Occurrence and Molecular Characterization of Recombinant *Potato virus Y*^{NTN} Isolates from Sulawesi, Indonesia

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Abstract

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The importance of potato has increased dramatically in Indonesia over the last three decades. During this period, ‘Granola’, a potato cultivar originally from Germany, has become the most common cultivar for fresh consumption in Indonesia. In August 2014, a survey was conducted in Sulawesi, where potato fields cultivated with Granola and its selection, ‘Super John’, were sampled for *Potato virus Y* (PVY) presence. PVY was found in Sulawesi for the first time. Samples determined to be positive for PVY were subsequently typed to strain using reverse-transcription polymerase chain reaction assays. All PVY isolates sampled were identified as PVY^{NTN} recombinants, with three recombination junctions in P3, VPg, and CP regions of the genome. Three local PVY isolates

were subjected to whole-genome sequencing and subsequent sequence analysis. The whole genomes of the Indonesian PVY^{NTN} isolates I-6, I-16, and I-17 were found to be closely related to the European PVY^{NTN}-A. This recombinant type was shown previously to cause potato tuber necrotic ringspot disease (PTNRD) in susceptible potato cultivars. The dependence of potato farmers on mostly a single cultivar, Granola, may have given a competitive advantage to PVY^{NTN} over other PVY strains, resulting in the predominance of the PVY^{NTN} recombinant. The dominance of PVY^{NTN} in Sulawesi, and possibly in Indonesia as a whole, represents a potential risk to any newly introduced potato cultivar to the country, especially cultivars susceptible to PTNRD.

Potato was introduced to Indonesia about 230 years ago by the Dutch and, since then, has become an important food staple for Indonesian people. In 2013, potato was cultivated on 62,900 ha and tuber production reached 1,023,374 tons (FAOSTAT 2015). Potato is grown mostly on small-scale farms at high elevation, mainly in Sumatra, Java, Sulawesi, and Nusa Tenggara (International Potato Center 2008). Due to the tropical climate, potato is cultivated year-round, with one or two crops annually in rotation with cabbage, spring onion, maize, or rice (International Potato Center 2008). The small-scale production and the high cost of certified potato seed limit the access of potato farmers to high-quality seed tubers. Consequently, farmers often produce their own seed potato tubers and purchase new seed only when the productivity of their fields declines considerably (International Potato Center 2008). Seed potato tubers are imported mainly from the Netherlands and Australia (International Potato Center 2008) and, in some instances, are produced locally by extension agents (D. Sembel, personal observation).

Potato viruses are one of the main biotic concerns for both seed and ware potato producers. Viruses cause the rapid degeneration of seed potato and are the main cause for seed rejection for certification in countries that have certification programs. Viruses also cause dramatic reductions of potato yield and quality. *Potato virus Y* (PVY), which it is the type member of the genus *Potyvirus*, family *Potyviridae* (Adams et al. 2012), is the most frequent and damaging potato virus worldwide. Currently, PVY isolates have been classified into at least nine strains according to their biological and genomic properties (Karasev and Gray 2013). PVY strains vary in their importance, economic impact, and prevalence in specific geographic areas (Anfoka et al. 2014; Baldauf et al. 2006; Boukhris-Bouhachem et al. 2010; Chikh Ali et al. 2016; Djilani-Khouadja et al. 2010; Fomitcheva et al. 2009;

