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Papaya Ringspot Virus (PRSV) Causing Ringspot Disease on Papaya in Bali

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Abstract

In a survey of papaya plantation in Tabanan, Bali, some papaya cultivars were found showing papaya ringspot disease symptomps i.e. yelowing and mottle on leaves; and ring spot on fruits. The analysis of enzyme linked immunosorbent assay (ELISA) of symptom leaf with papaya ringspot virus (PRSV) antiserum shown the disease was corresponding to the PRSV. The test results showed that PRSV infected papaya in Bali and detected in Tabanan. Previously, there has been no reports of the existence of this disease in the area of Bali. The objective of the study were to observe the variations of disease symptoms caused by PRSV, mapping the disease spread in Bali, and determine the molecular character of PRSV Bali isolate. Surveys were conducted to observe the variations of disease symptoms and mapping the disease spread. Molecular method was used to determine the coat protein (CP) sequence. Analysis of leaf samples from diseased plants by reverse transcription-polymerase chain reaction (RT-PCR) was conducted using a pair of primers specific to PRSV. Study result shown the disease was detected in Bangli village, Tabanan regency with the infection index 73,33% (11/15), whereas samples from other survey sites not found infected with PRSV. On samples that infected with PRSV were found yelowing and stunting on leaves; and ring spot on fruits. Amplicons of 905 bp were successfully amplified. Result from BLAST and sequence alignments showed that Bali isolate had considerable homology to the China (Accession No. KF 033092.1) isolate (99.45% na-level). Results of phylogenetic analysis using Maximum Parsimony approaches with 1000 bootstrap replications indicates that Bali isolate is in a clade with the China isolates (Accession No. KF 033092.1, KF 033089.1, KF 033078.1).

Keywords: papaya , symptom, infection index, disease spread, molecular character.

1. Introduction

Papaya (*Carica papaya*) is one of the most widely grown fruits in both tropical and subtropical countries. It is delicious in taste and rich in essential vitamins such as vitamin A, and is an excellent source of enzymes such as papain and chymopapain which are of benefi t as pharmaceuticals and in the food industry in terms of food processing (Gonsalves, 1998). In Indonesia, papaya is one of the staple foods and is grown both in commercial plantations as well as in backyard gardens.

Papaya ringspot disease, caused by the papaya infecting strain of Papaya Ringspot Virus (PRSV), is one of the most serious diseases of papaya in many countries of the word because of its effect on tree vigour and fruit set and quality (Gonsalves, 1998). The extensive list of countries known to have PRSV include USA, Australia, South America, India, Carribean, Taiwan, Thailand, Middle East, Sri Lanka, Germany, Mexico, Franch and Italy (Davis *et al.,* 2005, De La Rosa and Lastra, 1983; Purcifull *et al.,* 1984; Sukla *et al.,* 1994; Thomas and Dodman, 1993; Rod *et al.,* 2005).

PRSV is a member of *Potyvirus* genus in the family of *Potyviridae*. PRSV is a positive single stranded RNA virus in the potyvirus group (Purcifull *et al.* 1984.) with the virions being flexuous, filamentous particles of 780 × 12 nm.

PRSV isolates are devided into two strain, type P and W, which are serologically and morphologically identical and can only be differentiated on the basis of their host range (Gonsalves and Ishii, 1980). PRSV-W isolates naturally infect only cucurbits while PRSV-P isolates infect both papaya and cucurbits. The experimental host range of PRSV-P includes 15 species in three families (Caricaceae, Chenopodiaceae, Cucurbitaceae) while PRSV-W infects 38 species of 11 genera in two families (Cucurbitaceae, Chenopodiaceae) (Edwardson and Christie, 1986)

The infected papaya plants show a range of symptoms including yellowing and vein-clearing of younger leaves, mottling, leaves distortion and narrowing, ringed spots on the fruit and dark green streaks on the petioles and stems. Infected papaya plants exhibit significant stunting and a reduction in quality and quantity of fruit production (Kertbundit *et al.,* 2007; Purcifull *et al.,* 1984). Variation in symptoms is dependent on virus isolate, stage of infection, plant size and vigour, and temperature (Purcifull *et al.,* 1984).

In a survey of papaya plantation in Tabanan regency, Bali, some papaya cultivars were found showing papaya ringspot disease symptomps i.e. yelowing and mottle on leaves; and ring spot on fruits. The analysis of enzyme linked immunosorbent assay (ELISA) of symptom leaf with papaya ringspot virus (PRSV) antiserum shown the disease was corresponding to the PRSV. The test results showed that PRSV infected papaya in Bali and detected in Tabanan. Previously, there has been no reports of the existence of this disease in the area of Bali. Therefore, this study conducted a survey to observe the variations of disease symptoms caused by PRSV, mapping the disease spread in Bali and determine the molecular character of PRSV Bali isolate.

2. Methodology

2.1 Surveys

To evaluate the present of papaya ringspot virus (PRSV-P) base on occurance of ringspot symptoms, sampling was done at all of regency of Bali province. Leaf samples of papaya plants showing characteristic symptoms of ringspot disease were used for this study. Samples were tested in DAS-ELISA using PRSV antibody (Agdia Inc, USA). RNA was extracted for further molecular analysis from samples with positive reaction.

