Potato Virus Y (PVY) Isolates from *Solanum betaceum* Represent Three Novel Recombinants Within the PVY^N Strain Group and Are Unable to Systemically Spread in Potato

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ABSTRACT

Tamarillo, or tree tomato (*Solanum betaceum*), is a perennial small tree or shrub species cultivated in subtropical areas for fresh fruit and juice production. In Ecuador, tamarillo orchards are affected by several viruses, with one previously identified as potato virus Y (PVY); however, the specific strain composition of PVY in tamarillo was not determined. In 2015 and 2016, eight tamarillo plants exhibiting symptoms of leaf drop, mosaic, and mottled fruit were sampled near Tumbaco and Quito, Ecuador. These tamarillo PVY isolates were able to systemically infect tobacco, *Nicotiana benthamiana*, naranjilla, and tamarillo. Seven of the eight PVY isolates from tamarillo exhibited N-serotype, while one of the PVY isolates studied, Tam15, had no identifiable serotype. One isolate, Tam17, had N-serotype but produced asymptomatic systemic infection in tobacco. In tamarillo, four tamarillo isolates induced mosaic and slight

growth retardation and were unable to systemically infect pepper or potato. Tamarillo, on the other hand, was unable to support systemic infection of PVY isolates belonging to the PVY^O and PVY^{Eu-N} strains. The whole genomes of eight PVY isolates were sequenced from a series of overlapping RT-PCR fragments. Phylogenetically, tamarillo PVY isolates were found to belong to the large PVY^N lineage, in a new tamarillo clade. Recombination analysis revealed that these tamarillo PVY isolates represent at least three novel recombinant types not reported before. The combination of the biological and molecular properties found in these eight PVY isolates suggested the existence of a new tamarillo strain of PVY that may have coevolved with *S. betaceum*.

Keywords: ecology and epidemiology, genetics and resistance, virology

Tamarillo, also called tree tomato (tomate de árbol in Spanish) (*Solanum betaceum* Cav., synonym *Cyphomandra betacea* (Cav.) Sendtn.), is a small tree or shrub species cultivated for fresh fruit in many subtropical areas, including several countries in South America and Africa, as well as in India, Australia, and New Zealand. It is grown in Ecuador and Colombia as a perennial fruit crop for fresh fruit and juice production. Multiple viruses have been reported to affect tamarillo, including tamarillo mosaic virus, now reclassified as a strain of potato virus A (PVA), potato aucuba mosaic virus, alfalfa mosaic virus, cucumber mosaic virus (CMV), tomato spotted wilt virus (Eagles et al. 1994; Jaramillo et al. 2011), tomato mosaic virus and tomato ringspot virus (Jaramillo et al. 2011), potato virus Y (PVY), potato leafroll virus (PLRV) (Espinoza et al. 2017; Insuasti et al. 2016; Jaramillo et al. 2011), potato virus V (PVV), Peru tomato mosaic virus (PTMV) (Insuasti et al. 2016), and two recently identified tymoviruses, naranjilla chlorotic mosaic virus (NarCMV) (Green et al. 2018a), and naranjilla mild mosaic virus (NarMMV; Cornejo-Franco et al. 2019). PVA, formerly considered a distinct

tamarillo mosaic virus, represented a major problem in tamarillo cultivated in New Zealand (Eagles et al. 1994), while CMV, PLRV, and the two potyviruses, PVY and PVA, were common in tamarillo crops in Colombia (Jaramillo et al. 2011). In Ecuador, six viruses were reported to affect tamarillo: PVY, PLRV, PVV, PTMV (Espinoza et al. 2017; Insuasti et al. 2016), NarCMV (Green et al. 2018a), and NarMMV (Cornejo-Franco et al. 2019).

Virus-like symptoms in tamarillo orchards had been observed on multiple occasions during field surveys in Ecuador conducted since 2013, including mosaic, mottling, leaf deformation, and severe defoliation (Insuasti et al. 2016; D. Quito-Avila, *unpublished observations*). Of the six viruses found in tamarillo in Ecuador, PVY and PLRV were determined to be the most common, followed by NarMMV, PVV, and PTMV (Espinoza et al. 2017; Cornejo-Franco et al. 2019; Insuasti et al. 2016). Nevertheless, the prevalence and pathogenesis of PVY in tamarillo, as well as genetic diversity and strain composition of the virus in this crop in Ecuador have not been studied yet. A small, partial 0.9-kb nucleotide sequence from the P3 cistron region, determined for an isolate of PVY from tamarillo, was only 89% identical to the closest match in the GenBank database (Insuasti et al. 2016), suggesting possible presence of a new strain of the virus in this crop. Incidentally, various strains and recombinants of PVY were found in other crops, such as potato, cape gooseberry (*Physalis peruvianum*), and tomato, in neighboring Colombia and Peru (Alvarez et al. 2018; Fuentes et al. 2019; Munoz et al. 2016), although all this PVY diversity was represented by the known PVY strains and recombinants (Green et al. 2018b).

