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Phloem connectivity and transport are not involved in mature plant resistance (MPR) to Potato Virus Y in different potato cultivars, and MPR does not protect tubers from recombinant strains of the virus



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ABSTRACT

The aims of this study were: i) to investigate mature plant resistance (MPR) against four strains of Potato virus Y (PVY^O, PVY^N, PVY^{NTN} and PVY^{N-Wi}) in potato cultivars that differ in maturity (e.g. early or maincrop) at different developmental stages, and ii) to determine whether phloem translocation of photoassimilates at different stages including the source-sink transition influences MPR. The data showed that MPR was functional by the flowering stage in all cultivars, and that the host-pathogen interaction is highly complex, with all three variables (potato cultivar, virus strain and developmental stage of infection) having a significant effect on the outcome. However, virus strain was the most important factor, and MPR was less effective in protecting tubers from recombinant virus strains (PVY^{NTN} and PVY^{N-Wi}). Development of MPR was unrelated to foliar phloem unloading early in tuber development may be involved in the prevention of tuber infections with PVY^O. Recombinant virus strains were more infectious than parental strains and PVY^{NTN} has a more effective silencing suppressor than PVY^O, another factor that may contribute to the efficiency of MPR. The resistance conferred by MPR against PVY^O or PVY^N may be associated with or enhanced by the presence of the corresponding strain specific HR resistance gene in the cultivar.

1. Introduction

The aphid-transmitted potyvirus *Potato virus Y* (PVY) is the most important potato-infecting virus, causing major economic losses in potato production systems worldwide (Valkonen et al., 2007; Kerlan et al., 2008; Scholthof et al., 2011; Karasev and Gray, 2013; Torrance and Talianksy, 2020). Several major strain groups of PVY are recognised; these are based on symptoms induced in potatoes carrying different N (or hypersensitivity, HR) genes (pathotypes) and genome sequencing (phylogroups). The main groups include the ordinary (PVY^O) and common (PVY^C) strains which can induce systemic mosaics, or leaf crinkling, necrosis, leaf drop and dwarfing symptoms, and the necrotic strain (PVY^N) which causes a veinal necrosis reaction in tobacco, but relatively mild symptoms in potato. Two further groups comprising virus strains derived by recombination between genome sequences of PVY^{O} and PVY^{N} (Valkonen et al., 2007; Jones, 1990, 2014; Gibbs et al., 2017; Fuentes et al., 2019) have also been identified.

Recombinant strains, such as PVY^{NTN} and PVY^{N–Wi}, have been reported infecting potatoes since the 1980s (Glais et al., 2002; Schubert et al., 2007; Karasev et al., 2011; Visser et al., 2012) and since then their incidence has increased (Visser et al., 2012; Funke et al., 2017; Dupuis et al., 2018), displacing PVY^O as the most-prevalent strain in European

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Abbreviations: PVY, Potato virus Y; MPR, Mature Plant Resistance. * Corresponding author.

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and North American potato crops (Chikh-Ali et al., 2019; MacKenzie et al., 2019). It is thought that recombinant strains are becoming dominant because they often induce mild foliar symptoms that can be overlooked and not removed on visual inspection and they can be potent sources of inoculum (Gray and Power, 2018). Moreover, PVY^{NTN} is more efficiently transmitted by aphid vectors than PVY^O (Carroll et al., 2016). Although foliar symptoms from recombinant strains can be mild and foliar growth may not be significantly affected such strains can induce potato tuber necrotic ringspot disease (PTNRD) which decreases the yield and quality of the tubers (Beczner et al., 1984; Boonham et al., 2002; Le Romancer et al., 1994). Thus, improved control methods including alternatives to simple visual inspection are needed to better protect the tuber crop.

Application of insecticides to control virus-vector aphids is poorly effective in controlling PVY which is acquired and transmitted rapidly before insecticides take effect, and over-applications can lead to insecticide-resistant aphids. Farmers also rely on removal of symptombearing infected plants (roguing), however, this strategy also has limitations because, as mentioned above, PVY symptoms can be mild or indistinguishable in some cultivars, particularly for recombinant virus strains. Host resistance based on naturally-occurring resistance genes is an effective and environmentally benign way to control PVY, but many successful commercial cultivars do not carry such resistance genes, and even those cultivars listed as resistant to PVY often turn out to be susceptible based on recent studies using contemporary virus strains (Slater et al., 2020).

Another type of resistance to plant pathogens occurs as plants age, called mature plant resistance (MPR), where resistance develops in later growth stages. MPR has been shown to be effective against many plant pathogens (reviewed in Develey-Rivière and Galiana, 2007) including viruses in potatoes. Previous studies on MPR against PVY^O and PVY^N (e. g. Beemster, 1976, 1987; Sigvald, 1985, 1987; Gibson, 1991), revealed that the effectiveness of MPR varied with potato cultivar and virus strain. Gibson (1991) showed that MPR in field-grown potatoes was initiated at approximately four weeks after sprouts emerged from the ground, was fully effective at 8-10 weeks, and was observed to act against both PVY^O and PVY^N. MPR is often relied on in cool countries in Northern Europe to control virus in seed potato crops as the incidence of virus vector aphids occurs later in the growing season after MPR has been initiated (Beemster et al., 1987). However, there is a gap in knowledge of the effectiveness of MPR against the increasingly prevalent recombinant strains of PVY. Moreover, the underlying cell and molecular mechanisms of action of MPR are poorly understood.

Given the wide range of host-pathogen interactions in which MPR is observed, there are likely to be differences in mechanism depending on pathosystem, and work to date indicates that the response to MPR differs from the basal defence responses which induce HR and systemic acquired resistance (Develey-Rivière and Galiana, 2007). Studies to develop biochemical markers to detect onset of MPR in the field have been unsuccessful (Braber et al., 1982) and a better understanding of the mechanism would therefore be of considerable benefit towards developing such diagnostic tests.

