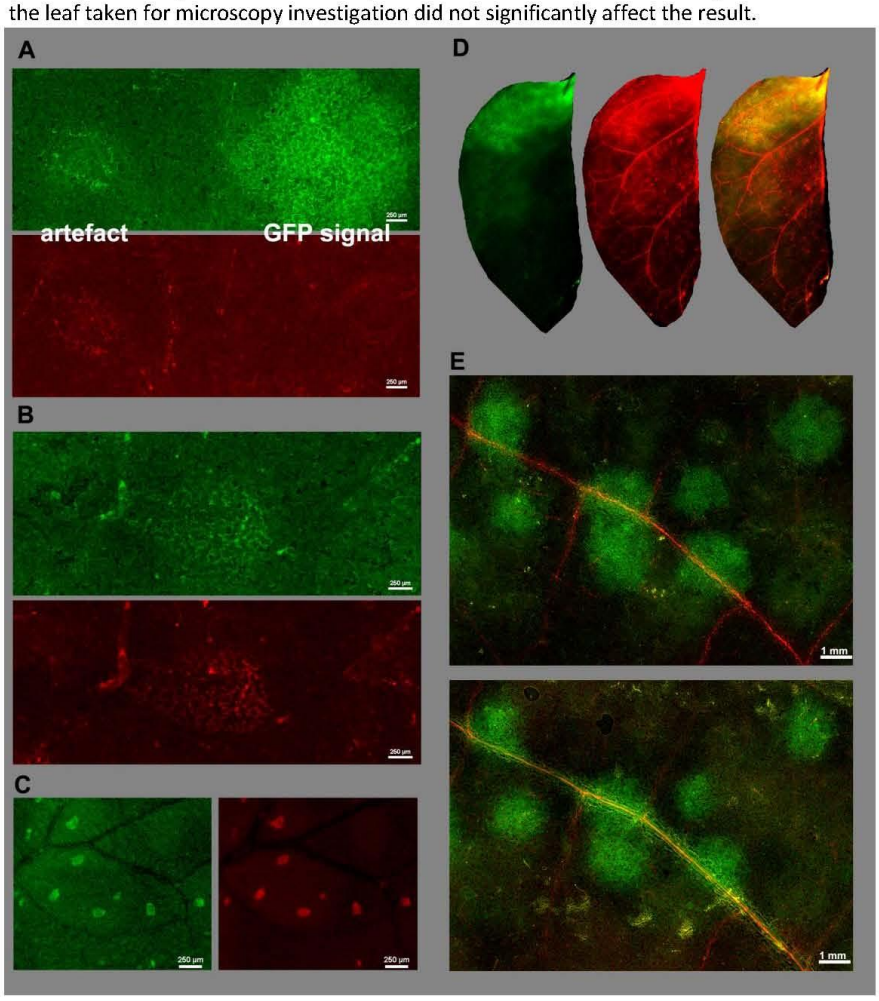


777
778
779

Appendix G. Affect of the"Look up table" (LUT) adjustment to visualise the fluorescence. The raw fluorescence images in the upper and LUT adjusted images in the lower part of the figure. Red, green and overlay of green and red channels are presented from left to right. The bar represents 5 mm.



Appendix H. Examples of controls used for proper interpretation of fluorescence observations on inoculated leaves. A) a genuine GFP signal is shown on the right side of the images (no signal in red channel). Signal similar to GFP is shown in the green channel on the left side of the image, however when red channel was analysed as well, it was clear that this is the case of an auto fluorescence artefact (signal in green and red). B) auto fluorescence artefact observed on plant inoculated with PVY N605-wt. C) fluorescent artefacts detected on healthy plants. D) Auto fluorescence caused by mechanical inoculation procedure on the leaves inoculated with the sap from healthy plants (mock). E) Overlay of red and green channels taken from the top of the leaf (upper), the bottom of the leaf (middle). The images obtained from top and bottom side of the leaf are very similar, thus the side of the leaf taken for microscopy investigation did not significantly affect the result.



797 Appendix I. Data from fluorescence detection and qPCR analysis of PVY N605 GFP inoculated samples for the stability study. Each sample was analysed with
798 four qPCR assays. Equations from Appendix F were used to calculate theoretical copy no. for each assay. The differences in the amount of plant tissue used
799 in different samples were normalised with COX.

