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A new potyvirus isolated from *Pennisetum alopecuroides* with the potential to infect cereal crops

Xuedong Liu[1,2†], Xi Chen[1†], Sijia Liu[1], Kaitong Du[1], Pei Wang[1], Tong Jiang[1], Mengji Cao[3], Xiangdong Li[4], Zaifeng Fan[1] and Tao Zhou[1*]

Abstract

Pennisetum plants (*Pennisetum alopecuroides* L.), displaying a dwarfing phenotype along with delayed flowering and mosaic symptom on leaves, were found in Beijing, China. Flexuous filamentous particles with a size of approximate 15 × 850 nm were observed in symptomatic leaves via transmission electron microscopy. Deep sequencing of small RNAs (sRNA) from symptomatic leaves and analysis of sRNA populations were then conducted to determine the genome sequence of the viral agent in diseased plant tissues. It showed that the viral agent had one positive-sense and single-stranded RNA genome, which consisted of 9717 nucleotides (nts) excluding poly(A) tail. The complete viral genome contained a large open reading frame, encoding a polyprotein of 3131 amino acids (aa). Sequence comparison and phylogenetic analysis demonstrated that the viral agent belonged to the genus *Potyvirus* in the family *Potyviridae*. In the cladogram it was most closely related to johnsongrass mosaic virus, sharing 72% nt and 65% aa sequence identity. This viral agent was provisionally named pennisetum alopecuroides mosaic virus (PalMV). Subsequently, it was confirmed that PalMV is the causal agent of this new disease in *P. alopecuroides* by Koch's postulates and reverse transcription-polymerase chain reaction analysis. Moreover, maize, millet, wheat, sorghum and rice plants were experimentally infected by PalMV via rub inoculation. Consequently, we proposed that PalMV could be a potentially dangerous virus threating a wide range of cereal crops.

Keywords: *Pennisetum alopecuroides*, *Potyvirus*, PalMV, Cereal crops, Koch's postulates, Maize, Millet

Background

Pennisetum grass (*Pennisetum alopecuroides* L.), a renewable prolific herbaceous plant species in the family Gramineae, can serve as a source for feed, knitting, medicine, paper-making and biofuel production (Wang et al. 2017). The annual dry matter yield of *Pennisetum* grass ranges from 40 to 50.2 t/ha, which is significantly higher than those of sugarcane and corn (13–21 t/ha) (Tang et al. 2019). Due to its rapid growth, high biomass yield, extensive adaptability and low cost, *P. alopecuroides* has been considered as an alternative and promising plant species that can adapt to different ecological environment (Kang et al. 2018).

The *Potyviridae* is the largest family of plant-infecting RNA viruses, including a number of agriculturally and economically important viral pathogens, and resulting in serious disease epidemics in a wide range of cultivated plants (Revers and Garcia 2015; Wylie et al. 2017). In terms of genomic organization, members of the family *Potyviridae* consist of monopartite and bipartite plant viruses with single-stranded and positive-sense RNA genomes, ranging in size from 8.2 kb to 11.3 kb with an average size of 9.7 kb. The flexuous filamentous particles with no envelope are 680–900 nm long and 11–20 nm in

* Correspondence: taozhoucau@cau.edu.cn
†Xuedong Liu and Xi Chen contributed equally to this work.
[1]State Key Laboratory for Agro-Biotechnology, and Ministry of Agriculture and Rural Affairs, Key Laboratory for Pest Monitoring and Green Management, Department of Plant Pathology, China Agricultural University, Beijing 100193, China
Full list of author information is available at the end of the article

diameter encompassed by a single core capsid protein (Wylie et al. 2017).

Currently, the family *Potyviridae* comprises 12 definitive genera (i.e., *Arepavirus*, *Bevemovirus*, *Brambyvirus*, *Bymovirus*, *Celavirus*, *Ipomovirus*, *Macluravirus*, *Poacevirus*, *Potyvirus*, *Roymovirus*, *Rymovirus* and *Tritimovirus*) distinguished by the host range, genomic features and phylogeny (Wylie et al. 2017; Walker et al. 2020). The species classification criteria, based upon their open reading frames (ORF) or polyprotein products, are normally accepted as < 76% nucleotide (nt) identity and < 82% amino acid (aa) identity. If the complete ORF sequence is not available, analogical criteria can be applied to the coat protein (CP) coding regions and their products (Wylie et al. 2017; Lefkowitz et al. 2018).

The *Potyvirus* is the type genus of the family *Potyviridae*. Most potyviruses infect dicots, and only a few can infect monocots (Revers and Garcia 2015; Wylie et al. 2017; Lefkowitz et al. 2018). The following potyviruses have been identified infecting monocots, including maize dwarf mosaic virus (MDMV), sorghum mosaic virus (SrMV), zea mosaic virus (ZeMV), sugarcane mosaic virus (SCMV), johnsongrass mosaic virus (JGMV), cocksfoot streak virus (CSV) and pennisetum mosaic virus (PenMV), which all belong to the SCMV subgroup (Barnett 1992; Shukla et al. 1992; Seifers et al. 2000; Gotz and Maiss 2002; Fan et al. 2003). Potyviruses usually induce longitudinal chlorotic or necrotic streaks in the leaves of monocotyledonous plant species, and chlorotic vein banding, mosaic mottling, necrosis, or distortion of leaves in dicotyledonous plant species along with alterations on flowers, fruits and seeds (Revers and Garcia 2015).