PVY exists as a complex of strains (Karasev and Gray 2013; Kerlan 2006; Quenouille et al. 2013; Singh et al. 2008), which may differ by up to 18% in their nucleotide sequences (Green et al.

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2018b). Phylogenetically, the currently known PVY isolates can be separated into four major lineages: PVY^O, PVY^N, Chile3, and PVY^C, two of which, PVY^O and PVY^N (Green et al. 2017b; Karasev and Gray 2013; Kehoe and Jones 2016; Quenouille et al. 2013; Singh et al. 2008), represent virus isolates from potato, while the PVY^C lineage comprises virus isolates from pepper, potato, and some other solanaceous hosts, such as tomato (Chikh-Ali et al. 2016; Green et al. 2017b; Singh et al. 2008) and cape gooseberry (Green et al. 2017b). A single sequence from the Chile3 lineage came from a pepper isolate of PVY (Moury 2010; Quenouille et al. 2013).

PVY infection is a serious problem in many solanaceous crops worldwide, but the most studied PVY-caused diseases are in potato, pepper, and tobacco (Kerlan 2006; Karasev and Gray 2013; Quenouille et al. 2013; Singh et al. 2008). Tamarillo is a specialty crop cultivated in the tropical and subtropical areas of the Andes, in Ecuador and Colombia, and the effects of PVY in this crop have not yet been elucidated. The objectives of this study were to investigate the genetic diversity of PVY isolates circulating in tamarillo orchards in Ecuador using sequencing and bioinformatics, and to determine the symptomatology and pathogenesis of a new PVY strain in tamarillo and several other solanaceous hosts using biological assays. We determined that the PVY isolates from tamarillo in Ecuador belong to a new strain that is apparently adapted to *S. betaceum*. This new PVY strain appears quite diverse, comprising at least three new PVY recombinants.

MATERIALS AND METHODS

Virus origin and maintenance. In early 2016, several tamarillo plants exhibiting defoliation, leaf mosaic, and discolored fruit were sampled at Tumbaco and Quito, Ecuador, as part of a PVY survey of local solanaceous hosts with virus-like symptoms. Symptomatic leaves were tested for PVY, PVV, PTMV, and PLRV as described (Insuasti et al. 2016). Leaves from eight samples, which were positive for PVY only, were selected and characterized at the University of Idaho. RNA samples were stored at -80°C and used for RT-PCR testing of two new tymoviruses, which were reported as this work was in progress (Cornejo-Franco et al. 2019; Green et al. 2018a). All eight samples tested negative for both tymoviruses.

Sap from the tamarillo leaf tissue was mechanically inoculated onto *Nicotiana benthamiana* plants, which developed virus-like symptoms and were confirmed PVY-positive by an immunostrip test (Agdia, Elkhart, IN). The isolates were transferred to and maintained in tobacco (*Nicotiana tabacum* L. 'Burley'), through periodic reinoculation into young healthy plants at the four- to six-leaf stage in an insect-free growth room at the University of Idaho, as part of the PVY isolate collection, according to previously described protocol (Green et al. 2017b). This collection was periodically subjected to serological (Nikolaeva et al. 2012) and RT-PCR (Chikh-Ali et al. 2013; Lorenzen et al. 2006b) typing as part of the maintenance protocol to avoid contamination or strain mixtures and ensure stable characteristics of the isolates.

Biological characterization. Infected tobacco (*N. tabacum* 'Burley') plants were used as a source for mechanical inoculations of five additional plant species: *N. benthamiana*, *S. betaceum* (tamarillo), *S. quitoense* (naranjilla), *Capsicum annuum* (pepper) 'Early California Wonder', and two *S. tuberosum* (potato) cultivars with known genetic background (Maris Bard and Russet Norkotah) (Rowley et al. 2015). The potato cultivars were maintained as virus-free tissue-culture lines at the Potato Tissue Culture Laboratory at the University of Idaho. All plants were grown in an insect-proof greenhouse before and after inoculations, some under artificial light provided by fluorescent and incandescent lamps, and some under natural and artificial light

with an 18 h day/6 h night cycle and maintained at 23 to 26°C. Plants were mechanically inoculated with sap from systemically infected tobacco at the four- to six-leaf stage as previously described (Chikh-Ali et al. 2016; Hu et al. 2009b). Symptom observations started between 3 to 10 days after inoculation, depending on the plant species, and were carried out for 3 to 5 weeks for all plant species, except tamarillo, which was observed and periodically tested for systemic virus infection for about 20 months. Two or three plants per species/cultivar were tested in a single experiment, which always included healthy control plants as well as PVY control isolates Oz (PVY^O) and Mont (PVY^{Eu-N}). Each plant was tested at 2 weeks postinoculation (wpi) for infection in inoculated leaves, except potato, which was tested at 3 wpi, and subsequently at 5 wpi and later for systemic infection, and subjected to strain typing to confirm the identity of the inoculated PVY strain, using triple-antibody sandwich (TAS) enzyme-linked immunosorbent assay (ELISA) and RT-PCR as described below.

Serological analysis, RT-PCR, and differentiating primers.