Galvino-Costa et al. 2012a,b; Gray et al. 2010; Karasev et al. 2011; Schubert et al. 2007, 2015). Certain PVY strains, such as PVY^{NTN} recombinants, are of major quality concern due to their ability to cause potato tuber necrotic ringspot disease (PTNRD), which can make tubers unmarketable (Gray et al. 2010). PVY^{NTN}, which was first reported in Hungary about three decades ago (Beczner et al. 1984), has become a significant concern to potato producers due to its effects on quality and its fast spread into most potato-producing areas of the world (Chikh Ali et al. 2016; Gray et al. 2010; Karasev and Gray 2013; Le Romancer et al. 1994; Singh et al. 2008). PVY^{NTN} recombinants belong to the PVY^Z strain (Karasev and Gray 2013) and have genomes with two recombination patterns. PVY^{NTN}-A type has three recombination junctions (RJ) in P3 protein (P3), VPg protein (VPg), and capsid protein (CP) regions while isolates of the PVY^{NTN}-B type have an additional RJ in the P1 coding region (Glais et al. 2002; Schubert et al. 2007). PVY^{NTN}-A recombinants are the most frequent, with almost worldwide distribution, while PVY^{NTN}-B isolates are endemic, and found only in Europe and, occasionally, some Mediterranean regions (Chikh Ali et al. 2008; Schubert et al. 2007).

In many countries, PVY decreases the productivity of locally produced potato seed and causes rapid degeneration of imported seed. This requires studies of predominant PVY strains and disease epidemiology in order to develop management programs and increase potato yield. Damayanti et al. (2014) recently reported PVY from West Java, Indonesia, found in an unknown potato cultivar; however, no reports on PVY strain composition in this important potato-growing area are available, which complicates any attempts to control PVY. To fill in this gap, we conducted a survey to determine whether PVY is present in the island of Sulawesi, one of the main potato production regions in Indonesia, where the German ‘Granola’ potato and a cultivar locally selected in North Sulawesi (‘Super John’) are grown, and where PVY had not yet been reported. Additionally, the PVY strains present in potato fields were identified using reverse-transcription polymerase chain reaction (RT-PCR) assays, and genome sequence analysis was carried out on three select isolates to characterize the PVY strains circulating in Indonesian potato fields.

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Materials and Methods

Survey and sample collection. A survey was conducted in the Modoinding subdistrict, province of North Sulawesi, Indonesia, where farmers produce potato on a small scale (Fig. 1). In late August 2014, five potato fields of various seed generations in three villages were assessed for PVY infection (Table 1). Potato fields were selected with the assistance of a local extension agent. Fields were at the flowering or shortly before flowering stage when examined and were generally free of weeds. The Modoinding area lies at elevations of 1,000 to 1,200 m, and fields sampled were 0.25 to 1 ha in size. Designation of a seed generation here refers to the number of times the seed was planted by a farmer after receiving it from a local extension agent. Because most of the farmers produce two potato crops a year, and replant their own seed, “generation 4” seed equals 2 years of planting (Table 1).

Incidence of visual virus symptoms was assessed by examining 10 randomly selected row sections (2 m long each) throughout each field. The number of plants exhibiting PVY symptoms was noted and divided by the total number of plants per row section and incidence averaged per field. In each field, leaves from four to seven plants showing virus-like symptoms, mainly mottle, were sampled and tested with the general PVY immuno-strip test (Agdia). PVY-positive samples were printed on FTA Classic Cards (Whatman) according to manufacturer’s instructions. The cards were dried at room temperature for 1 h, stored with silica gel, and transported to the University of Idaho (UI) virology laboratory for further analysis. In addition, plants in all fields were examined for the presence of aphid vectors of viruses. The abaxial surface of multiple leaves

from each of the plants sampled for PVY and 10 symptomless plants per field was examined for aphid presence.