In the past few years, the rapid development of next-generation sequencing (NGS) technology has advanced our ability to investigate diseases of unknown etiology and expedited the identification and characterization of new viral pathogens (Adams et al. 2009; Al Rwahnih et al. 2009; Kashif et al. 2012; Hadidi et al. 2016; Ramos-Gonzalez et al. 2017; Lan et al. 2019). Compared with some traditional techniques, such as enzyme-linked immunosorbent assay (ELISA), PCR assays and hybridization methods, the NGS technology does not require any virus-specific reagents (i.e., primers, probe or antibody) of the suspected pathogens. Due to antiviral defense, a mass of 20–22 nts small RNA (sRNA) fragments are abundant in virus-infected plant tissues (Wu et al. 2010). The sRNA reads are trimmed and assembled into larger contigs, with a combination of NGS analysis and PCR-based targeted resequencing to confirm some low coverage areas. NGS data provide a wide range of sequences in search of the pathogens involved in various diseases (Al Rwahnih et al. 2009; Kashif et al. 2012; Roy et al. 2015; Ramos-Gonzalez et al. 2017; Lan et al. 2019). Moreover, genomic information obtained from NGS data would accelerate the development of routine diagnostics and expedite the progress of epidemiological surveys aimed at carrying out disease control or eradication strategies (Adams et al. 2009; Hadidi et al. 2016).

In this study, we report a new viral disease on *P. alopecuroides*. The causing agent of this disease was provisionally named as pennisetum alopecuroides mosaic virus (PalMV), which was verified by Koch's postulates. NGS analysis of sRNA reads and 5′ and 3′ RACE PCR enabled assembly of the complete genomic sequence of PalMV. Sequence comparison and phylogenetic analysis indicated that PalMV was most closely related to JGMV. Moreover, maize, millet, wheat, sorghum and rice were experimentally infected by PalMV via rub inoculation. PalMV was proposed as a member of the genus *Potyvirus* on the basis of the biological and physiochemical characteristics determined by technologies available.

Results

A filamentous virus was observed in diseased *P. alopecuroides* tissues by transmission electron microscopy (TEM)

In June 2016, *P. alopecuroides* showing a dwarf phenotype along with leaf chlorosis and delayed flowering were found in Haidian District in Beijing (Fig. 1), and the plants were suspected to be infected with potential viral agents. To verify this hypothesis, TEM was utilized to visualize virus particles within the diseased plant tissues. Uniquely, flexuous filamentous particles with a size of approximate 15 × 850 nm were observed via TEM (Additional file 1: Figure S1), resembling those of potyviruses (Wylie et al. 2017; Yang et al. 2018; Cui and Wang 2019; Lan et al. 2019).

Deep sequencing of sRNA and determination of the full-length genomic sequence of the viral agent

Total RNA was isolated from symptomatic *P. alopecuroides* leaf samples and used for high-throughput sequencing of sRNAs. Approximately, 18 million reads ranging from 18 to 25 nts in length were obtained from sRNA library (Fig. 2a). These reads were mapped to the viral genome in sense and antisense orientations, meanwhile 21 and 22 nts virus-derived small interfering RNAs (vsiRNAs) accumulated to high levels (Fig. 2b). To examine the genomic distribution of vsiRNAs, vsiRNA sequences were mapped along the viral genome. The single nucleotide resolution maps indicated that vsiRNAs from both polarities were almost continuously but heterogeneously distributed throughout the entire genome, and the region corresponding to the *CP* gene contained more hotspots (Fig. 2c).

Subsequently, sRNA reads were trimmed and assembled into larger contigs using CLC Genomic Workbench

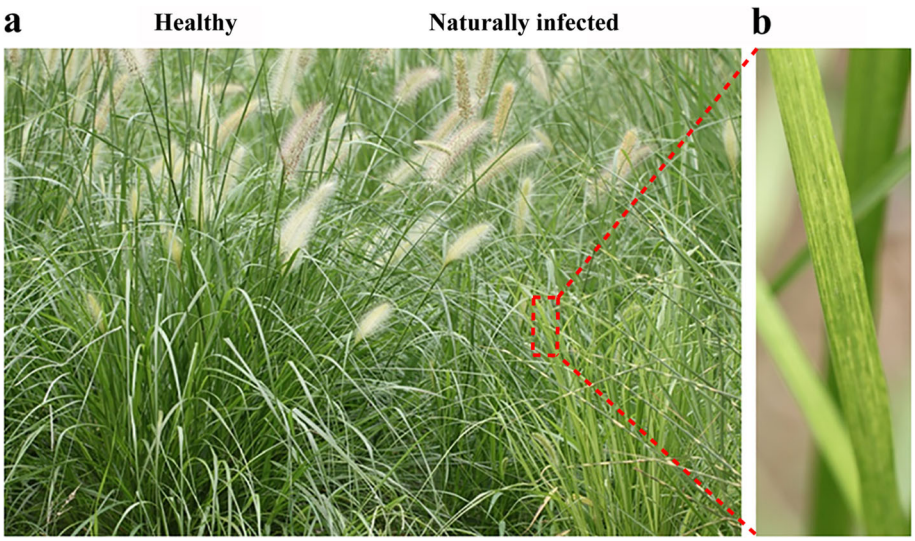

Fig. 1 Comparison of healthy and naturally infected *Pennisetum alopecuroides* plants. **a** Naturally infected plants (right) showing a dwarfing phenotype along with delayed flowering compared with healthy plants (left). **b** Close-up image showing mosaic symptom on leaves