The mechanism of MPR might be associated with changes in phloem or virus transport. For example, plants infected with PVY later in the season usually produce many fewer infected progeny tubers and this has been attributed to restriction or blockage of cell-to-cell movement to tubers (Beemster, 1976; Chick-Ali et al., 20219; Dupuis, 2017). Beemster (1976) found that PVY^N infected more tubers than PVY^O at later developmental stages and attributed this to faster translocation of PVY^N than PVY^O. Alterations in phloem transport can be observed at different developmental stages, for example, early maturing Arabidopsis plants escape infection with cauliflower mosaic virus (Leisner et al., 1993). Plant viruses are transported with photoassimilates in phloem (Leisner et al., 1993; Carrington et al., 1996; Gilbertson and Lucas, 1996), but the pattern of phloem transport changes as the sink-source status of tissues in a plant changes during development. This paper investigated the infectivity of four PVY strains in four potato cultivars that differed in N gene complement and maturity (early or main crop), to establish the developmental stage of onset of MPR and to test the hypothesis that virus movement to potato tubers was inhibited through changes in phloem transport at different developmental stages.

2. Materials and methods

Plant material and growth conditions: Potato cultivars were grown from certified virus-free micropropagated plants (GenTech, Dundee, UK initially in 10 cm diameter pots then 30 cm pots in compost: 85% (v/v) Irish moss peat, 7% (v/v) sand, 0.2% (w/v) magnesium limestone, 0.2% (w/v) calcium limestone, 0.15% (w/v) Osmocote Start controlled release fertilizer (Everris, ICL, UK), 7% (v/v) Perlite (Sinclair Pro, UK), 0.05% (w/v) Celcote wetting agent (Certis, Abington, UK) and 0.3% Osmocote Exact Standard fertilizer (Everris, ICL, UK). All experiments were conducted in a temperature-controlled, insect-proof greenhouse at 22 $^{\circ}$ C day/14 $^{\circ}$ C night and 16 h daylength.

PVY strains: PVY^O (GenBank AJ585196) and PVY^N (SCRI-N, GenBank AJ585197) were obtained from the James Hutton Institute virus collection; PVY^{N-Wi} (SASA207, Genbank AJ584851) and PVY^{NTN} (PVY^{EU-NTN}, SASA390) from SASA (Science Advice for Scottish Agriculture), Edinburgh. Strains were confirmed by sequencing coat protein (CP) RNA and inoculated and maintained on *Nicotiana tabacum* cultivar (cv.) White Burley; a new culture was started from freeze-dried stock every 12–16 weeks to maintain uniformity.

PVY infection of potato: Virus strains were maintained in continuous culture on glasshouse-grown N.tabacum plants. Inocula were prepared by macerating PVY-infected N. tabacum leaves in sterile water (1:5 w/v). A gloved finger was used to rub the sap extract onto potato source leaves dusted with aluminium oxide (Duralum Microgrits FEPA F-400; Washington Mills Ltd, Manchester, UK). The inoculum provided guaranteed infection on N.tabacum plants and so was assumed to have the potential to infect all susceptible plants. Control plants were mockinoculated with water. All experiments used single-stem plants to simplify the phylotaxy and solute transport pathways. Three or four terminal leaflets of compound source leaves were inoculated on each plant. Because the plants changed significantly between the four growth stages, phyllotactically identical leaves could not be utilised across all stages. At the 6-leaf stage, the lowest three or four leaves were inoculated, leaving the remaining upper (sink) leaves untouched. By later stages the lowest leaves had begun to age and senesce making them unsuitable for inoculation and so the lowest, healthy, well-expanded source leaves were selected at each developmental stage to maximise the likelihood of successful infection and spread to available sink tissue. Each infection experiment used between 5 and 8 plants per cultivar, with replications performed in years 2013-15. Inoculations were conducted at four developmental stages: 6-leaf (24-26 days post-planting), stolon development (36-40 days; stolon), tuber development (first tuber -1-2 cm in size; 50-52 days; tuber) and flowering (Shepody 67 days, other cultivars 77-81 days). See Supplementary Fig. S1. After inoculation, plants were grown until tubers matured. Tubers were harvested and stored at 4 °C for two months to break dormancy.

2.1. PVY detection

1. Double antibody sandwich (DAS) ELISA was used to detect PVY in tissue from sprouts removed from tubers and from inoculated and non-inoculated upper leaves, the latter taken at 21–24 days post inoculation (dpi), a timepoint determined to allow all viral strains to have achieved systemic infection in susceptible plants. Tissue was macerated using an electric press (MEKU Juice Press, Erich Pollähne Gmbh, Germany) and extracts mixed with PBS extract buffer (0.07 M phosphate buffered saline (PBS), 0.05% v/v Tween 20, 1% w/v Polyvinylpyrrolidone). PVY was detected using Anti-PVY all-conjugate antibodies (SASA, Edinburgh, UK) following manufacturer's

instructions. The SASA 'PVY-all' cell line was generated by inoculating balb-c mice with a mixture of PVY^O, PVY^N and PVY^C isolates and the successful hybridomas with the ability to detect all isolates were propagated. This antibody is the standard diagnostic detection for all PVY strains in the Scottish national seed certification scheme and assumed to detect all strains used in this study with equal efficiency since PVY^O, PVY^N and PVY^{N–Wi} all contain the conserved epitope in the coat protein recognised by the utilised monoclonal. The PVY^{NTN} strain utilised is published to be an EU-NTN strain with N-type serology and also detected with the same monoclonal antibody. Absorbance (A_{405nm}) was recorded after 1 h incubation at room temperature and after 16 h incubation at 4 °C using an ELISA microplate reader (Multiskan® Ascent; Thermo Fisher Scientific). Values that exceeded twice the mean control values of non-infected potato leaves were considered positive.

2. **RT-PCR.** Total RNA was prepared from leaf tissue using the RNeasy mini kit (Qiagen). RNA (1 μ g) samples were reverse transcribed using Random primers (ThermoFisher Scientific) and M-MLV RT (Promega) at 37 °C for 1 h prior to PCR amplification. PCR conditions (using G2 Taq; Promega) were denaturation at 95 °C for 2 min, then 35 cycles of 30s at 95 °C, 58 °C, 72 °C, followed by 10 min. at 72 °C. Primers for PVY detection were CPYFOR/CPYREV and for EF1 α were EF1 α FOR/EF1 α REV.