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_534	1	no	1	**15.9**	**19.3**	**19.6**	**34.3**	2.1E+05	9.9E+05	1.6E+06	6.2E+00	**4.8E+06**	**7.6E+06**	**3.0E+01**	0.001%
RM_535	1	no	1	**16.6**	**19.1**	**20.0**	**30.8**	1.4E+05	1.2E+06	1.1E+06	6.3E+01	**8.6E+06**	**8.4E+06**	**4.6E+02**	0.005%
RM_536	1	no	1	**14.9**	**17.6**	**18.3**	**30.1**	3.9E+05	3.2E+06	3.7E+06	1.0E+02	**8.2E+06**	**9.4E+06**	**2.5E+02**	0.003%
RM_537	1	no	1	**14.8**	**17.0**	**18.2**	**33.7**	4.1E+05	4.7E+06	4.1E+06	9.5E+00	**1.2E+07**	**1.0E+07**	**2.3E+01**	0.000%
RM_538	1	no	1	**15.1**	**18.9**	**20.0**	**34.2**	3.4E+05	1.3E+06	1.2E+06	6.7E+00	**3.9E+06**	**3.4E+06**	**2.0E+01**	0.001%
RM_539	1	no	1	**15.2**	**18.3**	**19.5**	**33.0**	3.1E+05	2.0E+06	1.7E+06	1.5E+01	**6.4E+06**	**5.3E+06**	**4.8E+01**	0.001%
RM_540	1	no	2	**13.2**	**22.8**	**24.1**	**40.7**	1.1E+06	9.4E+04	7.3E+04	*1.0E+00*	**8.3E+04**	**6.5E+04**	**8.9E-01**	0.001%
RM_541	1	no	2	**13.1**	**22.5**	**23.6**	**35.1**	1.2E+06	1.1E+05	1.0E+05	3.6E+00	**9.2E+04**	**8.2E+04**	**2.9E+00**	0.003%
RM_542	1	no	2	**14.3**	**24.6**	**25.8**	**34.8**	5.7E+05	2.7E+04	2.2E+04	4.7E+00	**4.7E+04**	**3.8E+04**	**8.2E+00**	0.017%
RM_543	1	no	2	**14.0**	**28.1**	**29.2**	**35.5**	6.8E+05	2.5E+03	2.1E+03	2.9E+00	**3.7E+03**	**3.1E+03**	**4.3E+00**	0.116%
RM_544	1	no	1	**15.5**	**19.4**	**20.5**	**30.9**	2.7E+05	9.2E+05	8.3E+05	6.0E+01	**3.4E+06**	**3.1E+06**	**2.2E+02**	0.006%
RM_545	1	no	1	**15.2**	**17.8**	**18.9**	**29.6**	3.2E+05	2.9E+06	2.5E+06	1.5E+02	**9.2E+06**	**7.8E+06**	**4.7E+02**	0.005%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_546	1	no	1	**15.3**	**18.7**	**19.0**	**30.5**	3.0E+05	1.6E+06	2.4E+06	7.7E+01	**5.3E+06**	**8.1E+06**	**2.6E+02**	0.005%
RM_554	2	no	1	**25.7**	**26.7**	**26.9**	**ND**	4.1E+02	6.7E+03	1.1E+04	*1.0E+00*	**1.6E+07**	**2.6E+07**	**2.4E+03**	0.015%
RM_555	2	yes	1	**24.3**	**25.3**	**25.7**	**ND**	1.0E+03	1.8E+04	2.5E+04	*1.0E+00*	**1.7E+07**	**2.4E+07**	**9.7E+02**	0.006%
RM_556	2	yes	1	**24.2**	**27.7**	**28.1**	**ND**	1.1E+03	3.4E+03	4.7E+03	*1.0E+00*	**3.0E+06**	**4.1E+06**	**8.9E+02**	0.029%
RM_557	2	no	1	**24.9**	**26.1**	**26.5**	**ND**	6.9E+02	1.0E+04	1.3E+04	*1.0E+00*	**1.5E+07**	**1.9E+07**	**1.4E+03**	0.010%
RM_558	2	no	1	**25.3**	**27.0**	**27.5**	**ND**	5.5E+02	5.4E+03	6.7E+03	*1.0E+00*	**9.9E+06**	**1.2E+07**	**1.8E+03**	0.018%
RM_559	2	no	1	**24.4**	**26.1**	**26.4**	**ND**	9.8E+02	9.7E+03	1.5E+04	*1.0E+00*	**1.0E+07**	**1.5E+07**	**1.0E+03**	0.010%
RM_560	2	no	1	**17.7**	**21.0**	**21.7**	**36.7**	6.7E+04	3.3E+05	3.8E+05	1.3E+00	**4.9E+06**	**5.6E+06**	**2.0E+01**	0.000%
RM_561	2	no	1	**15.8**	**19.6**	**20.5**	**ND**	2.3E+05	8.4E+05	8.3E+05	*1.0E+00*	**3.7E+06**	**3.7E+06**	**4.4E+00**	0.000%
RM_562	2	yes	1	**15.0**	**18.1**	**19.1**	**37.9**	3.8E+05	2.3E+06	2.2E+06	*1.0E+00*	**6.0E+06**	**5.7E+06**	**2.7E+00**	0.000%