RNA extraction, RT-PCR, and sequencing. RNA was extracted from the FTA cards according to the method described by Ndunguru et al. (2005). RNA pellets were dissolved in 30 μ l of nuclease-free water, heated at 65°C for 5 min, then chilled on ice for 3 min. RT was done according to the method of Chikh-Ali et al. (2013). For strain identification, two different multiplex RT-PCR assays were performed following the methods of Lorenzen et al. (2006) and Chikh Ali et al. (2010). Control isolates representing nine strains of PVY and additional recombinant variants were obtained from the UI laboratory collection (Chikh-Ali et al. 2013). In order to detect PVY^E isolates and differentiate them from other PVY strains, a new RT-PCR methodology was developed. To this end, two primers, E-F8985 (5'-CAAGTTGAATACCCACTCAAG-3') and E-R9495 (5'-TATACCACCCCTATCTGCC-3'), were designed. The PCR step was carried out using Green Taq DNA Polymerase (GenScript). Primers were used at a final concentration of 0.5 μ M each, and cDNA solution (2 μ l) was added to a PCR tube which contained 2 μ l of 10 $\times$ GenScript buffer, 0.52 μ l of dNTP (2.5 mM each), primers, and 0.3 μ l (5 U/ μ l) of GenScript Taq polymerase. The volume of the mixture was adjusted to 25 μ l using MilliQ-purified water. PCR amplification was performed in a DNA Engine Dyad Thermal Cycler (Bio-Rad Laboratories, Inc.) using the following PCR program: 1 cycle of initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s), and extension (72°C for 1 min). Final extension was performed at 72°C for 5 min. PCR products were separated in 2%

Fig. 1. Map of Sulawesi Island, Indonesia showing the location of seed potato fields where *Potato virus Y* (PVY) isolates were collected. Circle indicates the location of potato fields sampled.

agarose gels containing ethidium bromide at 10 µg/ml and visualized under UV illumination.

PVY isolates I-6, I-16, and I-17 (Table 1) were selected for whole-genome sequencing. The whole-genome sequencing was performed directly on a series of overlapping PCR fragments amplified on cDNA synthesized from these samples, as described by Karasev et al. (2011). Successfully amplified PCR products were treated with Exosap-It (Affymetrix) and submitted for Sanger sequencing to Genewiz, Inc. Individual sequence reads were assembled using the SeqMan Pro of the Lasergene 9 Suite (DNASTAR).

Sequence analysis. For the multiple sequence alignment, Clustal W (Thompson et al. 1994) implemented in the program MEGA version 4 (Tamura et al. 2007) was used with the default parameters. Sequence identity was checked using the BLAST program provided by the National Center for Biotechnology Information. Alignments were conducted based on the whole genome and a 440-nucleotide (nt) segment within the CP coding region of representative isolates of PVY strains. The aligned sequences were checked for recombination, using 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 Beta3 software (Martin et al. 2005). The analyses in RDP4 Beta3 package were done using default settings and a Bonferroni-corrected *P* value cut-off of 0.05. Only recombination points detected by more than four methods in the RDP4 Beta3 program were taken into consideration. Phylogeny inference was conducted based on the whole genomes and partial CP regions using the neighbor-joining method implemented in MEGA version 4 (Tamura et al. 2007) with 1,000 bootstrap replicates.

Results

All potato fields surveyed were planted with Granola potato and its local selection, Super John (Table 1). Incidence of visual symptoms, including mosaic, mottle, and crinkling, was found to be below 5% in all fields examined. In total, 25 samples were identified as PVY positive based on the ImmunoStrip assay (Agdia) producing two distinct bands on the test strip. All 25 samples were applied to the FTA cards for further analysis. Although PVY was found before in West Java (Damayanti et al. 2014), this is the first report of PVY presence in Sulawesi. No aphid vectors were found on the plants examined at any of the surveyed fields.

RT-PCR assay. Twenty-three PVY-positive samples were subjected to the multiplex RT-PCR assay developed by Lorenzen et al. (2006) and all produced two bands of 181 and 452 bp, expected for PVY^{NTN} (Table 1). In the second multiplex PCR assay, 17 samples produced bands of 1,307, 663, and 441 bp expected for PVY^{NTN}-A (Chikh Ali et al. 2010). Two samples produced bands of 1,307 and 441 bp expected for PVY^{NTN}-B (Chikh Ali et al. 2010). Three samples produced only two bands of 663 and 441 bp, expected for PVY^E (Chikh-Ali et al. 2013), and one sample produced a single faint band of 441 bp (Table 1). To confirm or disprove the PVY^E infection, a new PCR assay specific for PVY^E isolates only was developed to differentiate them from PVY^{NTN}. None of the samples tested, including the two samples identified as PVY^E with the second PCR assay, produced any bands while the PVY^E reference control produced a band of the expected size of 512 bp (Table 1).