3. Real-time PCR Quantification of PVY

Purified PVY^O virus was prepared according to Govier and Kassanis (1974) and viral RNA recovered using the PureLink Viral RNA Kit (Invitrogen). To generate a standard curve, cDNA was prepared with 20 ng of purified viral RNA using Random primers (Thermo-Fisher Scientific) and M-MLV RT (Promega) at 42 °C for 1h. A series of twelve, 2-fold serial dilutions (ranging from 20ng to 0.01ng) were used as a template for real-time PCR using FastStart TaqMan Probe Master Mix (supplemented with ROX reference dye; Roche) in a StepOnePlus Real-time PCR System (Applied Biosystems). Thermal cycling conditions were 95 $^{\circ}$ C for 10 min followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Primers used were PVYQPCRf and PVYQPCRr (Supp. Table 2) with UPL probe 21 (Roche). Using the standard curve the StepOne Software v.2.3 (Applied Biosystems) calculates the absolute quantity of PVY in plant samples that were prepared in the same way by interpolating their values from the standard curve. The total RNA concentration in the plant extracts was quantified using a NanoDrop 1000 microvolume spectrophotometer (ThermoFisher Scientific, USA) to allow the virus to be quantified from standardised plant samples.

Statistical analysis of infection data: The ELISA data gave counts of the number of infected tissues out of the total exposed. As such data does not follow a normal distribution, they cannot be analysed by the usual analysis-of-variance (ANOVA) model and instead they were analysed as a binomial generalised linear model (GLM) using Genstat software (VSN International, UK) with terms for cultivar, virus and developmental stage, and all interactions being tested. This gives an analysis of deviance table, with similar terms to an analysis of variance table for normal data. The treatment mean square terms are replaced by mean deviances and the variance ratio terms (of the mean deviance to the model dispersion). The model dispersion was estimated as part of the model fitting. Data were analysed for inoculated leaf infection, systemic leaf infection and tuber infection.

Phloem transport studies: Carboxyfluorescein diacetate (CFDA; Thermo Fisher Scientific, UK) was used as a phloem-mobile fluorescent probe to study changes in solute transport from source leaves to sink tissues (leaves and tubers) at time points before and after MPR develops; during early tuber development and at flowering respectively. Once inside the cell, CFDA is cleaved by endogenous esterase to release the membrane-impermeant CF moiety (Roberts et al., 1997). A 50 μ gml⁻¹ aqueous solution of CFDA was infiltrated into the

air spaces of source leaves through open stomata using a syringe (according to Viola et al., 2001). Due to the homeobaric nature of potato leaves, syringe infiltrations had to be carried out repeatedly on many portions of the leaf to ensure sufficient volume of dye was loaded to allow detection in sink tissues. CFDA was infiltrated into multiple neighbouring leaves at different radial positions on the stem and in the same position as would be chosen for virus inoculation at each developmental stage. Qualitative studies were used to understand and confirm that CF had successfully spread to both sink leaf and stolon/tuber tissue before a plant was utilised for imaging. (These studies also provided confidence that the leaves used for virus inoculations at each developmental stage were well selected to allow effective systemic virus spread.) Infiltrated leaves were wrapped loosely in plastic film and plants were watered well to encourage translocation of CFDA overnight (16 h). Sink tissues were then examined with a confocal microscope (either a Leica SP2; Leica Microsystems, Wetzlar, Germany, or an A1R; Nikon Instruments, Amsterdam, Netherlands) equipped with an argon laser. Tissues were excited using 488 nm light and CFDA emission collected between 505 and 530 nm, while tissue autofluorescence was collected between 570 and 630 nm.

Expression of sucrose transporters and invertases in tuber tissue: Potato cultivars Atlantic and Desiree were grown in pots in compost in a controlled temperature glasshouse (Dundee, UK, $56^{\circ}28'27''$ N; $3^{\circ}4'11''$ W) and minimum 16h daylength as above. Stolons and tubers were sampled at six harvest periods from 43 days (stolon formation) to 134 days (mature tubers) post-planting. At each stage, three representative tubers (within a median size range) from each of the 4 plants were selected, cut into 0.5 cm cubes (Further details in Supplementary Table 1), immediately frozen in liquid nitrogen and freeze-dried then ground in a Retsch mill PM400 (Verder Scientific UK Ltd) fitted with a 1 mm sieve, and stored at -80 °C.

Total RNA extraction from freeze-dried potato tubers Approximately 0.5 g of freeze-dried stolon or first sampling tubers, or 1.5g tuber (later samplings) tissue was extracted (according to Ducreux et al., 2008). RNA samples (100 μ g) were purified and genomic DNA contamination removed using an Ambion Turbo DNA-free kit according to the manufacturer's protocol. RNA samples were quantified using a NanoDrop 1000 microvolume spectrophotometer (ThermoFisher Scientific, USA) aliquoted into 25 μ g (1 μ g RNA μ l⁻¹) batches and stored at -80 °C.

2.2. Real-time PCR quantification of sucrose transporter gene expression

cDNA was synthesized by reverse transcription using 25 µg of total RNA in a 50 μ l reaction containing 2.5 μ M oligo d(T)₂₀, 0.5 mM each dATP, dCTP, dGTP, 0.2 mM dTTP, 10 mM DTT, and 500 U Superscript II (Invitrogen) in $1 \times$ reaction buffer. The RNA reaction mix was heated to 65 °C for 5 min followed by cooling on ice before addition of RT reagents, and the entire reaction incubated for 7 h at 42 °C, followed by incubation at 70 °C for 10 min and storage at 4 °C. To denature the remaining RNA, samples were treated with RNase (Promega) as per manufacturer's instructions. cDNA was used as template for real-time PCR. Reactions were performed in 10 μ l containing 1 \times Applied Biosystems PowerUp Sybr Green (supplemented with ROX reference dye) in a StepOnePlus machine. Gene-specific primers were used at a concentration of 0.2 µM. Thermal cycling conditions were: 2 min denaturation at 50 °C, 2 min denaturation at 95 °C followed by 40 cycles of 3 s at 95 °C, 30 s at 60 °C. Melting curve cycling conditions were: 15 s at 95 °C, 1 min at 60 °C then ramp at 2% to 90 °C, reading at 15s. The reactions were repeated in triplicate with independent cDNAs. Relative expression levels of 3 potato sucrose transporter genes were calculated using StepOne software v 2.3 (Applied Biosystems) using the $\Delta\Delta$ Ct method (Livak, 1997) and data obtained with the Cox (potato cytochrome oxidase) (Weller et al., 2000) specific primers as an internal control. Published sequences were used to design primers to amplify sucrose

transporters using NCBI/Primer-BLAST (Ye et al., 2012) against X69165.1 (Sut1), AY291289.1(Sut2) and AF237780.2 (Sut4). Primer sequences are available in Supplementary Table 2.