The serological reactivity of the eight tamarillo isolates of PVY was tested in a TAS-ELISA format, as described by Nikolaeva et al. (2012). All tests included control PVY isolates from the laboratory collection, with distinct serological patterns characteristic of PVY^O and PVY^N strains. In addition to a polyclonal antiserum, Asc5 (Funke et al. 2017; Karasev et al. 2010), three strain-specific monoclonal antibodies were used: SASA-O (Scottish Agriculture Science Agency [SASA], Edinburgh, Scotland), which recognize PVY^O, PVY^O-O5, PVY^{N-Wi/N:O}, and PVY^C; 1F5 (Agdia, Elkhart, IN), which reacts with PVY^N, PVY^O-O5, and PVY^{NTN}; and SASA-N (Scottish Agriculture Science Agency), which identifies PVY^N and PVY^{NTN}.

Two different multiplex RT-PCR assays were performed on the eight PVY isolates from tamarillo, following the methods of Lorenzen et al. (2006b) and Chikh-Ali et al. (2013). Immuno-capture reverse transcription (IC-RT-PCR) and PCR reactions were performed essentially as described previously (Chikh-Ali et al. 2013, 2016). Control strains of PVY and healthy controls were obtained from the University of Idaho laboratory collection, and assays were conducted at the same time on all samples.

Sanger sequencing and sequence analysis. The nearly whole genome sequences of PVY isolates Tam13 through Tam20, missing only 20 to 120 nt at the 5' end due to the sequencing methodology used, were obtained by sequencing overlapping RT-PCR products as described by Green et al. (2017a). All PCR products were purified using ExoSAP-IT for PCR Product Clean-Up (USB Products; Affymetrix Inc.), mixed with appropriate primers and submitted for Sanger sequencing to Genewiz, Inc. (South Plainfield, NJ). The contigs obtained were assembled using the SeqMan Pro program of the Lasergene 9 Suite (DNASTAR). Sequence identity was checked using the BLAST program provided by the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990). For the multiple sequence alignment, MUSCLE (Edgar 2004) implemented in the program MEGA version 7 (Kumar et al. 2015) was used with the default parameters. The alignment included all the isolates listed in Supplementary Table S1. The aligned sequences were checked for recombination using the RDP (Martin and Rybicki 2000), GENECONV (Sawyer 1999), BOOTSCAN (Salminen et al. 1995), MAXCHI (Maynard-Smith 1992), CHIMAERA (Posada and Crandall 2001), 3Seq (Boni et al. 2007), and SiScan (Gibbs et al. 2000) methods in RDP4 v4.61 software (Martin et al. 2005). Recombination analysis was conducted essentially as described in Green et al. (2017a, 2018b), with an isolate considered to be recombinant if all six programs of the RDP package (RDP, GENECONV, Chimaera, MaxChi, Bootscan, and SiScan) had significant support ($P < 0.0001$). The distance plot was constructed using the "manual distance plot" option in RDP4

(Martin et al. 2005). Model selection was done using the “Find Best DNA/Protein Models (ML)” option in MEGA 7 with the default parameters (Kumar et al. 2015). Phylogeny inference was conducted based on the nearly whole genomes using the maximum likelihood (ML) method implemented in MEGA 7 (Kumar et al. 2015), with the appropriate parameters changed to reflect the model selected.

RESULTS

Biological characterization. Six solanaceous species were used for biological assays (Table 1); while the eight tamarillo PVY isolates were tested in *N. benthamiana*, naranjilla, and in tobacco (Table 1), only isolates Tam13, Tam15, Tam17, and Tam18, were selected for additional biological assays based on the observed phylogenetic relationships (see below), and the assumption of close similarity of isolates Tam13, Tam14, Tam16, Tam19, and Tam20. A summary of symptoms observed on each plant species tested is given in Table 1.

In their original host, tamarillo, four tested tamarillo PVY isolates, Tam13, Tam15, Tam17, and Tam18, induced mosaic that was visible starting at 2 weeks postinoculation (Table 1). Because Tam18 isolate was found to be very similar to Tam13 in sequence (see below), only three of these isolates, Tam13, Tam15, and Tam17, were observed for a much longer period for up to 20 months postinoculation, and mosaic stayed quite visible through the several months of growth in the greenhouse, although the effect of the infection on plant growth was not very prominent (Fig. 1A). Of interest was discoloration observed on fruits of the PVY-positive plants, characteristic of all three tested isolates, Tam13, Tam15, and Tam17 (Fig. 1B and C; and not shown). The fruit discoloration observed in the greenhouse resembled the tamarillo fruit symptoms in orchards where the isolates were collected (not shown). The three tested isolates, Tam13, Tam15, and Tam17, induced mild mosaic in inoculated naranjilla plants, visible at 3 wpi (Fig. 1E). Seven of the eight tamarillo PVY isolates induced characteristic vein necrosis symptoms in tobacco (cultivar Burley), in addition to typical mosaic and vein clearing (Table 1); however, one isolate, Tam17, induced no symptoms in tobacco (Fig. 2), although full systemic infection was easily confirmed by detection methods described above (Table 2).