Sequencing and sequence analysis. One PVY isolate (I-17) originally typed as PVY^{NTN}-A and two isolates (I-6 and I-16) typed as PVY^{NTN}-B were selected randomly for whole-genome

Table 1. Strain identification of *Potato virus Y* (PVY) isolates collected from potato fields in Modoinding subdistrict, Sulawesi, Indonesia using multiplex reverse-transcription polymerase chain reaction (PCR) assays^a

PVY isolate ^b	Cultivar ^c	Location, field number ^d	Generation ^e	Multiplex PCR 1 (bp)	Multiplex PCR 2 (bp)	PCR 3 (bp)	Results
I-1	Granola	Makaruyen 1	Seed 4	181 + 452	441 + 633	...	?
I-3	Granola	Makaruyen 1	Seed 4	181 + 452	441 + 633	...	?
I-4	Granola	Makaruyen 1	Seed 4	181 + 452	441	...	?
I-5	Granola	Makaruyen 1	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-6	Granola	Makaruyen 1	Seed 4	181 + 452	441 + 1,307	...	NTN-B
I-7	Granola	Makaruyen 1	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-8	Granola	Makaruyen 1	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-9	Granola	Linelean 2	Seed 4	181 + 452	441 + 633	...	NTN
I-10	Granola	Linelean 2	Seed 4	nt	441 + 633 + 1,307	...	NTN-A
I-11	Granola	Linelean 2	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-12	Granola	Linelean 2	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-13	Granola	Linelean 2	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-14	Super John	Linelean 3	Seed 2	181 + 452	441 + 633 + 1,307	...	NTN-A
I-15	Super John	Linelean 3	Seed 2	181 + 452	441 + 633 + 1,307	...	NTN-A
I-16	Super John	Linelean 3	Seed 2	181 + 452	441 + 1,307	...	NTN-B
I-17	Super John	Linelean 3	Seed 2	181 + 452	441 + 633 + 1,307	...	NTN-A
I-18	Super John	Sinisir 4	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-19	Super John	Sinisir 4	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-20	Super John	Sinisir 4	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-21	Super John	Sinisir 4	Seed 4	181 + 452	nt	...	NTN
I-22	Super John	Sinisir 4	Seed 4	181 + 452	441 + 633	...	NTN
I-23	Super John	Sinisir 5	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-24	Super John	Sinisir 5	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-25	Super John	Sinisir 5	Seed 4	181 + 452	441 + 633 + 1,307	...	NTN-A
I-26	Super John	Sinisir 5	Seed 4	nt	441 + 633 + 1,307	...	NTN-A
HR-1	N/A	Idaho	...	181 + 452	441 + 633 + 1,307	...	NTN-A
MON	N/A	Idaho	...	181 + 452	441 + 633	512	E

^a PCR 1 is according to Lorenzen et al. (2006), PCR 2 is according to Chikh Ali et al. (2010, 2013), and PCR 3 is a uniplex PCR assay specific to the PVY^E strain (Chikh Ali et al. 2016); NT = not tested.

^b HR-1 and MON are reference isolates of PVY strains PVY^{NTN} and PVY^E, respectively, which are maintained with the PVY isolate collection of the University of Idaho.

^c N/A = not applicable.

^d Idaho = University of Idaho collection.