Entry of PVY into vascular tissue: PVY^N entry into phloem tissue was studied using a green fluorescent protein (GFP)-tagged infectious clone of PVY^N (PVY^{N605}; Jakab et al., 1997; Vassilakos et al., 2016). The infectious clone was assembled in the binary vector pCambia 1300 and created by duplicating the NIb-CP protease cleavage site at the C-terminus of the Nib. The GFP-coding region was inserted between the protease sites to allow excision of GFP by the PVY VPg protease (Vassilakos et al., 2016), such that post-translational processing releases GFP as a free protein in cells where the virus replicates. The complete $\ensuremath{\text{PVY}}^{N-GFP}$ infectious clone was assembled in the binary vector. Purified $\text{PVY}^{\text{N605-GFP}}$ (20 $\mu\text{g})$ was mechanically-inoculated onto 4–5 source leaves per plant of each cultivar and at each developmental stage. Fluorescent viral lesions were identified using a Leica SP2 confocal microscope at 6 dpi and thereafter each day until 12 dpi. Lesions were excised from leaves and embedded in 3% agar prior to the tissue being sectioned using a vibratome (Campden Instruments Ltd., (model 752M, Loughborough, UK). The presence of virus in class IV and V minor veins was observed by imaging GFP fluorescence using confocal microscopy (Leica SP2; Milton Keynes UK and Nikon A1R; Amsterdam, Netherlands) using argon-laser excitation at 488 nm, with emission collected between 505 and 530 nm).

HCPro Silencing Suppressor Assay: The isolate-specific HcProencoding sequences were amplified from PVY^O and PVY^{NTN} infected *Nicotiana tabacum* White Burley total RNA using attB adapter-flanked primers (YoHCFOR/YoHCREV and YntnHCFOR/YntnHCREV, respectively) and recombined into pDONR207 using Gateway BP Clonase II. The entry clones were recombined into plasmid pK7WG2D (Karimi et al., 2002) using Gateway LR Clonase II. Agrobacterium (strain AGL1) cultures carrying the plasmid constructs were prepared at OD600 = 0.1 and infiltrated into the lower surface of *N. benthamiana* leaves. Images were obtained of GFP fluorescence with an excitation wavelength (λ) of 488 nm and emission collected at λ 499-530 nm on a Zeiss LSM 710 confocal using x10 dry lens. A Gain setting of 800 was standard for all images. Mean pixel intensities were determined using ZEN 3.0 SR (black) software from ten images per construct over each of four leaves.

3. Results and discussion

3.1. Virus strain, host genotype and plant developmental stage all affect PVY infection

Tests were conducted to determine the developmental stage at which MPR occurs in glasshouse conditions against four strains of PVY (N, O, NTN and Wi) in four cultivars of potato; two early maincrop (Atlantic and Shepody) and two maincrop (Desiree and Maris Piper). The designation by maturity reflects that some cultivars take longer for tubers to mature than others; in the UK, early maincrops will typically mature from August onwards while maincrops are harvested through September and October. MPR is thought to occur in Desiree, Maris Piper and Atlantic, but not in Shepody (e.g Nolte et al., 2004; Hamm et al., 2010). Leaves were inoculated at the four different development stages: 6-leaf, stolon, tuber, and flowering. PVY was detected in the inoculated leaves of all cultivars at all stages with the exception of PVY^N in Desiree at flowering, while the extent of systemic infection decreased with plant maturity. Few plants became systemically infected when inoculations were carried out at flowering except in cultivar Shepody (Supplementary Fig. 1). This result indicates that the flowering stage was a key marker for onset of MPR. To understand the different effects of developmental stage, cultivar and virus strain, and to synthesise the large datasets from multiple experiments, a binomial generalised linear model (GLM) was applied to the PVY infection data derived from ELISA. The results are presented and explained in two sections: leaf infection (inoculated and systemic), and tuber infection.

3.2. Leaf infection

The GLM analysis showed that the virus strains were the most significant factor when evaluating virus accumulation in inoculated leaves from different cultivars inoculated at different developmental stages. However, the developmental stage, cultivar and interaction between developmental stage and cultivar were also statistically significant. When the insignificant terms were removed from the analysis, the significance of the remaining terms increased; further confirming their importance. This was true for both the inoculated and systemic leaves. The analysis of deviance tables with the significances of each factor are shown in Supplementary Table 4. In inoculated leaves both the virus strain and the cultivar were highly statistically significant (p = < 0.001) while the developmental stage and the interaction between developmental stage and cultivar were both statistically significant (p = 0.007and 0.022 respectively). In systemic leaves, the virus strain, developmental stage and cultivar were all highly significant factors in the amount of infection detected (p = <0.001) and the interaction between developmental stage and cultivar was highly significant at p = 0.001.

Fig. 1A shows the modelled, predicted proportion of inoculated leaves becoming infected with each of the four virus strains, irrespective of any other factor, while Fig. 1B shows the same for systemic leaves. The propensity for a given virus strain to cause infection differs between inoculated and systemic leaves, but PVY^O will always cause least infection. In inoculated leaves, PVY^O is predicted to cause the lowest level of infection (0.68, s.e. 0.036), followed by PVY^N (0.87, s.e. 0.027) and PVY^{NTN} (0.98, s.e. 0.012), with PVY^{N-Wi} (1.00, s.e. 0.0002) having the highest infectivity. The proportion of infections in systemic tissue for each virus changes such that PVYNTN (0.79, s.e. 0.03) and PVY N-Wi (0.82 s.e. 0.027) are both decreased by about 20%, PVY^N (0.62, s.e. 0.031) infectivity is decreased by about 30% and PVY^O (0.19, s.e. 0.027) is decreased by around 70%. These differences in response to the different virus strains suggests that the viruses differ in their infectivity or their ability to spread through the plant, or that the plant differs in its resistance response to different viral strains, or a combination of these factors. A summary table of the known resistance genes, time to maturity and published infectivity scores for the different cultivars is provided in Supplementary Table 5.