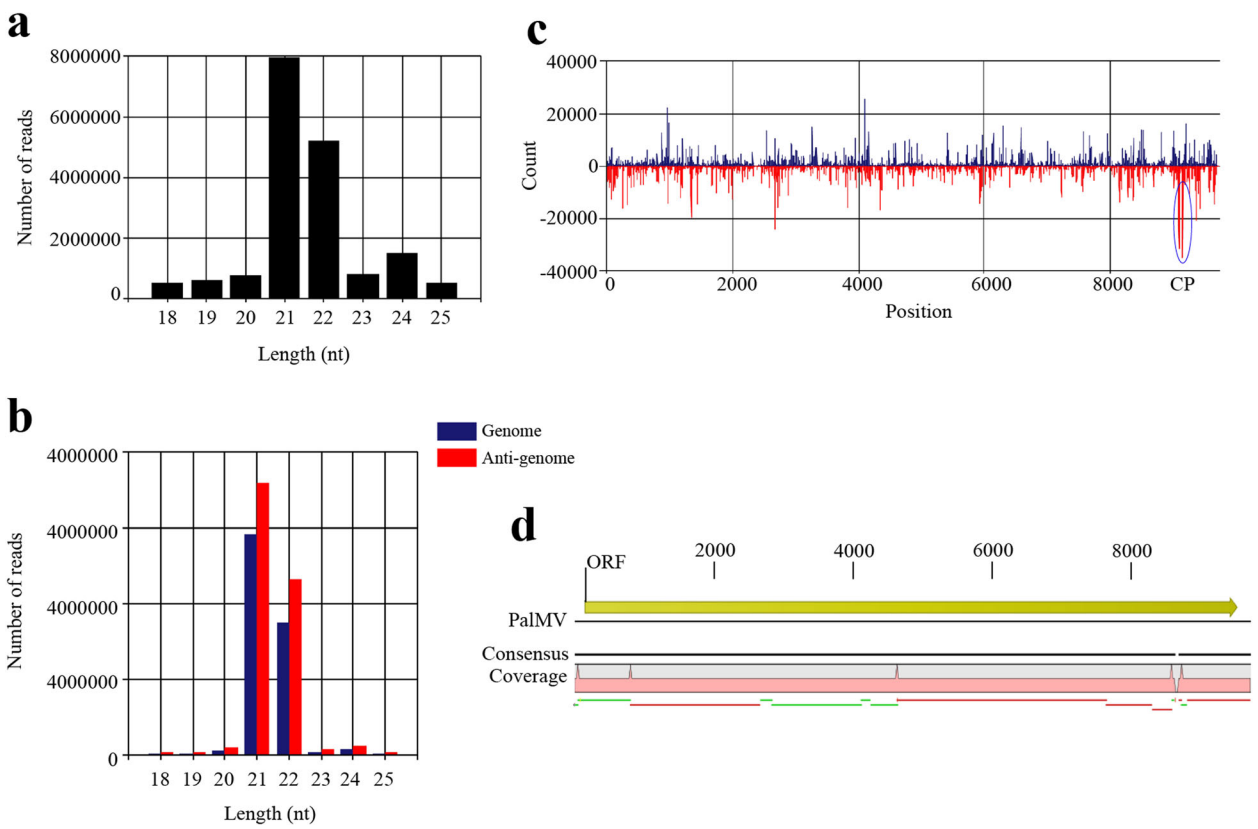

Fig. 2 Profile of vsiRNAs and contigs derived from library of PalMV-infected leaves. **a** Histogram representation of size distribution of total sRNAs in the library. **b** Histogram representation of total vsiRNA reads in each size class from genome and anti-genome. **c** Map of vsiRNAs reads at each nucleotide position of the PalMV genome. Bars above the axis represent sense reads starting at each respective position, and those below represent antisense reads ending at the respective position. **d** Map of continuous contigs covering most of the virus genomic sequence, except for a few gaps

(Liu and Di 2020). These contigs almost covered the entire viral genome, whereas there were still some gaps with low coverage regions in the genome (Fig. 2d). To confirm the NGS results and obtain the complete genomic sequence of the virus, multiple primers were designed on the basis of the known sequences (Additional file 2: Table S1). Viral RNA was extracted from the purified virions, and then applied to complementary DNA synthesis by reverse transcription-polymerase chain reaction (RT-PCR). The 5′ and 3′ RACE PCR were utilized to obtain the sequences of untranslated regions (data not shown). After sequencing, all the amplicons were trimmed and assembled into complete viral genomic sequence, which was entirely consistent with those of the above NGS data. Collectively, the complete genomic sequence was deposited in GenBank under the accession number MT790493. The virus was provisionally named pennisetum alopecuroides mosaic virus (PalMV).

Characterization of the PalMV genome

It showed that the viral agent had a positive-sense and single-stranded RNA genome, consisting of 9717 nts excluding the poly(A) tail. The complete viral genome contained a large ORF, encoding a polyprotein of 3131 aa, which began with an ATG codon at 143 nt and ended with a UAA termination codon at 9541 nt. The 5′ and 3′ untranslated regions (UTR) of PalMV were 142 nts and 176 nts in length, respectively (Fig. 3). In addition, a P3N-PIPO ORF resulted from viral RNA polymerase slippage, within the P3 cistron, was also predicted downstream of the $G_{1\text{-}2}A_{6-7}$ motif at position 2773–3048 nts. This motif was consistent with that of the highly conserved $G_{1\text{-}2}A_{6-7}$ motif known for other members in the family *Potyviridae* (Chung et al. 2008; Olspert et al. 2015; Rodamilans et al. 2015).