RM_563	2	no (H.B.N)	1	**15.3**	**19.4**	**20.5**	**39.3**	3.1E+05	9.4E+05	8.3E+05	*1.0E+00*	**3.0E+06**	**2.7E+06**	**3.2E+00**	0.000%
RM_564	2	yes	1	**15.2**	**18.9**	**20.0**	**38.5**	3.2E+05	1.3E+06	1.2E+06	*1.0E+00*	**4.1E+06**	**3.6E+06**	**3.1E+00**	0.000%
RM_565	2	no	2	**13.9**	**24.5**	**25.1**	**36.4**	7.5E+05	2.9E+04	3.6E+04	1.6E+00	**3.9E+04**	**4.9E+04**	**2.1E+00**	0.005%
RM_566	2	no	2	**14.5**	**25.0**	**25.6**	**ND**	5.1E+05	2.1E+04	2.5E+04	*1.0E+00*	**4.1E+04**	**4.9E+04**	**1.9E+00**	0.005%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_567	2	no	2	**14.4**	**23.5**	**24.5**	**35.4**	5.5E+05	5.9E+04	5.6E+04	3.0E+00	**1.1E+05**	**1.0E+05**	**5.4E+00**	0.005%
RM_568	2	no	2	**15.1**	**25.0**	**26.1**	**35.9**	3.5E+05	2.1E+04	1.8E+04	2.3E+00	**6.1E+04**	**5.1E+04**	**6.5E+00**	0.011%
RM_569	2	no	2	**15.4**	**24.7**	**25.9**	**36.5**	2.9E+05	2.6E+04	2.1E+04	1.5E+00	**8.9E+04**	**7.0E+04**	**5.1E+00**	0.006%
RM_570	2	no	3	**14.0**	**26.3**	**27.6**	**35.0**	7.0E+05	8.6E+03	6.4E+03	4.0E+00	**1.2E+04**	**9.2E+03**	**5.8E+00**	0.047%
RM_581	2	no (H.B.N)	1	**16.0**	**19.2**	**19.1**	**34.2**	2.0E+05	1.1E+06	2.2E+06	6.7E+00	**5.6E+06**	**1.1E+07**	**3.4E+01**	0.001%
RM_582	2	no (H.B.N)	1	**15.8**	**19.3**	**19.0**	**36.1**	2.3E+05	1.0E+06	2.3E+06	1.9E+00	**4.6E+06**	**1.0E+07**	**8.6E+00**	0.000%
RM_583	2	no (H.B.N)	1	**15.2**	**18.4**	**18.3**	**36.1**	3.3E+05	1.9E+06	3.8E+06	1.9E+00	**5.9E+06**	**1.2E+07**	**6.0E+00**	0.000%
RM_584	2	no (H.B.N)	1	**16.4**	**18.6**	**18.5**	**34.3**	1.6E+05	1.7E+06	3.2E+06	6.3E+00	**1.1E+07**	**2.1E+07**	**4.1E+01**	0.000%
RM_585	2	no	2	**14.5**	**22.6**	**22.3**	**35.6**	5.1E+05	1.1E+05	2.4E+05	2.7E+00	**2.1E+05**	**4.6E+05**	**5.3E+00**	0.003%
RM_586	2	no	2	**13.9**	**21.9**	**21.8**	**36.0**	7.5E+05	1.7E+05	3.4E+05	2.0E+00	**2.3E+05**	**4.5E+05**	**2.7E+00**	0.001%
RM_587	2	no	2	**14.4**	**19.3**	**19.2**	**35.0**	5.5E+05	1.0E+06	2.0E+06	4.0E+00	**1.8E+06**	**3.7E+06**	**7.3E+00**	0.000%
RM_588	2	no	2	**15.0**	**19.1**	**18.9**	**35.7**	3.7E+05	1.2E+06	2.6E+06	2.6E+00	**3.2E+06**	**7.0E+06**	**7.0E+00**	0.000%
RM_589	2	no	2	**15.9**	**19.1**	**19.0**	**36.3**	2.1E+05	1.2E+06	2.3E+06	1.7E+00	**5.7E+06**	**1.1E+07**	**8.2E+00**	0.000%
RM_590	2	no	3	**11.9**	**22.4**	**22.8**	**34.3**	2.6E+06	1.2E+05	1.8E+05	6.1E+00	**4.8E+04**	**6.8E+04**	**2.3E+00**	0.005%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_591	2	no	3	**12.6**	**22.4**	**21.6**	**35.0**	1.7E+06	1.2E+05	3.9E+05	4.0E+00	**7.1E+04**	**2.3E+05**	**2.3E+00**	0.003%
RM_592	3	no	3	**12.9**	**18.8**	**18.2**	**ND**	1.4E+06	1.4E+06	4.1E+06	*1.0E+00*	**1.0E+06**	**2.9E+06**	**7.1E-01**	0.000%
RM_593	3	no	2	**13.6**	**17.1**	**16.6**	**ND**	9.1E+05	4.4E+06	1.2E+07	*1.0E+00*	**4.8E+06**	**1.3E+07**	**1.1E+00**	0.000%
RM_594	3	no	2	**13.0**	**17.3**	**16.4**	**ND**	1.3E+06	4.0E+06	1.4E+07	*1.0E+00*	**3.2E+06**	**1.1E+07**	**7.9E-01**	0.000%