Separate laboratory testing of the inoculated and upper, uninoculated leaves for three isolates, Tam13, Tam15, and Tam17, in all tested solanaceous plants (Table 2) indicated that all three isolates were able to systemically infect tamarillo, naranjilla, tobacco, and *N. benthamiana*. In addition, all three were able to multiply in inoculated leaves of one of the tested potato cultivars, Russet Norkotah, while only one isolate, Tam17, was able to multiply in inoculated leaves of pepper (Table 2). None of the three

tested tamarillo PVY isolates was able to establish systemic infection in pepper and the two tested potato cultivars. Interestingly, potato isolates Oz (PVY^O) and Mont (PVY^{Eu-N}) were unable to establish systemic infection in tamarillo, being confined to inoculated leaves only (Table 2). At the same time, tamarillo and potato isolates did establish systemic infection in naranjilla (Table 2).

Serology and RT-PCR typing of tamarillo PVY isolates.

Serological typing of the eight tamarillo PVY isolates is summarized in Table 3. All tamarillo PVY isolates reacted with the strain nonspecific antibody Asc5 (Funke et al. 2017), while seven of them, excepting Tam15, reacted with the N-specific antibodies 1F5 and SASA-N provided by the commercial suppliers, Agdia and SASA, respectively. None of the eight tamarillo PVY isolates bound; however, the O-specific antibody, SASA-O, provided by SASA (Table 3). Based on these ELISA data, isolates Tam13, Tam14, and Tam16 to Tam 20 appeared to have an N serotype, similar to the control isolate Mont (PVY^{Eu-N}), while isolate Tam15 appeared to have a nonidentifiable serotype.

When subjected to the RT-PCR typing with the multiplex assay developed by Lorenzen et al. (2006b), all eight tamarillo PVY isolates produced one 328-bp band usually indicative of the presence of the PVY^{NA-N} or PVY-NE11 sequence (Table 4). The second RT-PCR typing assay (Chikh-Ali et al. 2013) produced a single, 1,307-bp band in seven of the eight tamarillo PVY isolates tested, and a single, 278-bp band in the Tam15 isolate (Table 4). The presence of a single 1,307-bp band supported the assignment of the seven tamarillo isolates as belonging to the PVY^{NA-N} strain, while the single small, band in Tam15 resembled a 278-bp characteristic of either the PVY-261-4 or PVY-SYR-III strains (Chikh-Ali et al. 2013; Chikh-Ali and Karasev 2015). However, the 261-4 strain would have been typed as PVY^{N-Wi} in the first assay of Lorenzen et al. (2006b) and produced two additional bands of 441 and 853 bp in the second assay (Chikh-Ali and Karasev 2015), while the SYR-III recombinant would have looked just like PVY^{NTN} in the first assay and produced two additional 441- and 1,076-bp bands in the second assay of Chikh-Ali et al. (2013). Thus, the RT-PCR-based typing was deemed as ‘inconsistent’: no recombinant type among the existing/known PVY isolates could account for or explain these banding patterns found in the eight tamarillo PVY isolates. The control isolate Oz (PVY^O) produced two characteristic bands in this assay, 532 and 853 bp, while isolate Mont (PVY^{Eu-N}) produced three characteristic bands, 398, 633, and 1,307 bp, as expected (Table 3).

Sequencing and genome analysis. Nearly whole genome sequences of all eight tamarillo PVY isolates were compared with each other and to the PVY sequences available in the GenBank database. When checked with the BLAST program provided by the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990), the isolates were found to have the closest identity to a group of PVY isolates collected from tomato in Colombia,

TABLE 1. Summary of symptoms induced by eight isolates of potato virus Y (PVY) from tamarillo, plus two control isolates Oz (PVY^O) and Mont (PVY^{Eu-N}) in different indicator plants at 4 weeks postinoculation^a

PVY isolate	<i>Nicotiana benthamiana</i>	Tobacco ('Burley')	Tamarillo	Naranjilla	Pepper ('ECW')	Potato	
						Maris Bard	Russet Norkotah
Tam13	Cr, St, Chl	Cr, Mo, vn	Mild mo, St, vc	Mild mo	NS	NS	NS
Tam14	Cr, St, Chl	Cr, Mo, vn	Nt	Mild mo	nt	nt	nt
Tam15	Cr, St, Chl	Cr, Mo, vn	Mild mo, vc	Mild mo	NS	NS	NS
Tam16	Cr, St, Chl	Cr, Mo, vn	Nt	Mild mo	nt	nt	nt
Tam17	Cr, St, Chl	NS	Mild mo, vc	Mild mo	NS	NS	NS
Tam18	Cr, St, Chl	Cr, Mo, vn	Mild mo, St, vc	Mild mo	NS	NS	NS
Tam19	Cr, St, Chl	Cr, Mo, vn	Nt	Mild mo	nt	nt	nt
Tam20	Cr, St, Chl	Cr, Mo, vn	nt	Mild mo	nt	nt	nt
Oz	Cr, St, Chl	Mo	NS	Mo	Mo	syst HR	Mo
Mont	Cr, Chl	Mo, vn	NS	NS	NS	Mo	NS