^e Seed generation refers to the number of times the seed was planted by a farmer; because most of the farmers produce two potato crops a year and replant their own seed, "Seed 4" indicates 2 years of planting.

sequencing (Table 1), because they exhibited a good yield of RT-PCR products in the typing assay. The sequences of their nearly complete genomes were determined by direct sequencing of overlapping RT-PCR fragments, and were found to be 9,583 nt for I-6, 9,612 nt for I-16, and 9,604 nt for I-17, excluding poly(A) tails and the extreme 5'-terminal sequences. These nearly whole-genome sequences were deposited in the GenBank database under accession numbers KT599906 (I-6), KT599907 (I-16), and KT599906 (I-17). All genomes coded for an open reading frame of 3,061 amino acids. Isolate I-6 shared a nucleotide sequence identity of 99.2 and 99.3% with I-16 and I-17, respectively, while I-16 and I-17 shared a nucleotide sequence identity of 99.2%. When compared with all previously published PVY whole genomes, I-6, I-16, and I-17 shared the highest nucleotide identity (99.5%) with PVY^{NTN} recombinants from the United Kingdom (KC634007) and Japan (AB702945).

In the recombination analysis, all three Indonesian PVY^{NTN} isolates had three recombination junctions closely resembling those of a typical European PVY^{NTN}-A (Fig. 2). The first recombination event (1-2415; Fig. 2) was detected by *P* values using RDP (1.841×10^{-114}), GENECONV (1.048×10^{-106}), BootScan (1.170×10^{-113}), MaxChi (M; 9.538×10^{-21}), Chimaera (7.236×10^{-22}), 3SEquation (2.944×10^{-212}), and SiScan (3.752×10^{-54}). The second recombination event in S2 (5840-9179; Fig. 2) was detected by *P* values using RDP (3.930×10^{-130}), GENECONV (2.920×10^{-115}), BootScan (6.872×10^{-129}), MaxChi (4.110×10^{-46}), Chimaera (5.363×10^{-41}), 3SEquation (4.394×10^{-219}), and SiScan (3.204×10^{-55}). The genomes of I-6, I-16, and I-17 isolates had O and N sequences identified as parents (Fig. 2). In the phylogenetic analysis of the whole genomes of the three Indonesian PVY isolates performed against representatives from major recombinant and nonrecombinant lineages of PVY strains, genomes of I-1, I-16, and I-17 grouped tightly with other recombinants of the PVY^{NTN}-A type, with a 100% bootstrap value (Fig. 3).

Discussion

The importance of potato has increased dramatically in Indonesia over the last three decades. Total production has increased more than four times, from about 230,000 tons in 1980 to more than one million tons in 2013, and the cultivated area has risen from about 25,000 to close to 63,000 ha during the same period (FAOSTAT 2015). Interestingly, during this period, the Granola became the most common potato cultivar for fresh consumption in Indonesia, cultivated on 80 to 85% of production area (Gunadi et al. 2011; Van der Burg

et al. 2013). The current survey of PVY strain composition was conducted in Sulawesi, one of the most important potato production areas of the country, where Granola and its local selection Super John are dominant. The absence of aphids on plants at the time of the survey and the low virus incidence in all fields suggest that virus spread within and among fields is low. Farmers in the Modonding region use insecticides for pest control on potato but do not spray specifically for aphids. Farmers in the region grow tomato and chili pepper but the role these crops might play in PVY epidemiology is unknown. Thus, it is uncertain at this time whether PVY is being perpetuated in this region in planting material or via other means.

Granola potato is described as moderate to highly resistant to PVY without specifying the PVY strain and as highly resistant to PVY^N (The European Cultivated Potato Database, <http://www.europotato.org/menu.php?>). In the description sheet released by one of the producer companies (Solana GmbH & Co. KG), Granola is described as resistant to PVY^{NTN}. However, in the current study, all PVY isolates collected from Granola and its selection Super John were identified as PVY^{NTN}, which indicates that this cultivar can be infected with PVY^{NTN}. It is possible that the description of Granola as resistant to PVY^{NTN} refers to low susceptibility to PTNRD. In a recent report, Damayanti et al. (2014) found PVY in potato in West Java, Indonesia and, although unable to identify the strain of the virus, reported a small fragment of the CP genes of four isolates (GenBank accession numbers KF261310 to KF261313). These small fragments belong to the PVY^N sequence lineage, consistent with the possible PVY^{NTN} presence in West Java as well (Fig. 4).