Putative proteolytic cleavage sites were identified according to BLAST results, and thus proteolytically processed into 10 mature proteins P1, HC-Pro, P3, 6 K1, CI, 6 K2, NIa-VPg, NIa-Pro, NIb and CP, at aa positions 262, 723, 1070, 1122, 1775, 1828, 2017, 2259 and 2776 (Fig. 3). It appeared that the dipeptide at the putative NIa-Pro/NIb junction of PalMV was V/K, which was different from those of other members in genus *Potyvirus*. Except for NIa-Pro/NIb junction, other cleavage sites were relatively conserved (Table 1). All these site changes were confirmed by RT-PCR assays and sequencing (data not shown). Furthermore, conserved motifs that are typical for potyviruses were found, including ^{220}GSSG^{223} (protease activity) in P1; ^{317}KITC^{320} (aphid transmission), ^{462}CDNQLD^{467} (symptomatology), ^{557}CSCVT^{561} (long distance movement), ^{575}PTK^{577} (aphid transmission) and ^{607}GYCY^{610} (cysteine proteinases) in HC-Pro; ^{1230}VLVLEPTRPL^{1239} (potential helicase activity), ^{1443}VATNIIENGVTL^{1454} (potential helicase activity) and ^{1494}GRVGR^{1498} in CI; ^{2446}FTAAP^{2450}, ^{2460}CVDD^{2463}, ^{2564}GNNSGQPSTVVD NTLMV^{2580} (RNA-dependent polymerase activity), ^{2608}GDD^{2610} (RNA-dependent polymerase activity) and ^{2650}WFMS^{2653} in NIb; ^{2870}DAG^{2872} (aphid transmission), ^{2982}MVWCIENG^{2989} and ^{3084}QMKAAA^{3090} in CP (Lan et al. 2019; Worrall et al. 2019).

PalMV is a distinct new species in the genus *Potyvirus* based upon sequence comparison and phylogenetic analysis

To analyze the taxonomic position of PalMV, pairwise comparison of potyviral sequences identities was initially carried out with the CLUSTALX program (Aiyar 2000). A total of 42 representative potyviral nt and aa sequences were retrieved from GenBank database (Additional file 2: Table S2). The results of sequence comparison indicated that PalMV was most closely related to JGMV. The genome sequence of PalMV showed 72 and 65% identity, at the nt and aa level, respectively, with the genome of JGMV. Moreover, the CP sequences of PalMV and JGMV shared 64% aa identity. These values are below the corresponding threshold values for species demarcation in the *Potyviridae* family (Wylie et al. 2017).

Subsequently, phylogenetic trees were constructed based on the polyprotein (Fig. 4a) and CP (Fig. 4b) of PalMV and the 42 representative members of family *Potyviridae* by neighbor-joining method with 1000 bootstraps replicates using MEGA7.0.2 software (Kumar et al. 2016). Phylogenetic reconstructions based on the polyprotein and CP protein distinguished PalMV from other potyviruses in the analogical position of the

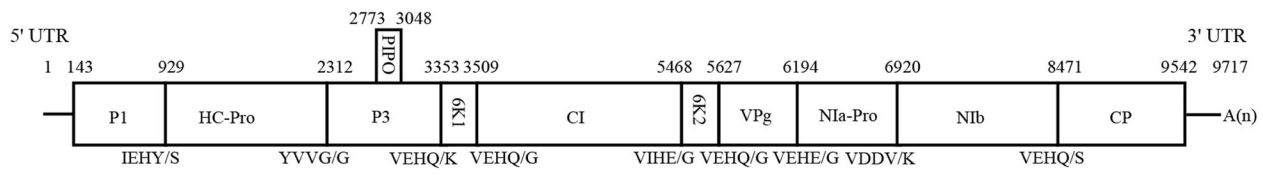

Fig. 3 Schematic diagram of the genomic organization of PalMV. Two short horizontal lines represent 5′ and 3′ UTRs, respectively. The largest box represents the long ORF. The smaller boxes represent the mature proteins. The smallest box represents PIPO derived from RNA polymerase slippage on the P3 cistron. The putative cleavage sites are marked below the ORF

Table 1 Comparison of the cleavage sites of the polyprotein among PalMV and some related potyviruses

Virus[a]	P1/HC-Pro	HC-Pro/P3	P3/6K1	6K1/CI	CI/6K2	6K2/VPg	VPg/NIa-Pro	NIa-Pro/NIb	NIb/CP
SrMV	NEIDHF/S	REYVVG/G	TGVIHE/A	TQVTHQ/S	TTVIHQ/G	MKVCHQ/G	VEVEHE/S	CEVTEQ/G	IDVFHQ/A
PVY	NSMIQF/S	KHYRVG/G	YDVRHQ/R	YEVRHQ/S	QFVHHQ/A	ETVSHQ/G	QEVEHE/A	DVVVEQ/A	YEVHHQ/A
CSV	NGMVEY/S	KNYQVG/G	PMVKHQ/A	QSVHHQ/M	NIVQHQ/T	EPVHHQ/G	SYVDHE/T	QFVSMQ/S	PYVSHQ/A
MDMV	QEIEHY/A	REYAVG/G	VGVIHE/G	SQVTHQ/S	VTVIHQ/G	TDVKHE/A	VEVEHE/A	FDVTEQ/G	IDVKHQ/A
PenMV	LDINHY/A	RDYIVG/G	TGVIHE/H	RNVVHQ/S	NTVIHQ/G	QDVTHQ/G	EGVTHE/A	DDVQEQ/G	EDVYHQ/S
SCMV	MEIEHY/A	REYIVG/G	TGVIHE/G	PPVTQQ/S	NTVIHQ/G	TEVSHQ/G	AGVAHE/S	MSVEEQ/C	EDVFHQ/S
JGMV	KQICHY/S	KEYIVG/G	TEVEHE/R	QEVKHE/G	QTVIHE/N	TEVEHE/G	PEVEHE/G	ERISNE/S	VDVEHQ/S
CaYSV	SLIDHY/A	KGYVVG/G	DEVEHQ/K	TTVEHQ/G	QTVIHE/T	MDVEHQ/A	ADVEHE/G	MHNVSE/D	LEVEHQ/A
PalMV	REIEHY/S	KDYVVG/G	EEVEHQ/K	TEVEHQ/G	QTVIHE/G	TEVEHQ/G	VNVEHE/G	HSVDDV/K	IEVEHQ/S

[a] SrMV, sorghum mosaic virus; PVY, potato virus Y; CSV, cocksfoot streak virus; MDMV, maize dwarf mosaic virus; PenMV, pennisetum mosaic virus; SCMV, sugarcane mosaic virus; JGMV, johnsongrass mosaic virus; CaYSV, canna yellow streak virus

cladogram (Fig. 4). We therefore concluded that PalMV is a distinct new species in the genus *Potyvirus*.