^a NS, no visible symptoms; mo, mosaic; vc, vein clearing; Chl, chlorosis; Cr, crinkling; vn, vein necrosis; HR, hypersensitive resistance response, local necrotic lesions on inoculated leaves; syst HR, systemic HR; St, stunting; and nt, not tested. Images of some symptoms are presented in Figures 1 and 2.

including mar7 (KR270797) (95% identity for Tam isolates 13, 14, 16, 18, 19, and 20; 92% identity for isolates 15 and 17). When subjected to a phylogenetic analysis (Fig. 3), all tamarillo PVY isolates were placed in a separate clade within the PVY^N lineage (Green et al. 2017a, b) that included previously described clades PVY^{Eu-N}, PVY^{NA-N}, and PVY-NE11 (Green et al. 2017a, b), and also a new clade, described recently and named N2 (Green et al. 2020; Fuentes et al. 2019). To better distinguish this new clade from other clades comprising the PVY^N lineage, we propose to slightly change this name to SA-N, following the arbitrary designations of other clades as European N (PVY^{Eu-N}) and North American N (PVY^{NA-N}) (Fig. 3).

Since the PVY^N lineage includes recombinants, such as PVY-NE11 (Lorenzen et al. 2008; Green et al. 2017a, 2018b), all whole genome PVY sequences comprising this lineage were subjected to a thorough recombination analysis together with the newly determined tamarillo PVY sequences. The eight tamarillo PVY isolates analyzed were found to represent at least three distinct recombinant patterns, Tam13, Tam15, and Tam17 (Table 5), that suggested exchanges of extended segments of their genomes with each other and also with PVY isolates from the PVY^{SA-N} clade (Fig. 3). Unlike most of the PVY recombinants found in potato PVY isolates, where the parental sequences were identified as coming from PVY^O and PVY^{Eu-N} lineages (Glais et al. 2002;

Fig. 1. A, Foliar symptoms induced by the three tamarillo isolates of potato virus Y (PVY), Tam13, Tam15, and Tam17, in tamarillo at 25 weeks postinoculation (wpi). Fruit color B, in healthy and C, in Tam17-infected tamarillo, 12 months postinoculation. D, Prominent mosaic and vein clearing induced by Tam13 isolate in tamarillo foliage at 25 wpi, visible from the leaf underside. E, Foliar symptoms of mild mosaic in naranjilla induced by Tam 13, Tam15, and Tam17 at 8 wpi. All inoculated plants were found fully systemically infected using triple-antibody sandwich enzyme-linked immunosorbent assay and RT-PCR as described in Materials and Methods. H designates a healthy, uninoculated plant used as control.

Green et al. 2017a, 2018b; Hu et al. 2009a; Lorenzen et al. 2006a), the three tamarillo recombinant patterns involved the mar7 parental sequence found in a PVY isolate from tomato in Colombia, and also presumably novel parental sequences from yet undefined nonrecombinant PVY isolates. The distance plot analysis of Tam13, Tam15, and Tam17 genomes with the two of the PVY^{SA-N} sequences, mar7 and VarB, is presented on Figure 4A, and the schematic diagram of the identified recombinant structures of the five sequences is presented in Figure 4B.

It is clear from this distance plot that the VarB sequence from the SA-N clade is a recombinant carrying a long section of the genome encompassing P3, 6K1, and CI cistrons of PVY closely related to the mar7 sequence which is here (arbitrarily) assumed to be a parental sequence (Fig. 4A). A section of the VarB genome upstream of the P3 cistron is less similar although still related to

the mar7 sequence (Fig. 4A), while the section of the VarB genome downstream of the CI cistron is coming from another, yet unknown parental PVY genome (Fig. 4A and B). In two of the tamarillo PVY sequences presented, Tam13 and Tam17, a section of the genome spanning 6K2 to N1b cistrons is also very similar to the mar7 sequence, assumed here to be a parental sequence (Fig. 4A). All three tamarillo PVY genomes, Tam13, Tam15, and Tam17, have a long section spanning P3 to CI cistrons distinct from mar7/VarB sequences in this area, apparently coming from another parental sequence, which here (arbitrarily) is assumed to be Tam17 (Fig. 4A and B). Thus, the Tam17 genome can be viewed as a relatively simple PVY recombinant carrying its own (parental) section between positions 1 to 5862, which is followed by a mar7-specific section between positions 5862 to 9219, and a small section spanning a 3'-terminal half of the CP cistron coming from yet another unknown parental genome (Fig. 4A and B). Assuming these arbitrary assignments of the parental status to the entire mar7 genome, and to the partial Tam17 genome (Fig. 4B), Tam13 has a more complex recombinant structure, where it is very close to the recombinant genome of Tam17 between positions 2201-3'-end, but carries a mar7-specific section between positions 1 to 2201 (Fig. 4B). With the parental assignments described above, the Tam15 recombinant has the most complicated structure, resembling Tam13 genome between positions 1 to 5862 and 9090-3'-end, but with one insert of a small section from an unknown parent between positions 556 to 696, and another long insert from another unknown parent between positions 5846 to 9090 (Fig. 4B).