RM_595	3	no	2	**14.0**	**20.6**	**19.4**	**ND**	6.8E+05	4.2E+05	1.8E+06	*1.0E+00*	**6.2E+05**	**2.7E+06**	**1.5E+00**	0.000%
RM_596	3	no	2	**15.1**	**24.1**	**23.7**	**39.3**	3.4E+05	3.9E+04	9.6E+04	*1.0E+00*	**1.2E+05**	**2.9E+05**	**3.0E+00**	0.003%
RM_597	3	no (H.B.N)	1	**15.9**	**21.2**	**20.7**	**ND**	2.0E+05	2.8E+05	7.2E+05	*1.0E+00*	**1.4E+06**	**3.5E+06**	**4.9E+00**	0.000%
RM_598	3	yes	1	**16.1**	**18.0**	**17.6**	**ND**	1.9E+05	2.4E+06	6.3E+06	*1.0E+00*	**1.3E+07**	**3.4E+07**	**5.3E+00**	0.000%
RM_599	3	yes	1	**16.9**	**22.1**	**21.4**	**ND**	1.1E+05	1.5E+05	4.4E+05	*1.0E+00*	**1.4E+06**	**4.1E+06**	**9.3E+00**	0.001%
RM_600	3	yes	1	**15.9**	**19.7**	**19.1**	**ND**	2.1E+05	7.9E+05	2.2E+06	*1.0E+00*	**3.7E+06**	**1.1E+07**	**4.7E+00**	0.000%
RM_601	3	no	1	**19.0**	**24.9**	**23.9**	**ND**	2.9E+04	2.3E+04	8.0E+04	*1.0E+00*	**7.7E+05**	**2.7E+06**	**3.4E+01**	0.004%
RM_602	3	no	1	**18.6**	**22.9**	**21.9**	**42.5**	3.8E+04	8.6E+04	3.2E+05	*1.0E+00*	**2.3E+06**	**8.5E+06**	**2.6E+01**	0.001%
RM_603	3	no	1	**18.9**	**24.1**	**23.4**	**39.3**	3.1E+04	4.0E+04	1.1E+05	*1.0E+00*	**1.3E+06**	**3.6E+06**	**3.2E+01**	0.003%
RM_604	3	yes	1	**18.6**	**18.2**	**17.3**	**38.4**	3.7E+04	2.1E+06	7.3E+06	*1.0E+00*	**5.8E+07**	**2.0E+08**	**2.7E+01**	0.000%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_605	3	no	1	**18.9**	**22.7**	**21.5**	**ND**	3.1E+04	9.9E+04	4.3E+05	*1.0E+00*	**3.2E+06**	**1.4E+07**	**3.2E+01**	0.001%
RM_606	3	no	1	**21.7**	**27.7**	**26.4**	**ND**	5.2E+03	3.3E+03	1.5E+04	*1.0E+00*	**6.2E+05**	**2.8E+06**	**1.9E+02**	0.031%
RM_649	4	yes	3	**14.9**	**16.4**	**17.0**	**36.4**	4.0E+05	7.5E+06	9.1E+06	1.6E+00	**1.9E+07**	**2.3E+07**	**4.0E+00**	0.000%
RM_651	4	yes	3	**15.5**	**16.2**	**14.6**	**36.5**	2.6E+05	8.6E+06	4.7E+07	1.5E+00	**3.3E+07**	**1.8E+08**	**5.7E+00**	0.000%
RM_653	4	yes	3	**16.0**	**18.4**	**18.4**	**36.7**	1.9E+05	1.9E+06	3.5E+06	1.3E+00	**9.6E+06**	**1.8E+07**	**6.8E+00**	0.000%
RM_654	4	no	3	**16.0**	**24.9**	**24.9**	**35.7**	1.9E+05	2.3E+04	4.2E+04	2.6E+00	**1.2E+05**	**2.2E+05**	**1.3E+01**	0.011%
RM_659	4	yes	3	**15.1**	**16.1**	**16.4**	**36.1**	3.4E+05	8.8E+06	1.4E+07	1.9E+00	**2.6E+07**	**4.1E+07**	**5.7E+00**	0.000%
RM_660	4	no	3	**16.4**	**21.5**	**21.8**	**33.8**	1.5E+05	2.3E+05	3.4E+05	8.8E+00	**1.6E+06**	**2.3E+06**	**5.9E+01**	0.004%
RM_663	4	yes	3	**15.5**	**16.2**	**16.7**	**35.1**	2.6E+05	8.3E+06	1.1E+07	3.7E+00	**3.1E+07**	**4.2E+07**	**1.4E+01**	0.000%
RM_665	4	yes	3	**13.2**	**17.5**	**18.3**	**36.8**	1.2E+06	3.4E+06	3.7E+06	1.2E+00	**2.9E+06**	**3.2E+06**	**1.0E+00**	0.000%
RM_666	4	yes	4	**13.5**	**17.6**	**16.5**	**35.4**	9.2E+05	3.2E+06	1.3E+07	3.0E+00	**3.5E+06**	**1.4E+07**	**3.2E+00**	0.000%
RM_667	4	yes	4	**13.4**	**17.3**	**18.0**	**35.0**	1.0E+06	3.8E+06	4.6E+06	4.0E+00	**3.8E+06**	**4.5E+06**	**4.0E+00**	0.000%