The interaction between cultivar and developmental stage is illustrated for inoculated (Fig. 1C) and systemic (Fig. 1D) tissue, with infections being less prevalent in systemic tissue. Taken together, the data show that cv Shepody is the most susceptible cultivar to PVY infection, with Maris Piper and Atlantic showing intermediate levels of infection and Desiree being most resistant. Note that in these graphs, the virus strains are considered together as there was no statistically significant interaction between virus and the other factors. This analysis also shows that when plants reach flowering stage there is a significant reduction in infection in both inoculated and systemic tissues, but particularly in systemic tissue. All cultivars show a clear trend towards reduction in infection as the plants mature, and MPR is developed and effective by the flowering stage.

3.3. Tuber infection

Similar statistical analysis was carried out on progeny tubers. Due to the large scale of the experiments, tubers were not analysed for every permutation of virus strain-cultivar-developmental stage, but data was gathered to allow PVY^{O} , PVY^{N} and PVY^{NTN} to be analysed at 6-leaf and flowering stages (the youngest and oldest stages studied), while data was gathered for all four developmental stages with PVY^{O} and PVY^{N} . These sets were analysed separately. Graphs shown in Fig. 2A–F displays the most informative relationships from the different statistical models that were investigated.

For the tuber data for three virus strains and two stages, all single factor and two-way interactions were statistically significant (*p*-values provided in Supplementary Table 4), showing that the virus strain, the



Fig. 1. Statistical models illustrate the effect of potato cultivar and virus strain on MPR in leaf tissue.

(A) shows the proportion of inoculated leaves of all cultivars that become infected with each of the four virus strains, irrespective of any other factor (i.e. cultivar or developmental stage). Variation in the propensity to cause infection in inoculated leaves varied from 68% for PVYO to 100% for PVY^{N-Wi} . (**B**) shows the same information for systemic leaves. The likelihood of infection drops in systemic tissue and ranges from 19% for $\ensuremath{\text{PVY}^{\text{O}}}$ to 81% for PVYWi; systemic infections can be reduced by around 70% compared to inoculated-leaf infections depending on the virus strain. Analysis of the interaction between cultivar and developmental stage also showed differences between inoculated (C) and systemic (D) tissue, with infection again being less prevalent in systemic tissue, and a significant reduction in infection in both inoculated and systemic tissues by flowering (c.f. C and D). Error bars show ±standard errors.

cultivar and the developmental stage all significantly influence the propensity for infection. The three-way interaction between virus strain, cultivar and developmental stage was not statistically significant. Similar to leaf data, the virus strain alone was the most significant factor (Fig. 2A), with a clear differential between the ability of PVY^{O} (0.11, s.e. 0.015), PVY^{N} (0.63, s.e. 0.017) and PVY^{NTN} (0.94, s.e. 0.015) to cause tuber infections. When the developmental stage (6-leaf or flowering) was added into this model with three virus strains (Fig. 2B), a similar trend can be seen, with least infection at flowering and greatest at 6-leaf irrespective of the viral strain. Importantly, for PVY^{O} , complete resistance to this viral strain was observed at the flowering stage, while for PVY^{NTN} , flowering has very little effect and more than 90% of tubers were infected at this stage.

The model was next used to study the effects of all four developmental stages with PVY^{O} and PVY^{N} . All single factors were statistically significant, and also the two-way interactions of virus strain with cultivar and virus strain with developmental stage. The model shows that in general, irrespective of the interactions studied, a decline in infection is observed as plants grow older. When the virus strains (PVY^{O} or PVY^{N}) are separated out (Fig. 2C) and the interaction between strain and stage is studied, the difference in infectivity between virus strains become more obvious. While PVY^{O} can be predicted to fail to infect tubers at flowering, resistance is much less effective against PVY^{N} .

The model was finally used to study the effect of potato cultivar on the tuber infection process. When the effect of cultivar alone was studied (Fig. 2D), as seen in leaf infections, Desiree showed greatest resistance to PVY, Shepody had least resistance, and Maris Piper and Atlantic showed intermediate levels of tuber infection. Taken together, PVY^N and recombinant strains can be expected to cause tuber infection across the cultivars studied and at any developmental stage. When the developmental stage and cultivar were combined (Fig. 2E), we see that, similar to the leaf infection results, the propensity for tuber infection is lower at flowering than at the 6-leaf stage, but some infection would be predicted at any time point. The significant interaction between cultivar and virus (Fig. 2F) shows that the virus strain strongly affects the likelihood of tuber infection in all cultivars, and highlights the reduced infection across all varieties with PVY^O. In general, PVY^{NTN} will result in the greatest tuber infection irrespective of cultivar, PVY^N will cause some infection in all potato cultivars, and PVY^O will cause relatively little infection in cultivars Shepody, Maris Piper and Atlantic, and no infection in cv. Desiree. While MPR can reduce tuber infection for PVY^O and PVY^N in some cultivars, it has little effect against PVY^{NTN}, which therefore appears to overcome MPR.

The cultivars Desiree and Atlantic carry the Ny_{tbr} resistance gene and local and systemic hypersensitive reactions respectively are elicited on infection with PVY^O (Jones, 1990; Jones and Vincent, 2018; Kehoe and Jones, 2016; Rosario Herrera et al., 2018). Although cultivars Desiree and Atlantic carry similar resistance genes (see Suppl Table 5), in the present study cv Desiree was consistently more resistant to PVY infection. This may be due to variation in N-gene efficacy in different genetic backgrounds (Jones and Vincent, 2018). Notably, in the cv. Yukon Gold, which is known to possess the same N-genes as Desiree and Atlantic (Jones and Vincent, 2018) and is susceptible to PTNRD, Chikh-Ali et al. (2019) found that systemic infection and PTNRD symptoms in tubers were apparent in early season infections but rarely occurred later in the season. These results suggest that MPR may be effective in association with, or additive to other resistance responses. Taken together, these studies indicate that the effect of MPR may be modified by the



Fig. 2. Statistical models illustrate the effect of potato cultivar and virus strain on MPR in tubers.