DISCUSSION

Genetic diversity of PVY was established in the past based, first, on biological and, later, molecular data that defined distinct strains or strain groups differing in their host range and molecular properties (Cockerham 1970; Jones 1990; Singh et al. 2008). Until very recently, these genetic diversity studies were largely confined to two major agricultural hosts affected by PVY, potato and pepper. Three main ‘strain groups’ were distinguished, PVY^C, PVY^O, and PVY^N (Karasev and Gray 2013; Kehoe and Jones 2016; Quenouille et al. 2013; Singh et al. 2008), while a single PVY sequence from pepper, Chile3, was defined as a sister clade to the three main strain groups (Moury 2010; Quenouille et al. 2013). Only recently it became clear that solanaceous species other than potato and pepper may support replication of other PVY strains

Fig. 2. Comparison between symptoms induced by two isolates of potato virus Y (PVY), Tam13 and Tam17, in tobacco ('Burley'), 5 weeks postinoculation. Note a prominent vein necrosis induced by the Tam13 isolate, and the completely asymptomatic appearance of the tobacco plant infected with the Tam17 isolate. Both plants were found fully systemically infected using triple-antibody sandwich enzyme-linked immunosorbent assay and RT-PCR as described in Materials and Methods.

TABLE 2. Spread of the three tamarillo isolates of potato virus Y (PVY), Tam13, Tam15, and Tam17, and two control PVY isolates, Oz (PVY^O) and Mont (PVY^{Eu-N}), in inoculated and noninoculated leaves of six plant species from the family Solanaceae, as tested by two laboratory methods, enzyme-linked immunosorbent assay (ELISA) and RT-PCR^a

Host tested	Tam13		Tam15		Tam17		Oz		Mont	
	Inoculated leaves	Uninoculated leaves	Inoculated leaves	Uninoculated leaves	Inoculated leaves	Uninoculated leaves	Inoculated leaves	Uninoculated leaves	Inoculated leaves	Uninoculated leaves
Tamarillo	+++	+++	+++	+++	+++	+++	+	–	+	–
Naranjilla	+++++	+++++	+++++	+++++	+++++	+++++	++	++	++	++
Nicotiana benthamiana	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
Tobacco 'Burley'	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Pepper 'Early California Wonder'	–	–	–	–	+	–	+	+++	++	–
Potato 'Maris Bard'	–	–	–	–	–	–	+++	–	+++	++
Potato 'Russet Norkotah'	++	–	++	–	+++	–	+++	+++	++	+++

^a Virus presence was detected using triple-antibody sandwich (TAS)-ELISA and typed to strain in TAS-ELISA using three monoclonal antibodies, and in RT-PCR as described in Materials and Methods; + indicates an ELISA signal (optical density at 405 nm) exceeding the noninfected control threefold or more, with the number of plusses reflecting the number of plants found virus-positive, and – indicates an ELISA signal equal to the noninfected background (<0.2) 18 to 24 h after adding the developing solution into the wells. Three to five plants were used for each inoculation, and inoculated leaves were tested 2 weeks postinoculation (wpi), except potato plants that were tested at 3 wpi, and uninoculated leaves were tested at 5 wpi.

adapted to these hosts, and these new, nonpotato and nonpepper PVY isolates may display an impressive genetic diversity as well (Green et al. 2017b). In this study, it was shown that tamarillo (*S. betaceum*) is yet another solanaceous host originating from the Andes that contributed to the evolution and diversification of PVY, through both accumulation of mutations (Fig. 3) and recombination (Fig. 4).

Biological properties of the eight tamarillo isolates of PVY collected in Ecuador were found distinct from a PVY^O isolate Oz (GenBank accession EF026074; Baldauf et al. 2006), because seven out of eight PVY isolates from tamarillo induced prominent vein necrosis in tobacco cultivar Burley (Table 1, Fig. 2), and three tested isolates, Tam13, Tam15, and Tam17, were able to infect tamarillo plants systemically (Table 2). PVY isolate Oz, like all PVY^O isolates, induced mosaic and vein clearing in tobacco, and was unable to move systemically in tamarillo, being restricted to inoculated leaves. In pepper (cultivar Early California Wonder), only one tamarillo isolate, Tam17, was found multiplying in inoculated leaves of this host (Table 2), while PVY isolate Oz, like other PVY^O isolates, was able to establish a systemic infection in pepper (Chikh-Ali et al. 2016) (Table 2). The eight tamarillo PVY isolates were also found distinct from a PVY^N isolate PVY-Mont (AY884983), collected from potato (Lorenzen et al. 2006a). Unlike the PVY-Mont isolate, tamarillo PVY isolates were unable to systemically infect two cultivars of potato (Tables 1 and 2), with only one tamarillo PVY isolate, Tam17, able to multiply in inoculated leaves of one potato cultivar, Russet Norkotah

(Table 2). All PVY isolates tested easily established systemic infection in naranjilla plants clearly visible at 5 wpi (Tables 1 and 2). Together with distinct serological and molecular properties (Tables 3 and 4; Fig. 3), these biological differences suggest that the eight tamarillo PVY isolates studied represent at least one additional strain of PVY that perhaps evolved as a tamarillo-adapted PVY lineage.