RM_668	4	yes	4	**13.5**	**16.5**	**17.3**	**33.9**	9.5E+05	6.8E+06	7.3E+06	8.5E+00	**7.2E+06**	**7.8E+06**	**8.9E+00**	0.000%
RM_669	4	yes	4	**13.8**	**16.8**	**17.0**	**35.2**	7.7E+05	5.5E+06	9.3E+06	3.5E+00	**7.0E+06**	**1.2E+07**	**4.6E+00**	0.000%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_670	4	yes	4	**15.7**	**17.3**	**17.9**	**36.4**	2.4E+05	4.0E+06	4.8E+06	1.6E+00	**1.7E+07**	**2.0E+07**	**6.6E+00**	0.000%
RM_671	4	yes	4	**15.7**	**16.9**	**17.0**	**35.1**	2.4E+05	5.3E+06	9.4E+06	3.8E+00	**2.3E+07**	**4.0E+07**	**1.6E+01**	0.000%
RM_135	5	yes	1	**17.6**	**15.4**	**15.9**	**ND**	7.0E+04	1.4E+07	1.9E+07	*1.0E+00*	**2.0E+08**	**2.8E+08**	**1.4E+01**	0.000%
RM_136	5	yes	1	**16.6**	**14.6**	**15.5**	**ND**	1.3E+05	2.4E+07	2.6E+07	*1.0E+00*	**1.9E+08**	**2.0E+08**	**7.7E+00**	0.000%
RM_137	5	yes	1	**16.5**	**14.8**	**15.3**	**38.0**	1.5E+05	2.2E+07	3.0E+07	*1.0E+00*	**1.5E+08**	**2.1E+08**	**6.8E+00**	0.000%
RM_138	5	yes	2	**15.9**	**13.8**	**13.8**	**39.0**	2.1E+05	4.3E+07	8.1E+07	*1.0E+00*	**2.1E+08**	**3.9E+08**	**4.8E+00**	0.000%
RM_139	5	yes	2	**16.1**	**13.4**	**13.9**	**38.7**	1.9E+05	5.7E+07	7.8E+07	*1.0E+00*	**3.0E+08**	**4.2E+08**	**5.3E+00**	0.000%
RM_140	5	yes	2	**15.7**	**15.5**	**16.2**	**40.9**	2.4E+05	1.3E+07	1.6E+07	*1.0E+00*	**5.5E+07**	**6.8E+07**	**4.2E+00**	0.000%
RM_141	5	yes	3	**15.4**	**13.9**	**14.7**	**40.2**	2.9E+05	4.0E+07	4.5E+07	*1.0E+00*	**1.4E+08**	**1.6E+08**	**3.5E+00**	0.000%
RM_142	5	no	3	**18.1**	**27.1**	**27.2**	**ND**	5.2E+04	4.9E+03	8.4E+03	*1.0E+00*	**9.4E+04**	**1.6E+05**	**1.9E+01**	0.020%
RM_143	5	yes	3	**15.2**	**16.0**	**16.5**	**ND**	3.1E+05	9.9E+06	1.3E+07	*1.0E+00*	**3.1E+07**	**4.0E+07**	**3.2E+00**	0.000%
RM_144	5	no	3	**15.6**	**24.9**	**25.4**	**ND**	2.5E+05	2.3E+04	2.9E+04	*1.0E+00*	**9.1E+04**	**1.2E+05**	**4.0E+00**	0.004%
RM_145	5	yes	3	**15.4**	**15.4**	**15.9**	**42.5**	2.9E+05	1.4E+07	2.0E+07	*1.0E+00*	**4.9E+07**	**6.9E+07**	**3.5E+00**	0.000%
RM_146	5	yes	4	**13.6**	**13.1**	**13.2**	**36.1**	9.1E+05	7.0E+07	1.2E+08	1.9E+00	**7.7E+07**	**1.3E+08**	**2.1E+00**	0.000%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_161	5	yes	3	**15.7**	**15.7**	**16.6**	**36.4**	2.3E+05	1.1E+07	1.2E+07	1.6E+00	**5.0E+07**	**5.3E+07**	**6.9E+00**	0.000%
RM_162	5	no	3	**15.6**	**23.8**	**24.8**	**44.1**	2.5E+05	4.9E+04	4.5E+04	*1.0E+00*	**1.9E+05**	**1.8E+05**	**4.0E+00**	0.002%
RM_163	5	no	3	**20.6**	**22.9**	**23.3**	**34.4**	1.1E+04	9.0E+04	1.2E+05	6.1E+00	**8.2E+06**	**1.1E+07**	**5.6E+02**	0.007%
RM_164	5	yes	4	**12.9**	**14.6**	**15.4**	**40.1**	1.4E+06	2.5E+07	2.7E+07	*1.0E+00*	**1.9E+07**	**2.0E+07**	**7.3E-01**	0.000%
RM_222	7	no	5	**15.5**	**20.2**	**21.3**	**36.3**	2.7E+05	5.6E+05	4.9E+05	1.6E+00	**2.1E+06**	**1.8E+06**	**6.2E+00**	0.000%