Confirmation of PalMV as the etiological agent causing dwarf and mosaic phenotypes on *P. alopecuroides* by Koch's postulates and RT-PCR analysis

It was verified that PalMV is the causal agent inducing leaf mottling and chlorosis, delay in flowering, and dwarfing in *P. alopecuroides* by Koch's postulates and RT-PCR analysis. Symptomatic leaf samples were collected and homogenized with phosphate buffer (pH 7.0), then mechanically inoculated onto healthy *Pennisetum* plants. Most inoculated plants developed severe mosaic symptom on upper leaves (Fig. 5a), similar with those observed in naturally diseased plants (Fig. 1). Virus specific RT-PCR assay confirmed the presence of PalMV. RNA extracts from different leaf tissues were subjected to RT-PCR assay using the primer pair of CP-F and CP-R (Additional file 2: Table S1). Among 24 inoculated *P. alopecuroides* plants in two independent experiments, 14 samples tested positive (Additional file 1: Figure S2). All the amplified fragments were subjected to sequencing, and they shared more than 98% nt sequence identity with PalMV partial *CP* gene (data not shown).

Determination of host range of PalMV

To investigate the host range of PalMV, the crude virus extracts were mechanically inoculated to some common monocotyledonous plants and indicator plants. On the upper leaves of PalMV-inoculated maize (*Zea mays*) and millet (*Setaria italica*) plants, obvious mosaic symptoms were observed (Fig. 5b, c). Meanwhile, slight mosaic symptoms were observed on upper leaves of PalMV-inoculated wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*) plants (Fig. 5d). Further, RT-PCR assay tested positive for PalMV in symptomatic

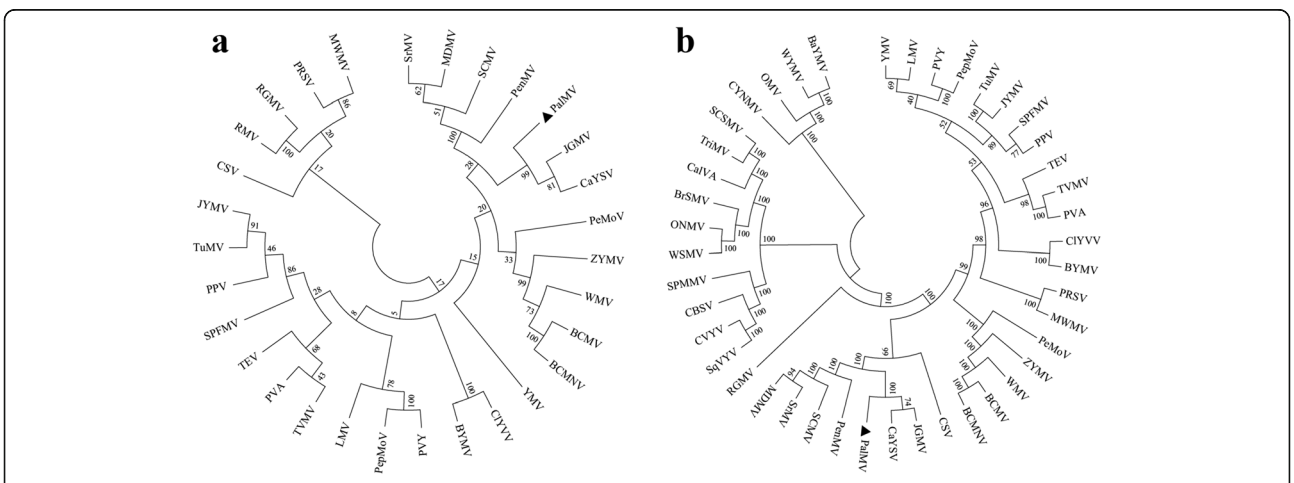

Fig. 4 Phylogenetic trees illustrating the position of PalMV among the members of the family *Potyviridae*. Phylogenetic analysis of the deduced aa sequences of polyprotein (**a**) and coat protein (**b**) of PalMV and other potyviruses. Sequence sources are listed in Additional file 2: Table S2. Phylogenetic tree was generated by neighbor-joining method with 1000 bootstraps replicates using MEGA7.0.2 software. The numbers at the nodes indicate the percentage of 1000 bootstraps occurred in this group. PalMV is highlighted by triangle in black