The eight tamarillo PVY isolates studied formed a distinct clade within the PVY^N lineage (Fig. 3), separate from the four clades established earlier (Fuentes et al. 2019; Green et al. 2017a, b, 2020; Hu et al. 2009a, b; Lorenzen et al. 2006a, 2008). Of the five clades identified in the PVY^N lineage (Fig. 3), three represented recombinants. These are clades NE-11 (Green et al. 2018b, 2020; Lorenzen et al. 2008), N2/SA-N (Fuentes et al. 2019; Green et al. 2020) (Fig. 3), and now tamarillo (Fig. 3). All three recombinant types from the PVY^N lineage are composed of parental sequences belonging to the same PVY^N lineage (Green et al. 2018b; Lorenzen et al. 2008) (Fig. 4), a somewhat unusual situation, since most of the other PVY recombinants identified earlier were composed of only two parental sequences, PVY^N and PVY^O (Green et al. 2018b; Hu et al. 2009a). Interestingly, the PVY^N lineage includes only two clades, N2/SA-N and tamarillo, comprising nonpotato PVY isolates, coming from either tomato or tamarillo (Fig. 3), with all other sequences from this PVY^N lineage coming from potato isolates.

It is clear from the comparison of the phylogenetic position of the tamarillo PVY isolates (Fig. 3) and the results of their molecular typing using RT-PCR (Table 4) that existing methodologies of strain identification (Chikh-Ali et al. 2013; Chikh-Ali and Karasev 2015; Lorenzen et al. 2006b) may not work for the isolates representing the tamarillo PVY strain. These existing typing methodologies were developed based on sequence information coming almost exclusively from PVY isolates found in potato, and almost exclusively representing recombinants between PVY^O and PVY^N parents, targeting several conserved recombinant junctions identified in PVY recombinants in the past (Chikh-Ali et al. 2013; Green et al. 2017a, 2018b). If one would like to identify the tamarillo isolates specifically, new approaches and primers will be needed to reliably distinguish tamarillo PVY isolates from other known PVY recombinant and nonrecombinant isolates. We believe that once the number of sequences in this tamarillo clade increases, the diversity accumulated will allow to develop additional primers to differentiate PVY isolates infecting hosts other than potato. Inspection of the recombinant structures of the three tamarillo isolates, Tam13, Tam15, and Tam17, and two tomato isolates, mar7 and VarB (Fig. 4B), suggested a considerable diversity among the PVY isolates from the N2/SA-N clade (Fig. 3), and possible circulation in solanaceous hosts of yet undiscovered parental PVY genomes producing the recombinants revealed here (Fig. 4B).

TABLE 3. Serological reactivity of the eight tamarillo PVY isolates, marked Tam13 through Tam20, and two control isolates, Oz (PVY^O) and Mont (PVY^{Eu-N}), with three different monoclonal antibodies tested in triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA)^a

Isolates (strain)	TAS-ELISA			
	Asc5 ^b	SASA-O ^c	1F5 ^c	SASA-N ^c
Tam13	+	–	+	+
Tam14	+	–	+	+
Tam15	+	–	–	–
Tam16	+	–	+	+
Tam17	+	–	+	+
Tam18	+	–	+	+
Tam19	+	–	+	+
Tam20	+	–	+	+
Oz (O)	+	+	–	–
Mont (N)	+	–	+	+

^a Positive sign (+) indicates the OD₄₀₅ signal in ELISA exceeding the healthy control signal threefold or more, and negative sign (–) indicates the OD₄₀₅ signal that is equal to the healthy control.

^b Polyclonal antibody: Asc5 reacts with all PVY strains (Funke et al. 2017).

^c Monoclonal antibodies: SASA-O specific to PVY^O or PVY^C, 1F5 specific to PVY^O-O5 or PVY^{N/NTN}, and SASA-N specific to PVY^{N/NTN}.

TABLE 4. RT-PCR-based strain typing of the eight tamarillo PVY isolates, labeled Tam13 to Tam20, using two multiplex RT-PCR assays, in comparison with the two control isolates, Oz (PVY^O) and Mont (PVY^N)

PVY isolate	Multiplex PCR 1 ^a bands	Multiplex PCR 2 ^b bands	Strain typing results
Tam13	328 bp	1,307 bp	NA-N
Tam14	328 bp	1,307 bp	NA-N
Tam15	328 bp	278 bp	?
Tam16	328 bp	1,307 bp	NA-N
Tam17	328 bp	1,307 bp	NA-N
Tam18	328 bp	1,307 bp	NA-N
Tam19	328 bp	1,307 bp	NA-N
Tam20	328 bp	1,307 bp	NA-N
Oz ^c	267 + 689 bp	532 + 853 bp	O
Mont ^c	328 + 398 bp	398 + 633 + 1,307 bp	N

^a PCR 1 according to Lorenzen et al. (2006b).