RM_223	7	no	5	**16.3**	**19.2**	**20.6**	**31.9**	1.6E+05	1.1E+06	7.9E+05	3.2E+01	**6.7E+06**	**4.9E+06**	**1.9E+02**	0.003%
RM_224	7	yes	5	**16.3**	**14.6**	**15.7**	**40.7**	1.6E+05	2.4E+07	2.3E+07	*1.0E+00*	**1.5E+08**	**1.5E+08**	**6.4E+00**	0.000%
RM_225	7	no	4	**17.3**	**20.5**	**21.7**	**36.1**	8.4E+04	4.5E+05	3.6E+05	2.0E+00	**5.4E+06**	**4.3E+06**	**2.3E+01**	0.000%
RM_226	7	no	4	**17.0**	**21.8**	**22.7**	**35.4**	1.1E+05	1.9E+05	1.9E+05	3.1E+00	**1.8E+06**	**1.7E+06**	**2.9E+01**	0.002%
RM_227	7	no	4	**16.4**	**18.7**	**20.5**	**33.9**	1.5E+05	1.5E+06	8.4E+05	8.5E+00	**1.0E+07**	**5.6E+06**	**5.6E+01**	0.001%
RM_228	7	no	4	**16.0**	**18.8**	**20.2**	**35.4**	1.9E+05	1.4E+06	1.0E+06	3.1E+00	**7.5E+06**	**5.5E+06**	**1.6E+01**	0.000%
RM_229	7	yes	4	**16.0**	**15.0**	**15.9**	**34.4**	1.9E+05	1.9E+07	2.0E+07	6.1E+00	**1.0E+08**	**1.1E+08**	**3.2E+01**	0.000%
RM_230	7	yes	4	**15.4**	**14.4**	**15.3**	**33.7**	2.8E+05	2.8E+07	2.9E+07	9.2E+00	**1.0E+08**	**1.0E+08**	**3.3E+01**	0.000%
RM_231	7	yes	4	**15.9**	**13.1**	**14.0**	**34.1**	2.0E+05	7.0E+07	7.3E+07	7.4E+00	**3.4E+08**	**3.6E+08**	**3.6E+01**	0.000%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_232	7	yes	4	**16.0**	**13.1**	**13.8**	**33.7**	1.9E+05	6.9E+07	8.4E+07	9.4E+00	**3.7E+08**	**4.4E+08**	**5.0E+01**	0.000%
RM_233	7	yes	4	**17.0**	**13.5**	**13.5**	**34.7**	1.1E+05	5.4E+07	9.8E+07	5.0E+00	**5.1E+08**	**9.3E+08**	**4.7E+01**	0.000%
RM_234	7	yes	4	**17.1**	**13.1**	**14.4**	**34.4**	9.9E+04	6.9E+07	5.6E+07	5.9E+00	**7.0E+08**	**5.6E+08**	**6.0E+01**	0.000%
RM_235	7	yes	4	**17.0**	**12.8**	**14.2**	**37.2**	1.0E+05	8.4E+07	6.4E+07	*1.0E+00*	**8.2E+08**	**6.3E+08**	**9.8E+00**	0.000%
RM_236	7	yes	4	**17.6**	**13.0**	**14.4**	**34.5**	7.1E+04	7.2E+07	5.3E+07	5.7E+00	**1.0E+09**	**7.5E+08**	**8.0E+01**	0.000%
RM_251	10	n.t.	1	**16.5**	**31.1**	**31.3**	**ND**	1.4E+05	3.4E+02	5.1E+02	*1.0E+00*	**2.4E+03**	**3.5E+03**	**7.0E+00**	0.292%
RM_252	10	n.t.	2	**16.9**	**11.6**	**12.0**	**37.2**	1.1E+05	1.9E+08	2.8E+08	*1.0E+00*	**1.8E+09**	**2.6E+09**	**9.3E+00**	0.000%
RM_253	10	n.t.	2	**17.1**	**28.9**	**29.3**	**ND**	9.8E+04	1.5E+03	2.1E+03	*1.0E+00*	**1.5E+04**	**2.1E+04**	**1.0E+01**	0.069%
RM_254	10	n.t.	2	**17.5**	**33.9**	**32.0**	**ND**	7.7E+04	4.9E+01	3.1E+02	*1.0E+00*	**6.4E+02**	**4.0E+03**	**1.3E+01**	2.025%
RM_255	10	n.t.	3	**17.6**	**36.2**	**34.1**	**ND**	7.1E+04	1.0E+01	7.7E+01	*1.0E+00*	**1.5E+02**	**1.1E+03**	**1.4E+01**	9.676%
RM_256	10	n.t.	3	**16.9**	**11.5**	**12.2**	**36.6**	1.1E+05	2.0E+08	2.4E+08	1.4E+00	**1.8E+09**	**2.2E+09**	**1.3E+01**	0.000%
RM_257	10	n.t.	3	**16.61**	**11.3**	**11.8**	**ND**	1.3E+05	2.4E+08	3.2E+08	*1.0E+00*	**1.8E+09**	**2.4E+09**	**7.6E+00**	0.000%
RM_258	10	yes	4	**14.2**	**12.4**	**13.4**	**33.8**	6.0E+05	1.1E+08	1.1E+08	8.9E+00	**1.9E+08**	**1.8E+08**	**1.5E+01**	0.000%