(A-F) display the most informative relationships illustrated in the different statistical models. The virus strain alone was the most significant factor (A), with a clear differential between the ability of PVY^O (0.11), PVY^N (0.63) and PVY^{NTN} (0.94) to cause tuber infections irrespective of cultivar or stage of development. (B) shows the model of developmental stage (6-leaf or flowering) and virus strain; a similar effect of viral strain can be seen (c.f. A and B), but with less infection at the later developmental stage for PVY^O and PVY^N. Flowering has little effect on PVY^{NTN} infection of tubers. Models of all four developmental stages (6-leaf, stolon, tuber, and flowering (C and D) show a decline in tuber infection as plants grow older. When the virus strains (PVY^O or PVY^N) are separated out (C), the difference in infectivity between virus strains is evident: resistance to tuber infections at flowering can be predicted for PVY^O, but is less effective against PVY^N. (**D**) illustrates the effect of potato cultivar on the tuber infection process; similar to leaf infections (Fig. 1C and D), Desiree showed greatest resistance, Shepody had least resistance, and Maris Piper and Atlantic showed intermediate levels of tuber resistance. When the developmental stage and virus were combined (E), the propensity for tuber infection is lower at flowering than at the 6-leaf stage, but some infection would be predicted at any time point. If the individual virus strains are added to the model (F), the significance of virus strain is clear, but cultivar is also important.

complement of strain specific N genes carried by the potato cultivar.

3.4. Phloem transport alters with maturity and flowering reduces symplastic unloading in sink tissues

Since there must be a route for PVY movement into tubers, carboxyfluorescein (CF) tracer experiments were used to study phloem transport pathways. When carboxyfluorescein diacetate (CFDA) is loaded into the phloem, endogenous esterases cleave the diacetate molecule, leaving the fluorescent CF molecule trapped in the symplast of the phloem where it can be imaged to show phloem connectivity and unloading. These experiments were performed at both the tuber development stage and at flowering, i.e. before and after MPR was initiated. At the tuber development stage, when most potato cultivars showed PVY infection in systemic leaves, CF was detected in the phloem of sink leaves of all cultivars (Fig. 3A–D). A functional solute transport pathway from source to sink leaves was therefore active at this stage and could be utilised by PVY. Similarly, extensive unloading of CF was detected in tubers at this stage (Fig. 3E–H), although solute unloading was more limited in tubers of cv Desiree than those of cultivars Atlantic, Maris Piper and Shepody (c.f. Fig. 3E with F, G and H). Therefore, at the tuber development stage, an active phloem transport pathway exists to carry PVY to both non-inoculated leaves and tubers, but the symplastic phloem unloading capacity of Desiree appears to be much lower than the other cultivars.

In contrast, at the flowering stage, CF unloading was not detected in

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Fig. 3. Phloem transport alters as plants mature

Confocal microscope images of leaves showing CF fluorescence as a measure of phloem transport. Images show for each of the four cultivars (Desiree, Atlantic, Maris Piper, Shepody): A to D sink leaves; E to H developing tubers; I to L tubers at flowering stage; M to P as I to L at higher magnification. (A to D) CFDA was detected in the phloem of sink leaves of all potato cultivars at the tuber development stage (i.e. before MPR is active), when most cultivars showed some degree of PVY infection in systemic leaves. Extensive unloading of CFDA was also detected in tubers at this stage (E to H), although solute unloading was more limited in tubers of cv Desiree than those of cultivar Atlantic, Maris Piper and Shepody (c.f. E with F, G and H).

apical sink leaves that corresponded to the non-inoculated leaves sampled for PVY infections previously (data not shown; blank images). CF translocation into tubers was detected at the flowering stage (Fig 3 I to L), but the quantity of CF detected was much less than that found at the earlier developmental stage. When higher magnification images of this tuber tissue were collected (Fig 3 M to P) the dve was seen to be limited to within the phloem strands; extensive phloem unloading was not as visible as in young tubers and in fact looked similar to the pattern of unloading seen in Desiree at the earlier developmental stage. These results suggest that a significant reduction in symplastic phloem unloading to both sink leaves and tubers occurs around the time of flowering, and that Desiree shows less symplastic unloading than the other three cultivars, a fact that is particularly interesting given the apparently greater resistance of Desiree to PVY (c.f. the reduced infection rates of both foliar and tuber tissues of Desiree infected with PVY^O in Fig. 2E and F and Supplemental Fig. 1). It may be that Desiree utilises apoplastic phloem unloading at an earlier developmental and that this may be a mechanism associated with other resistance genes found in Desiree.

3.5. The latter stages of tuber filling occur using an apoplastic phloem unloading mechanism

A reduction in phloem unloading appears to explain the reduction in virus observed in mature plants but seems inconsistent with the continuing development of potato tubers, shown in Fig. 4A. Flowering occurred in the glasshouse experiments between 77 and 81 days post planting (dpp), but at this stage tubers were relatively small and had yet to reach maturity. Fig. 4A shows the mean tuber weights for each of the four potato cultivars sampled at six time points, starting as the stolons began to show signs of swelling. The final time point was collected at plant maturity, as foliage senesced in the pot-grown plants, and tubers had reached maximum size. The different cultivars produce varying tuber sizes; some make fewer large tubers and some more numerous smaller tubers, but in each case, most of the tuber filling can be seen to occur after flowering. The four developmental stages used to sample tissue for the results shown in Figs. 1 and 2 are shown along the x-axis of Fig. 4A to aid comparison with days post planting, or days postemergence which is used in some potato publications.

If tubers continue to grow once symplastic phloem unloading has



Fig. 4. The latter stages of tuber filling occur using an apoplastic phloem unloading mechanism.

(A) Mean tuber fresh weights for each of the four potato cultivars sampled at six time points (43, 49, 64, 78, 98 and 134 days post-planting), beginning as stolons showed signs of swelling The final time point at plant maturity (senescence of foliar tissue) was considered to be when tubers had reached their maximum size. The different cultivars produce varying tuber sizes, but in each case, the majority of tuber filling occurs after flowering. The four developmental stages used to sample tissue for the results shown in Figs. 1 and 2 are shown along the x-axis of the graph; 6-leaf stage = 24-26 dpp, stolon stage = 36-40 dpp, tuberisation stage = 50-52 dpp and flowering stage = 77-81 dpp. (B) Relative expression levels of 3 sucrose transporters; Sut1, Sut2 and Sut4 in tuber tissue from cultivars Desiree and Atlantic, sampled at the same six time points shown in Fig. 4A. For both cultivars and all three transporters studied, increased expression was observed in the latter stages of tuber filling; timepoints which fall after flowering and the onset of MPR.

ceased, there must be an alternative pathway available to unload sucrose accumulated from foliar photosynthesis for subsequent starch conversion and storage. We therefore investigated sucrose transporter expression during tuber development. Tuber tissue from cultivars Desiree and Atlantic were sampled at the same six time points shown in Fig. 4A and qRT-PCR was used to study the relative expression levels of 3 sucrose transporters known to be important in potatoes; Sut1, Sut2 and