^b PCR 2 according to Chikh-Ali et al. (2013).

^c Oz and Mont are reference isolates of PVY strains PVY^O and PVY^N, respectively, maintained in the PVY isolate collection of the University of Idaho.

The original definition of the PVY^N strain group was based on the ability of PVY^N isolates to overcome three resistance genes, *Nc_{ibr}*, *Ny_{ibr}*, and *Nz_{ibr}*, in potato (Cockerham 1970; Jones 1990; Singh et al. 2008). The tamarillo PVY isolates described in this study were unable to overcome these three genes present in cultivar Maris Bard, *Nc_{ibr}*,

Ny_{ibr}, and *Nz_{ibr}* (Table 2), and thus clearly distinguished themselves from the ordinary PVY^N isolates defined biologically (Cockerham 1970; Jones 1990; Singh et al. 2008). In addition, tamarillo PVY isolates were unable to move systemically in potato cultivar Russet Norkotah, which is known to have no *N* genes conferring resistance

Fig. 3. Phylogenetic analysis of nucleotide sequences of the whole genomes for the eight tamarillo and 29 additional, representative potato virus Y (PVY) isolates, mainly from the PVY^N clade, conducted with the maximum likelihood algorithm. Only nodes with bootstrap values of 70% or higher were retained. Brackets designate individual strains and variants of PVY as described in Green et al. (2018b). The new tamarillo clade is underlined. Bold font designates the tamarillo sequences determined in this work. Arrow designates a single isolate, Chile3, from a clade distant to all other PVY clades. Both an isolate name and a corresponding accession number in the GenBank database are given for each reference sequence.

TABLE 5. Recombinant junction positions in genomes of all tamarillo PVY isolates, as determined in RDP4, with the common NTNa and NE-11 strains included for comparison^a

Isolate or strain	GenBank accession	Breakpoint position(s) in alignment ^b	Reference
Tam13	MT380736	2201, 2495, 5862, 9184	This paper
Tam14	MT380737	2201, 5862, 9184	This paper
Tam15	MT380738	556, 696, (2108–2626?), 5846, 9090	This paper
Tam16	MT380739	2201, 5862, 9066	This paper
Tam17	MT380740	5862, 9219	This paper
Tam18	MT380741	2201, 5862, 9066	This paper
Tam19	MT380742	2201, 5862, (9066?, 9220?)	This paper
Tam20	MT380743	2201, 5862, 9184	This paper
VarB	KT290512	328, 2201, 2495, 5862	This paper
NE-11 (short)	HQ912867	2220, 2857	(Green et al. 2017a)
NTNa	KY847944	2390, 5850, 9200	(Green et al. 2017a)

^a Instances where a breakpoint was unclear are indicated with a ? inside parentheses. All breakpoints and parental strains of recombinant sections are visualized in Figure 4A and B. For each isolate a Reference is provided if the authors submitted the isolate in association with a manuscript.
^b Recombination analysis was conducted essentially as described in Green et al. (2017a, 2018b), with an isolate considered to be recombinant if all six programs of the RDP package (RDP, GENCONV, Chimaera, MaxChi, Bootscan, and SiScan) had significant support ($P < 0.0001$).

Fig. 4. Recombination analysis of the three tamarillo (Tam13, Tam15, and Tam17) isolates of potato virus Y (PVY) in comparison with the two control isolates of PVY, mar7 (GenBank accession number KR270797) and VarB (KT290512). **A**, Manual distance plot based on the aligned full-length nucleotide sequences of PVY isolates mar7, VarB, Tam13, Tam15, and Tam17; mar7 (KR270797) was used as the reference sequence. x axis represents nucleotide position in the alignment, and y axis represents relative distance from the reference sequence, calculated using Kimura model (Kimura 1980). **B**, Schematic diagram showing putative PVY recombination structures for PVY isolates mar7, VarB, Tam13, Tam15, and Tam17. Designations R1 to R3 refer to the recombinant structure types. The diagram reflects distance plot data presented in A. Similar shading of the rectangles indicates homologous sequences present in PVY genomes; assignment of parental status to specific tamarillo and tomato PVY isolates is arbitrary. A schematic of the PVY genome is presented at the top of each figure, approximately to scale.

against PVY (Chikh-Ali et al. 2014; Rowley et al. 2015), remaining confined to inoculated leaves (Table 2). This suggests another possible type of resistance present in cultivar Russet Norkotah, restricting systemic movement of the tamarillo strain of the virus. Until very recently, the PVY^N lineage was considered one of the least diverse among the PVY strain groups (Green et al. 2017b). However, new sequences found in the Andes in potato and tomato (Fuentes et al. 2019; Green et al. 2020), and in tamarillo (this work; Fig. 3), suggests a much more diverse composition of the PVY^N lineage. It appears that the genetic diversity within the PVY^N lineage was underestimated in the past, and studies of the PVY strain composition in other solanaceous plants in South America might be warranted to uncover new strains of the virus.

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