RM_259	10	yes	4	**14.9**	**12.6**	**13.2**	**39.6**	3.8E+05	1.0E+08	1.2E+08	*1.0E+00*	**2.6E+08**	**3.2E+08**	**2.6E+00**	0.000%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_260	10	yes	4	**15.6**	**11.7**	**12.1**	**27.8**	2.4E+05	1.8E+08	2.6E+08	4.6E+02	**7.3E+08**	**1.1E+09**	**1.9E+03**	0.000%
RM_261	10	no	5	**13.4**	**21.7**	**22.3**	**ND**	9.8E+05	2.0E+05	2.5E+05	*1.0E+00*	**2.0E+05**	**2.5E+05**	**1.0E+00**	0.001%
RM_262	10	yes	5	**15.5**	**13.4**	**14.1**	**34.1**	2.7E+05	5.7E+07	6.8E+07	7.2E+00	**2.2E+08**	**2.6E+08**	**2.7E+01**	0.000%
RM_263	10	no	6	**13.3**	**22.3**	**22.8**	**34.3**	1.1E+06	1.3E+05	1.7E+05	6.3E+00	**1.2E+05**	**1.6E+05**	**5.7E+00**	0.005%
RM_264	10	yes	6	**14.5**	**18.1**	**18.6**	**ND**	5.0E+05	2.3E+06	3.0E+06	*1.0E+00*	**4.7E+06**	**6.1E+06**	**2.0E+00**	0.000%
RM_265	10	no	7	**14.8**	**24.3**	**24.5**	**ND**	4.2E+05	3.3E+04	5.3E+04	*1.0E+00*	**7.9E+04**	**1.3E+05**	**2.4E+00**	0.003%
RM_266	10	yes	5	**13.3**	**16.1**	**16.5**	**ND**	1.1E+06	8.7E+06	1.3E+07	*1.0E+00*	**8.2E+06**	**1.2E+07**	**9.4E-01**	0.000%
RM_744	14	n.t.	4	**13.6**	**8.2**	**8.6**	**ND**	8.7E+05	2.0E+09	2.9E+09	*1.0E+00*	**2.3E+09**	**3.4E+09**	**1.2E+00**	0.000%
RM_745	14	n.t.	5	**12.3**	**7.9**	**8.8**	**ND**	2.0E+06	2.4E+09	2.6E+09	*1.0E+00*	**1.2E+09**	**1.3E+09**	**4.9E-01**	0.000%
RM_746	14	n.t.	6	**13.2**	**9.8**	**10.2**	**ND**	1.1E+06	6.5E+08	9.9E+08	*1.0E+00*	**5.8E+08**	**8.9E+08**	**8.9E-01**	0.000%
RM_747	14	n.t.	7	**13.7**	**13.5**	**14.1**	**32.3**	8.1E+05	5.1E+07	6.7E+07	2.4E+01	**6.3E+07**	**8.2E+07**	**3.0E+01**	0.000%
RM_748	14	n.t.	8	**14.3**	**16.3**	**17.3**	**34.6**	5.6E+05	7.9E+06	7.5E+06	5.1E+00	**1.4E+07**	**1.3E+07**	**9.1E+00**	0.000%
RM_753	14	n.t.	4	**13.6**	**9.8**	**9.7**	**ND**	8.6E+05	6.7E+08	1.3E+09	*1.0E+00*	**7.7E+08**	**1.6E+09**	**1.2E+00**	0.000%
RM_754	14	n.t.	5	**11.9**	**12.0**	**12.7**	**ND**	2.5E+06	1.5E+08	1.8E+08	*1.0E+00*	**5.9E+07**	**6.9E+07**	**3.9E-01**	0.000%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_755	14	n.t.	6	**12.8**	**13.1**	**13.2**	**ND**	1.5E+06	6.9E+07	1.2E+08	*1.0E+00*	**4.6E+07**	**8.2E+07**	**6.7E-01**	0.000%
RM_756	14	n.t.	7	**12.3**	**19.8**	**20.7**	**36.0**	2.0E+06	7.2E+05	7.5E+05	2.1E+00	**3.6E+05**	**3.7E+05**	**1.1E+00**	0.000%
RM_757	14	n.t.	8	**12.5**	**21.1**	**21.6**	**36.1**	1.8E+06	3.0E+05	4.0E+05	1.9E+00	**1.7E+05**	**2.2E+05**	**1.1E+00**	0.001%
RM_772	120	no	1	**14.9**	**16.1**	**16.8**	**ND**	4.0E+05	9.0E+06	1.0E+07	*1.0E+00*	**2.2E+07**	**2.5E+07**	**2.5E+00**	0.000%
RM_773	120	yes	2	**14.2**	**9.4**	**10.2**	**ND**	6.2E+05	8.3E+08	9.5E+08	*1.0E+00*	**1.3E+09**	**1.5E+09**	**1.6E+00**	0.000%
RM_774	120	yes	3	**14.4**	**13.3**	**14.0**	**ND**	5.4E+05	6.1E+07	7.3E+07	*1.0E+00*	**1.1E+08**	**1.4E+08**	**1.9E+00**	0.000%
RM_775	120	no	4	**14.0**	**16.1**	**16.8**	**ND**	6.9E+05	8.9E+06	1.1E+07	*1.0E+00*	**1.3E+07**	**1.5E+07**	**1.5E+00**	0.000%