Sut4 (Fig. 4B). For both cultivars and all three transporters studied, very large increases in expression were observed in the latter stages of tuber filling; timepoints which fall after flowering and the onset of MPR. Tuber filling therefore occurs apoplastically and the symplastic unloading pathway is not functional when MPR is functional, which will prevent PVY unloading and restrict the virus to phloem cells. Tuber filling was assumed to occur symplastically (Viola et al., 2001, 2007; Hancock et al., 2008), but we show that this switch occurs much earlier, while the tuber is developing. This switch to apoplastic unloading may represent an evolutionary strategy which benefits potato plants by restricting pathogen access to their vegetative reproductive tissue; but one that is ineffective for any virus that can gain access to the tuber phloem strands and wait until symplastic connections are re-established at bud break, allowing virus to enter daughter sprouts. Our data shows that the virus has a functional pathway to exit the inoculated leaf and move through the transport phloem while MPR is functional, but virus unloading is hampered by an apoplastic unloading pathway and restriction of unloading in tubers again appears to be the most likely mechanism for MPR.

3.6. The transport phloem is active and symplastically connected

Having studied phloem unloading, experiments to determine the functionality of the transport phloem were conducted. While small quantities of CF were detected transporting to, and unloading into tubers at the flowering stage, none was detected in leaves of the potato cultivars (data not shown; blank images). At the flowering stage, stem sections imaged above the CFDA-infiltrated leaves showed that CF was negligible in phloem tissue to the apical part of the plant (Fig. 5 A & B show representative sections). In contrast, stem sections below the CFDA-infiltrated leaves showed strong evidence of CF in the transport phloem (Fig. 5C and D), indicating that the majority of phloem translocation occurs downwards at flowering; and that at this stage, tubers are a greater sink than apical leaves as published by Fernie and Willmitzer (2001). Since the same leaves were used to inoculate virus and infiltrate CFDA, the presence of CF in transport phloem is taken to represent the expected pathway available for virus movement.

Many plant viruses utilize the solute transport pathway to move through to other parts of the plant (Kappagantu et al., 2020; Tilsner et al., 2014). Therefore, any change in solute movement might be expected to result in failure of long distance movement of PVY. However, MPR was still elicited against PVY^O even when only a small amount of CF was transported and unloaded to tubers in cv Atlantic at the flowering stage (Fig. 5B). Hence even this reduced level of solute transport and unloading may be enough to express MPR against PVY^O. However, it is intriguing that other strains of PVY (PVY^N, PVY^{NTN} and PVY^{NWi}) overcome such a change in solute transport and successfully cause infection in the tuber.

3.7. PVY can exit the inoculated leaves at the flowering stage

PVY moves through the phloem, utilising the existing metabolite transport pathway and involves entry of viruses into sieve elements, passive (bulk) flow of virus in the transport phloem and exit (unloading) of viruses in sink tissue (Dupuis, 2017). The most likely possible points to control long distance movement of virus are at entry into and exit from SEs in sources/sinks respectively. It is clear that a functional phloem transport pathway for solutes continues to operate after MPR begins, but lack of phloem entry, as opposed to transport, could be a mechanism for MPR against viruses. Experiments were therefore carried out to investigate the ability of PVY to access the transport phloem at the flowering stage using a full-length infectious clone of PVY^N tagged with GFP (PVY^{N–GFP}). Confocal imaging was used to investigate movement of the fluorescent virus into phloem cells on the inoculated leaf at the same four growth stages as studied previously. The time taken for the virus to enter phloem cells varied with cultivars; $PVY^{N–GFP}$



Fig. 5. The transport phloem is active and symplastically connected, and PVY can enter the phloem of inoculated leaves at the flowering stage.

CF fluorescence is shown in green and cell wall or lignin autofluorescence is shown in magenta. (A and B) Stem sections above the CFDA-infiltrated leaves show CF transport was negligible to the apical part of the plant. (C & D) stem sections below the CFDAinfiltrated leaves showed strong evidence of CF in the transport phloem indicating that the majority of phloem translocation occurs towards sink tubers at this developmental stage. A and C = Desiree; B and D = Maris Piper. The same phenotype was seen in all cultivars. (E to H) Confocal imaging of a full-length infectious clone of PVY^N tagged with GFP (PVY^{N-GFP}) shows that PVY can access the transport phloem at both 6-leaf and flowering stages in all cultivars. PVY^{N-GFP} can enter the phloem at flowering, as seen by GFP fluorescence in companion cells (arrows), showing that restricted entry into the phloem does not contribute to MPR. Representative images show 6-leaf stage (E,F) or flowering stage (G, H). E and G = Desiree; F and H = Maris Piper. Bar in A = 50 um for A and B: Bar in E = 30 um for E to H. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in approx. 5–6 days in cultivars Shepody and Atlantic while in cv Desiree PVY^{N–GFP} it was detected in phloem tissues at 7–9 days post inoculation (data not shown). These time periods for detection of virus in the phloem corresponded to the window in which systemic infection was expected to occur. These studies showed PVY^{N–GFP} entry/loading into phloem cells at all growth stages of each cultivar, including the flowering stage. Representative images of fluorescent virus in 6-leaf (Fig. 5E and F) and flowering stage (Fig. 5G and H) plants are shown and indicate that restricting entry of the virus into the phloem is not a feature of MPR. In addition, PVY^O was assayed in tissues above and below the inoculated leaves in cv. Atlantic, and in stolons and tubers using RT-PCR to detect coat protein (Fig. 6A). PVY^O was strongly detected in the inoculated leaf and weakly in the inoculated petiole (i.e. in the transport phloem as the virus exited the inoculated leaf) and also leaves below the inoculated

leaf. Traces of virus were detected in the apical (sink) leaf and roots, but no virus was found in the (likely source) leaves above the inoculated leaf or in tubers; likely due to the fluorescence being below the detection limit of the confocal microscope, particularly against the autofluorescent tissue of tubers. The results show that PVY^O moves down into sink tissue but not upwards into source tissue at the flowering stage, indicating that tubers are the strongest sink in relation to the inoculated leaves at this stage and that virus access mirrors CF transport at each developmental phase.

3.8. PVY^{NTN} is more infectious than PVY^{O} and has a stronger silencing suppressor

To determine whether differences in virus replication could account



Fig. 6. The distribution of PVY in different tissues and replication efficiency of different strains at flowering stage.