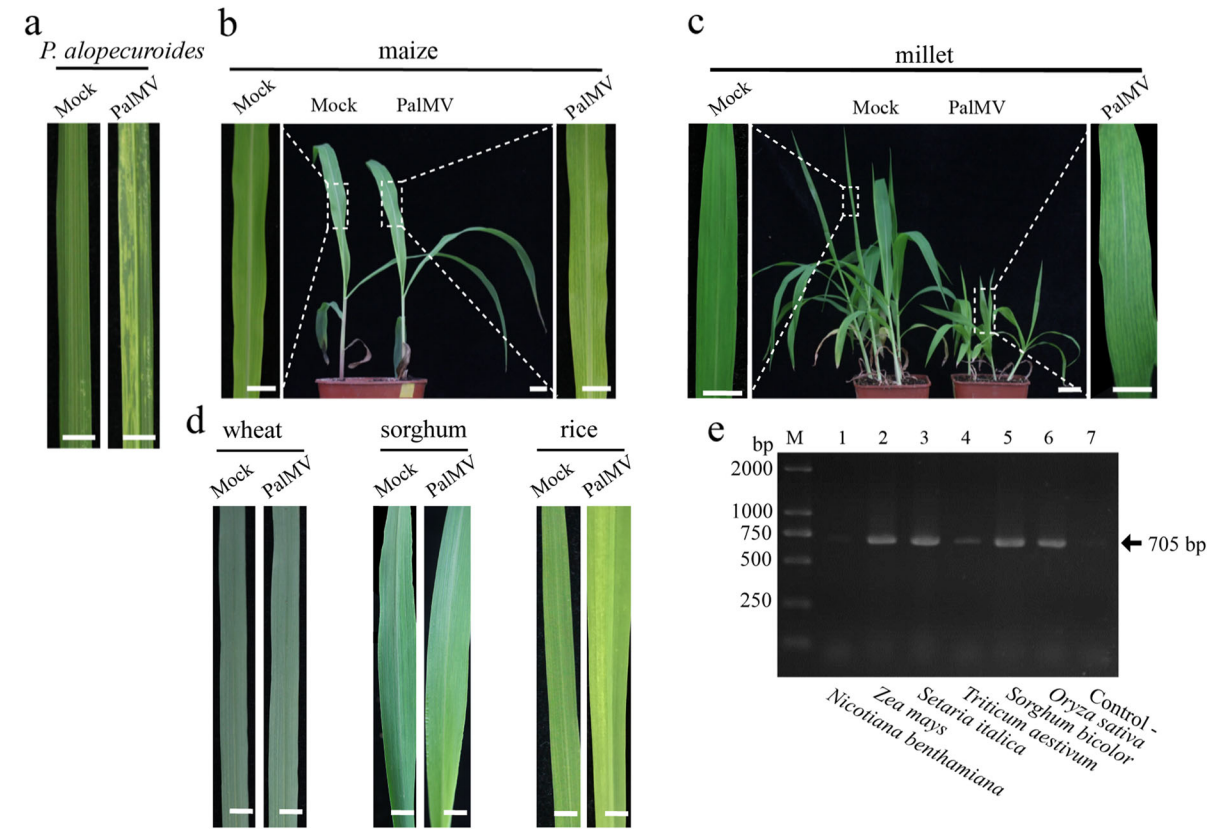

Fig. 5 Determination of systemic infection in inoculated plants by PalMV. **a** PalMV-inoculated *P. alopecuroides* plants displayed severe mosaic symptom on upper leaves, while the mock-inoculated plants were healthy. **b** PalMV-inoculated maize plants showed severe mosaic symptom in systemically infected leaves, while the mock-inoculated plants were healthy. **c** PalMV-inoculated millet plants showed a dwarfing phenotype and severe mosaic symptom on leaves. **d** Slight mosaic symptoms were observed on upper leaves of PalMV-inoculated wheat, sorghum, and rice plants. **e** Detection of PalMV by RT-PCR with the primer pair of CP-F and CP-R in inoculated plants. Lane M, DL2000 DNA ladder (TSINGKE, Beijing). Lanes 1 to 6 indicate PCR products amplified from infected leave samples of *Nicotiana benthamiana*, *Zea mays*, *Setaria italica*, *Triticum aestivum*, *Sorghum bicolor* and *Oryza sativa*, respectively. Lane 7, negative control. Leaves mechanically inoculated with phosphate buffer were used as negative control (Mock). Bars = 1 cm

plants (Fig. 5e). All the amplified fragments were sent for sequence analysis, which shared more than 98% nt sequence identity with partial *CP* gene of PalMV (data not shown). Subsequently, we chose some indicator plants to assess the infectivity of PalMV, and no diseased symptom appeared on the plants of *Chenopodium amaranticolor*, *C. quinoa*, *Nicotiana tabacum* or *N. clevelandii*. In addition, no amplicons were produced in these PalMV-inoculated indicator plants (data not shown). In conclusion, PalMV was identified to experimentally infect maize, millet, wheat, sorghum and rice via rub inoculation, and we proposed PalMV as a potentially harmful virus for a wide range of cereal crops.

Discussion

In this study, we characterized a distinct potyvirus, PalMV, from *P. alopecuroides* plants by deep sequencing, TEM and RT-PCR. Koch's postulates assay proved that PalMV is the causal agent inducing dwarf and mosaic symptom on *P. alopecuroides* plants. The complete sequence of PalMV shares the highest nt sequence identity (72%) and aa sequence identity (65%) with JGMV. In our mechanical inoculation assays, PalMV infected maize, millet, sorghum, wheat and rice, suggesting that this virus could be a potential pathogen threating a wide range of cereal crops.

As previously reported, weeds have significance in contribution to both the outbreaks of viral diseases on crops, and survival and evolution of viruses in nature (Malmstrom et al. 2005; Kaliciak and Syller 2009; Zhao et al. 2019). Being an omnipresent and perennial grass species, *P. alopecuroides* may serve as a reservoir for PalMV and play a significant role in the epidemiology of diseases caused by this virus (Roossinck 2012). In nature, potyviruses are transmitted by aphids in a non-persistent manner, which is one of the reasons why they cause serious disease epidemics in a wide range of cultivated plants (Ng and Perry 2004). PalMV is likely transmitted

by aphids because its proteins contain several conserved motifs, including a ^{317}KITC^{320} motif and a ^{575}PTK^{577} motif in HC-Pro, as well as a ^{2870}DAG^{2872} motif in CP. Those motifs have been considered to play vital roles in aphid transmission of potyviruses (Revers and Garcia 2015). However, even though all these conserved motifs are present, aphid transmission demands species-specific interactions (Gibbs et al. 2008; Pelletier et al. 2012). Further examination is needed to identify which aphid species can transmit PalMV.