PVY^{NTN} can infect most potato cultivars but not all of these cultivars are susceptible to PTNRD. It is well known that some potato cultivars such as 'Yukon Gold', 'Nadine', and 'Nicola' are more susceptible to PTNRD than other cultivars (Gray et al. 2010; Kerlan 2006). The dominance of PVY^{NTN} in Sulawesi and, possibly, other Indonesian islands represents a potential risk to any potato cultivar newly introduced to the country, especially cultivars susceptible to PTNRD. Therefore, susceptibility to PTNRD should be considered an important trait when selecting or introducing new cultivars due to the prevalence of PVY^{NTN} in Sulawesi. The dependence of potato farmers on a single cultivar and its derivative (Granola and Super John, respectively, in this case) may have played a substantial role in PVY strain selection in Indonesia, giving a competitive advantage to PVY^{NTN} relative to other PVY strains. This is a good example of the role of potato cultivars in shaping PVY strains

Fig. 2. Recombinant structure of I-6, I-16, and I-17 isolates of *Potato virus Y* (PVY) as determined based on nucleotide sequence analysis. Rectangles designate virus genomes and shading corresponds to the relatedness to the parental sequences PVY^O and PVY^N, indicated below the diagram; nt = nucleotides.

circulating in potato fields, and helps to highlight the importance of the use of multiple cultivars with variable genetic backgrounds to place similar pressure on PVY strains, especially the more damaging ones such as PVY^{NTN}. Two sources of resistance have been identified to PVY^{NTN}, the *Nz_{tblr}* and *Ry_{chc}* genes (Chikh-Ali et al. 2013, 2014;

Kerlan et al. 2011; Sato et al. 2006). Potato cultivars carrying the *Nz_{tblr}* react with hypersensitive reaction to PVY^{NTN}; therefore, using potato cultivars carrying the *Nz_{tblr}* resistance gene could contribute to the control of this damaging PVY strain. On the other hand, potato cultivars carrying the *Ry_{chc}* resistance gene (such as the two Japanese potato

Fig. 3. Phylogenetic analysis of nucleotide sequences of the whole genomes for representative *Potato virus Y* (PVY) isolates inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. Isolate name was followed by a corresponding accession number in the GenBank database. Brackets indicate strains of PVY according to Karasev and Gray (2013); Non-P = nonpotato PVY sequence.

cultivars, ‘Konafubuki’ and ‘Sakurafubuki’) showed extreme resistance to PVY^{NTN} (Chikh-Ali et al. 2013; Sato et al. 2006), which makes breeding for potato cultivars with the *Ry^{chc}* resistance gene important to control PVY and, in particular, PVY^{NTN}.

In conclusion, PVY^{NTN} appears to be the dominant PVY strain in Sulawesi and additional research is needed to reveal whether the same strain is dominant in other regions of Indonesia. It is not clear when and how PVY arrived in Indonesia. However, the dependence

on a limited number of cultivars for potato production has most likely selected for and led to the prevalence of the PVY^{NTN} strain. Additional research in Sulawesi and other potato-producing regions of Indonesia is merited to determine whether PVY is widespread and to identify the strains circulating in potato fields. Similarly, studies are needed to examine the factors contributing to disease epidemiology and to assess the economic impact that this important virus is having on potato production by small-scale farmers.

Fig. 4. Phylogenetic analysis of nucleotide sequences of a 440-nucleotide segment within the capsid protein coding region of representative *Potato virus Y* (PVY) isolates, including previously reported PVY isolates from West Java, Indonesia (Damayanti et al. 2014) inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Only bootstrap values higher than 70% are retained. Evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. Brackets indicate strains of PVY according to Karasev and Gray (2013); Non-P = nonpotato PVY sequence.

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