(A) Detection of PVY^O in leaves, stolons and tubers using RT-PCR to the viral coat protein. Virus was detected strongly in the inoculated leaf and present in the inoculated petiole (likely representing transport phloem) and leaves below the inoculated leaf. Traces of virus were detected in the tip leaf and roots, but no virus was found in the (likely source) leaves above the inoculated leaf or in tubers. U1 and U2 = Upper Leaves; one and two leaves respectively above the inoculated leaves. L1 and L2 = Lower leaves; one and two leaves respectively below the inoculated leaves. IP = Inoculated Leaf Petiole. S = Stem. R = Root, T = Tip leaves at apex of plant. Tu = Tuber tissue. +ve and -ve lanes indicate template from PVY^O-infected N.tabacum White Burley or a mock-inoculated control respectively. (B) Relative quantities of PVY^O and PVY^{NTN} in inoculated leaves of six-leaf or flowering Atlantic plants. In young leaves, the amount of PVY^{NTN} was taken to equal 100% infection, mock-inoculated leaves gave an index of zero and PVY^O achieved only 15.6% of the infection caused by PVY^{NTN}. In comparison, at the flowering stage, mock, $\ensuremath{\text{PVY}^{\text{O}}}$ and $\ensuremath{\text{PVY}^{\text{NTN}}}$ achieved 0%, 2% and 14% infections respectively. Comparison of the efficiency of the HCPro silencing suppressors was achieved by comparing the level of GFP expression when co-expressed with PVY^{O} HCPro (C) and PVY^{NTN} HCPro (D). Images were collected and presented at identical microscope and software settings and the difference in fluorescence was obvious between the virus strains. Mean pixel intensities were calculated and showed that the PVYNTN silencing suppressor is more efficient than that of PVY^O, as evidenced by lower fluorescence in the PVY^{O} -infected tissue. Bar in $C = 100 \ \mu m$ for C and D.

for differences in the effectiveness of MPR, given that the virus strain is the most significant factor in the infection model, the relative quantities of virus at 8dpi in cv. Atlantic inoculated leaves at flowering was measured (Fig. 6B). Eight days post-inoculation was selected to allow time for abundant inoculated-leaf spread to occur, and around the time when systemic infection is expected to occur. Following the standardised inoculation procedure, relative amounts of virus were estimated by qRT-PCR, and the amount of PVY^{NTN} at the 6-leaf stage was taken to equal 100% infection, with mock-inoculated leaves at 0% infection at both the 6-leaf and flowering stage. In comparison, PVY^O achieved only 15.6% of the amount of PVY^{NTN} at the 6-leaf stage and PVY^O and PVY^{NTN} achieved 9.1% and 21.7% infections respectively at the flowering stage. It is possible that the inherent infectivity of the virus strains may account for some of this variation between PVY^O and PVY^{NTN}, but all infections were initiated with an excess virus load to maximise infection in susceptible plants and allow cultivar and strain differences to be studied with confidence. This data shows that PVY^{NTN} has a replication advantage over PVY^O irrespective of the growth stage, but also that older, flowering plants are significantly more resistant to both virus strains.

The efficiency of the isolate-specific HcPro silencing suppressors was tested using an agrobacterium-mediated GFP patch assay (Fig. 6 C and D). There was a clear difference in fluorescence between the samples of tissue co-expressing either PVY^O HCPro (Fig. 6C) and PVY^{NTN} HCPro (Fig. 6D). GFP fluorescence was imaged and quantified and the results showed a significant difference (p = 0.008) between the mean pixel intensities (15.21 and 32.78 for PVY^O and PVY^{NTN} respectively) indicating that the PVY^{NTN} silencing suppressor is more efficient than that of PVY^O.

4. Conclusions

The virus strain is the most significant factor in predicting likelihood of PVY infection, but differences in cultivars also significantly affect whether MPR is functional in potato. In cultivars where MPR is functional, it provides protection to some viral strains by the time the plant is flowering, but MPR is not functional against recombinant PVY strains which are more infectious and have a stronger silencing suppressor than PVY^O. It appears that the effectiveness of MPR may be affected by the complement of host N-genes. If PVY^N or recombinant virus strains are in the environment, irrespective of which cultivar is grown, tuber infection is almost assured if foliar PVY infection is present during the growing season. Strain-specific N-genes have been widely utilised in breeding programs and usually confer field resistance to PVY but with the decline in incidence of PVY^O and increase in recombinant strains worldwide, controlling the prevalent strains of PVY may require the deployment of dominant R-genes which confer extreme resistance against most strains of PVY.

The sink-source transition and phloem transport are not involved in the mechanism of MPR. All potato cultivars which were inoculated with recombinant PVY strains produced infected tubers irrespective of the developmental stage at which they were inoculated, and irrespective of whether the virus achieved systemic leaf infection. We showed that tuber filling occurred apoplastically at an earlier time point than previously known. This switch to apoplastic unloading may represent a strategy utilised by potato plants to restrict pathogen access to vegetative tissues, and as was seen for Desiree inoculated with PVY^O, it may be sufficient to prevent tuber infections. However, the pathway into tuber phloem strands is present and functional for N-type and recombinant virus strains where the virus can remain until tubers sprout. Restriction of unloading in tubers appears to be the most likely mechanism for MPR in those plants where it is effective.

CFDA translocation into tubers was detected at the flowering stage, after the onset of MPR (I to L), but the quantity of CFDA detected was much less than that found at the earlier developmental stage (compare E-H with I-L). (M to P) Higher magnification images of tissue shown in I to J show the dye was limited to within the phloem strands (arrows), in contrast to the extensive phloem unloading seen in young tubers (E to H). Scale bar in A = 5mm for A to D. Bar in E = 5mm for E to L. Bar in M = 1mm for M to P.

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CRediT authorship contribution statement

Pankaj Kumar: Investigation, Methodology, Writing – original draft, Visualization. **Graham H. Cowan:** Investigation, Methodology, Visualization. **Julie N. Squires:** Investigation. **Christine A. Hackett:** Formal analysis, Writing – original draft. **Alyson K. Tobin:** Conceptualization, Writing – original draft, Supervision, Funding acquisition. **Lesley Torrance:** Conceptualization, Writing – original draft, Supervision, Project administration, Funding acquisition. **Alison G. Roberts:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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