PalMV induces systemically dwarf mosaic symptom on maize plants, which is similar to the maize dwarf mosaic disease caused by several potyviruses including MDMV, SCMV, JGMV, ZeMV, PenMV and SrMV (Janson and Ellett 1963; Williams and Alexander 1965; Taylor and Pares 1968; Shukla et al. 1989; Seifers et al. 2000; Fan et al. 2003). Importantly, these maize-infecting potyviruses were found to cause maize lethal necrosis disease (MLND) when synergistic co-infection with maize chlorotic mottle virus (MCMV) (Scheets 1998; Stenger et al. 2007; Wangai et al. 2012; Stewart et al. 2017; Wamaitha et al. 2018) occurred. More recently, MLND outbreaks in the sub-Saharan East Africa, Southeastern Asia and Ecuador, posing a menace to maize production and food security (Mahuku et al. 2015). It is reasonable to speculate that PalMV could co-infect with MCMV leading to MLND. Furthermore, in this study, PalMV-infected millet plants showed serious dwarfing and mosaic symptom during early growth stages, resulting in inability to flowering, which would seriously damage crop yield and quality. Further studies are intended to evaluate the potential impact of PalMV on the growth and production of some cereal crops.

The host adaptation of some potyviruses may be due to P1 protein, the most divergent ones in terms of length and aa sequence among the 11 mature proteins (Valli et al. 2007; Salvador et al. 2008; Maliogka et al. 2012). P1 self-cleavage activity is indispensable for both potyviral viability and host range specificity (Valli et al. 2007; Maliogka et al. 2012; Pasin et al. 2014; Shan et al. 2015; Yahaya et al. 2019). Sequence comparison shows that the P1 aa sequence of PalMV shares less than 30% identities with those of other potyviruses. It is assumed that P1 protein has a similar effect in determining the host range of PalMV. Hence, not only the actual host range of PalMV, but also the relationship between PalMV infection and mosaic symptom are yet to be investigated in the future.

Further, we developed a specific RT-PCR diagnostic assay for detection of PalMV using the primer pair of CP-F and CP-R. It is widely known that credible and effective diagnosis of plant viruses is very crucial for generating disease management strategy. Meanwhile, this molecular test should be useful in supervising viral distribution and epidemiology (Marais et al. 2015; Rajbanshi and Ali 2019; Yahaya et al. 2019).

Conclusions

In conclusion, the biological, pathological and molecular data we obtained here support that PalMV is assigned to a distinct new species within the genus *Potyvirus*. Members of *Potyvirus* are distributed worldwide and cause severe losses on crops. Although limited numbers of potyviruses have been reported in monocots, cereal-infecting potyviruses pose great damage on grain production. In this study, we proved that maize, millet, wheat, sorghum and rice were experimentally infected by PalMV via rub inoculation, suggesting that PalMV is a potentially dangerous virus for a wide range of cereal crops. Considering the danger to cereal crops posed by PalMV, we report it for the development of disease control strategies in the future.

Methods

Plant growth and virus inoculation

Pennisetum plants showing severe leaf chlorosis and growth abnormalities (particularly severe dwarfism and delayed flowering) were sampled, and the potential viral agent was maintained by periodical sap inoculation of the diseased *Pennisetum* on the first true leaves of maize (*Z. mays* L. cv inbred line B73) plants. The inoculated maize plants were grown in a greenhouse (24 °C/16 h day and 22 °C/8 h night cycles).

For inoculation experiments, crude virus extracts were prepared by homogenizing the symptomatic maize leaf tissues in 0.01 M phosphate buffer (pH 7.0) at 1:1 (wt/vol) ratio. Plant sap was filtered and the crude extracts were rub-inoculated individually to the tested plant leaves. For mock control, plants were inoculated with phosphate buffer. Carborundum was utilized as an abrasive, and the inoculated leaves were rinsed with double distilled H_2O (ddH_2O) to remove impurities. After inoculation, the plants were kept in a greenhouse under natural conditions for symptom development (Dijkstra and de Jager, 1998; Addy et al. 2017).

Virus purification and RNA extraction

Virus particles were isolated from systemically infected maize leaves following the method for filamentous virion purification (Dijkstra and de Jager, 1998). We obtained crude purified virus particles from the supernatant. Virus yield in purified preparation was estimated spectrophotometrically (Hema et al. 1999).

Viral RNA was extracted from purified virus particles according to the previous methods (Dijkstra and de Jager, 1998; Zhu et al. 2014). The precipitated viral RNA was resuspended in 30 μL of DEPC-treated sterile ddH_2O, and stored at − 70 °C for future use.

TEM observation

Purified virion sample was dropped onto 200-mesh Formvar-coated copper grids, followed by absorption for 2 min and negative staining with 1% phosphotungstic acid (pH 7.0). Then the copper grids were dried under tungsten lamp for about 30 min. Observations and micrographs were done on a TEM (Hitachi HT-7700) (Damsteegt et al. 2013; Yang et al. 2018).