RM_776	120	yes	1	**14.0**	**8.6**	**9.0**	**ND**	7.0E+05	1.5E+09	2.2E+09	*1.0E+00*	**2.1E+09**	**3.2E+09**	**1.4E+00**	0.000%
RM_777	120	yes	2	**14.6**	**10.4**	**11.0**	**ND**	4.7E+05	4.2E+08	5.6E+08	*1.0E+00*	**8.9E+08**	**1.2E+09**	**2.1E+00**	0.000%
RM_778	120	yes	3	**14.2**	**10.4**	**11.2**	**ND**	6.2E+05	4.3E+08	4.8E+08	*1.0E+00*	**6.9E+08**	**7.7E+08**	**1.6E+00**	0.000%
RM_779	120	yes	4	**13.5**	**17.3**	**17.7**	**ND**	9.4E+05	3.8E+06	5.5E+06	*1.0E+00*	**4.1E+06**	**5.9E+06**	**1.1E+00**	0.000%
RM_780	120	yes	1	**14.8**	**9.5**	**9.4**	**ND**	4.1E+05	7.8E+08	1.7E+09	*1.0E+00*	**1.9E+09**	**4.1E+09**	**2.4E+00**	0.000%
RM_781	120	no	2	**14.8**	**17.8**	**17.0**	**ND**	4.2E+05	2.9E+06	9.1E+06	*1.0E+00*	**6.9E+06**	**2.2E+07**	**2.4E+00**	0.000%
RM_782	120	yes	3	**14.7**	**11.8**	**12.2**	**ND**	4.6E+05	1.7E+08	2.5E+08	*1.0E+00*	**3.7E+08**	**5.5E+08**	**2.2E+00**	0.000%

Sample nr.	DPI	GFP observed	Leaf nr.	Average Cq value				Theoretical copy no. (TCN)				Normalised TCN (per 10^6 COX copies)			Percent of PVY –wt in total PVY population
				COX	UNI	PVY GFP	PVY wt	COX	UNI	PVY-GFP	PVY-wt	UNI	PVY-GFP	PVY-wt	
RM_783	120	yes	4	**13.9**	**9.9**	**9.9**	**ND**	7.2E+05	6.1E+08	1.2E+09	*1.0E+00*	**8.5E+08**	**1.6E+09**	**1.4E+00**	0.000%
RM_784	120	yes	5	**14.0**	**14.9**	**15.0**	**ND**	7.0E+05	2.1E+07	3.5E+07	*1.0E+00*	**3.0E+07**	**5.1E+07**	**1.4E+00**	0.000%
RM_785	120	yes	1	**14.8**	**9.6**	**10.6**	**ND**	4.2E+05	7.4E+08	7.3E+08	*1.0E+00*	**1.8E+09**	**1.8E+09**	**2.4E+00**	0.000%
RM_786	120	no	2	**15.2**	**18.2**	**17.4**	**ND**	3.1E+05	2.1E+06	7.2E+06	*1.0E+00*	**6.6E+06**	**2.3E+07**	**3.2E+00**	0.000%
RM_787	120	yes	3	**16.7**	**11.3**	**10.8**	**ND**	1.2E+05	2.4E+08	6.5E+08	*1.0E+00*	**1.9E+09**	**5.2E+09**	**8.0E+00**	0.000%
RM_788	120	yes	4	**15.5**	**10.8**	**10.3**	**ND**	2.7E+05	3.3E+08	9.2E+08	*1.0E+00*	**1.2E+09**	**3.5E+09**	**3.8E+00**	0.000%
RM_789	120	yes	5	**15.0**	**12.0**	**12.7**	**ND**	3.7E+05	1.4E+08	1.8E+08	*1.0E+00*	**3.9E+08**	**4.8E+08**	**2.7E+00**	0.000%
RM_790	120	yes	1	**14.0**	**8.8**	**9.6**	**ND**	6.7E+05	1.3E+09	1.4E+09	*1.0E+00*	**1.9E+09**	**2.2E+09**	**1.5E+00**	0.000%
RM_791	120	yes	2	**16.0**	**16.8**	**17.5**	**ND**	2.0E+05	5.4E+06	6.3E+06	*1.0E+00*	**2.7E+07**	**3.2E+07**	**5.0E+00**	0.000%
RM_792	120	yes	3	**15.8**	**11.7**	**11.6**	**ND**	2.2E+05	1.8E+08	3.7E+08	*1.0E+00*	**7.9E+08**	**1.7E+09**	**4.5E+00**	0.000%
RM_793	120	no	4	**17.0**	**19.9**	**19.6**	**ND**	1.0E+05	6.7E+05	1.6E+06	*1.0E+00*	**6.6E+06**	**1.5E+07**	**9.8E+00**	0.000%
RM_794	120	yes	5	**15.3**	**13.0**	**13.6**	**ND**	3.0E+05	7.1E+07	9.6E+07	*1.0E+00*	**2.4E+08**	**3.3E+08**	**3.4E+00**	0.000%

UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Matevž RUPAR

LOKALIZACIJA IN SLEDENJE KROMPIRJEVEGA VIRUSA Y V RASTLINAH KROMPIRJA (*SOLANUM TUBEROSUM* L.)

DOKTORSKA DISERTACIJA

Ljubljana, 2014