Next generation sequencing

To identify the potential viruses involved in the disease, leaf samples were collected and total RNA was extracted using TRIzol reagent (Tiangen, Beijing, China). A sRNA library was constructed using a TruSeq Small RNA Library Prep Kit (Illumina), followed by deep sequencing with an Illumina HiSeqTM 2500 system. Raw Illumina sRNA reads were filtered for quality, reduced to unique reads and de novo assembled into larger contigs using CLC Genomic Workbench 9.5 (Qiagen, USA). Following assembly, the obtained contigs were aligned to the non-redundant protein GenBank database (nr) using the BLAST programs at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Adams et al. 2009).

Determination of the complete genome sequence of the viral agent

To confirm the full genome of the virus associated with *P. alopecuroides* disease in this study, multiple primers (Additional file 2: Table S1) were designed on the basis of the sequences and relative positions of contigs mapped in the genomic RNAs. The primers were utilized to obtain the gaps between the various contigs and terminal untranslated region sequences. The 5′ and 3′ terminal sequences were determined following manufacturer's protocol of the 5′/3′ RACE Kit 2nd Generation (Roche, Shanghai, China). The 5′ and 3′ RACE PCR amplicons were obtained by amplifying the terminal mRNA sequences via RT-PCR. The RACE amplicons were cloned into the pMD™18-T vector (Takara, Dalian, China) for DNA automated sequencing. Overlapping sequences were assembled using Seqman Pro 7.1.0 to confirm the genomic regions with low coverage and obtain the complete genomic sequence (Minutillo et al. 2015).

Sequence comparisons and phylogenetic analyses

All nt and aa sequences used in sequence comparisons and phylogenetic analyses were retrieved from GenBank database (Additional file 2: Table S2). The sequences of PalMV were compared with those in GenBank database using the BLAST programs (Johnson et al. 2008). Pairwise comparison and calculation of sequence identities were carried out with CLUSTALX (http://www.clustal.org) (Aiyar 2000). Average pairwise distances were computed using MEGA7.0.2 (https://www.megasoftware.net/). Phylogenetic trees were constructed using the Neighbor-joining method with strict nt or aa distances and randomized bootstrapping evaluation of branching validity using MEGA7.0.2. The robustness of the inferred evolutionary relationships was assessed by 1000 bootstrap replicates (Kumar et al. 2016).

RNA extraction and RT-PCR assay for PalMV-specific detection

Total RNA was extracted from leaf tissues using Trizol reagent (Tiangen, Beijing, China) and reverse transcribed into cDNA using random primers. PalMV-specific primers (Additional file 2: Table S1) were designed on the basis of the *CP* gene. The cDNA (1 μL) was then amplified in a 25 μL reaction volume, containing ddH_2O (18.25 μL), 10× *Taq* buffer (2.5 μL), dNTPs (10 mM, 1 μL), *Taq* DNA Polymerase (0.25 μL) (FunGenome, Beijing, China), 1 μL each of CP-F and CP-R primers (10 μM). PCR program consists of an initial denaturation (94 °C, 5 min), 35 cycles made up by denaturation (94 °C, 1 min), annealing (58 °C, 30 s) and extension (72 °C, 1 min), followed by a final extension (72 °C, 10 min). The amplified fragments were subjected to agarose gel electrophoresis and then visualized in UV illuminator after ethidium bromide staining. PCR bands from positive samples were excised from the gel and purified by gel-extraction purification kit (Tiangen, Beijing, China), and the nt sequences were determined by sequencing (TSINGKE, Beijing, China) (Rajbanshi and Ali 2019).

Abbreviations

aa: Amino acid; CP: Coat protein; ddH_2O: Double distilled H_2O; DEPC: Diethyl pyrocarbonate; ELISA: Enzyme-linked immunosorbent assay; JGMV: Johnsongrass mosaic virus; MLND: Maize lethal necrosis disease; NGS: Next-generation sequencing; nt: Nucleotide; ORF: Open reading frame; PalMV: Pennisetum alopecuroides mosaic virus; PCR: Polymerase chain reaction; PTGS: Post-transcriptional gene silencing; RT-PCR: Reverse transcription-polymerase chain reaction; sRNA: Small RNA; TEM: Transmission electron microscopy; UTR: Untranslated region; vsiRNA: Virus-derived small interfering RNA

Supplementary Information

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Additional file 1: Figure S1. Transmission electron micrograph of flexuous filamentous viral particles observed at a magnification of 100,000×. Bar = 200 nm. **Figure S2.** Detection of PalMV by RT-PCR with the primer pair of CP-F and CP-*R*. Lane M, DL2000 DNA ladder (TSINGKE, Beijing). Lanes 1 to 24, PCR products amplified from samples of inoculated *P. alopecuroides* plants. Lanes 25 and 26, negative and positive controls, respectively.

Additional file 2: Table S1. Primers used in this study. **Table S2.** The accession numbers of the potyviruses for sequence comparisons and phylogenetic analyses from NCBI GenBank

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Authors' contributions
XL, XC and TZ designed the research. XL and XC performed the experiments. SL, KD, PW, TJ, MC, XL and ZF collected and analyzed the data. XL, XC and TZ wrote the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
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Declarations

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Competing interests
The authors declare that they have no competing interests.

Author details
[1]State Key Laboratory for Agro-Biotechnology, and Ministry of Agriculture and Rural Affairs, Key Laboratory for Pest Monitoring and Green Management, Department of Plant Pathology, China Agricultural University, Beijing 100193, China. [2]Guangdong Provincial Key Laboratory for Plant Epigenetics, Longhua Institute of Innovative Biotechnology, College of Life Sciences and Oceanography, Shenzhen University, Shenzhen 518060, China. [3]National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing 400712, China. [4]Department of Plant Pathology, Shandong Agricultural University, Taian 271018, China.

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