2.2 Detection of PRSV using Enzyme Linked Immunosorbent Assay (ELISA)

A standard plate-trapped ELISA method was used as described by Stack & Macmillan (2005). Tissue (100 mg) was ground in 1 mL sample extraction buffer (8 g sodium chloride, 0.2 g monobasic potassium phosphate, 1.15 g dibasic sodium phosphate, 0.2 g potassium chloride, 0.2 g sodium azide, and 0.5 g tween-20, 2 g Polyvinyl pyrrolidone diluted in 1000 mL H2O, pH 7.4) and 100 mL aliquots were coated onto the wells of immunosorbs microtiter plates. The plates were incubates at 4 ^{o}C overnignt. Primary antibody was diluted 1:1000 in ECI buffer (0.2 g bovine serum albumin, 2 g polyvinylpyrrolidone, 0.02 g sodium azide diluted in 100 mL PBST, pH 7.4). The wells were washed 6 times with PBST (8 g sodium chloride, 0.2 g monobasic potassium phosphate, 1.15 g dibasic sodium phosphate, 0.2 g potassium chloride, 0.2 g sodium azide and 0.5 g Tween-20 diluted in 1000 mL H_2O, pH 7.4) and 100 mL of primary antibody solution was added and incubated for 2 hr at room temperature. The wells were washed thorougly 4-5 times with PBST and 100 mL of conjugate (anti rabbit-IgG, Sigma, USA) diluted 1:1000 in ECI buffer was added and incubated at room temperature for 2 hr. The wells were washed again and reactions measured colorimetrically using p-nitrophenyl phosphate (PNP) substrat diluted in PNP buffer (97 mL diethanolamine, 600 m H2O, 0.2 g sodium azide diluted in 1000 ml H_2O, pH 9.8). Results were measured spectrophotometrically at A_{405nm} after 30 min using ELISA reader. Readings were considered positive when absorbance values were at least two times greater than the healthy control.

2.3 Isolation and detection of PRSV using specific primer (coat protein of PRSV)

Total RNA was reverse-transcribed using the PRSV coat protein specific primers (MB12, 5'-GGATCCGCCCGACAAACACACAAGTGCGATG-3'; dan MB 11, 5'- GGATCCATGTCCAAAAATG AAGCTGTGGATGCT-3') (Marion *et al.* 1994). Total RNA were extracted from 100 mg of fresh or frozen leaf tissue using *Rneasy Plant Mini Kits* (Qiagen, Germany). They were used directly for cDNA synthesis. cDNA was synthesized using Moloney Murine Leukemia Virus (MMuLV) reverse transcriptase (New England BioLabs) and used immediately for PCR amplification or stored at -20 °C.

A 905 bp fragment representing the coat protein gene of PRSV was amplified from 3 µl cDNA mixture using 1 unit *taqDNA polimerase* (New England BioLabs) and 0,4mM each of synthetic primers MB12 and MB11. The DNA RNA hybrid was denatured at 94 °C for 5 min and the cDNA amplified for 35 cycles by denaturing at 94 °C for 2 min, annealing at 55 °C for 1 min and extending at 72 °C for 1 min with a final extension cycle at 72 °C for 10 min. PCR products were analysed by electrophoresis in 1.2% agarose gels and stained with ethidium bromide. The CP gene was subsequently sequenced using the primer walking strategy and an automated DNA sequencing system ABI Prism 3100 (Applied Biosystems, USA).

DNA sequence databases searching by BLAST (www.ncbi.nlm.nih.gov) to identify the most closely related Coat Protein-sequences of viruses from which sequence data are available. Sequences editing using MEGA6 program and multiple sequence alignment was carried out using ClustalW. Phyllogenetic analyses was conducted using the maximum parsimony character-based method in PAUP program version 4.0 beta 10 win.

3. Results and Discussion

3.1 Ringspot Disease Symptoms

In a survey of papaya plantation in at all of regency of Bali province, Indonesia, some papaya varieties were found showing ringspot disease symptomps on red lady and Calina Primakal-11 varieties: i.e. yelowing and stunting of the crown; and a mottling of the foliage (Fig. 1). Ringspot symptoms were found in 6 regencies of Bali province. In a total 101 samples with ringspot disease symptomps were taken from 8 fields. Eleven (10,89%) samples tested positive for PRSV-P, on Calina Primakal-11 variety. Infection percentages of PRSV in the village of Bangli, regency of Baturiti, Tabanan regency is 73.33%, while in the other survey sites are not found infection of PRSV on papaya (Table 1). Ronald *et al.* (2002) describe that papaya ringspot disease symptoms characterized by a yellowing and stunting of the crown of papaya trees, a mottling of the foliage, shoe-stringing of younger leaves, water-soaked streaking of the petioles (stalks), and small darkened rings on the surface of fruit. Purcifull *et al.* (1984) have reported that ringspot disease symptoms included mottling, ringspot and distortion of leaves, rings and spots of fruit, and streaks with a greasy or water-soaked appearance on stem and petioles Other studies have reported on the reduction in quality and quantity of fruit production (Kertbundit *et al.,* 2007).

Fig. 1. Ringspot disease symptoms on papaya. A : a mottling of the foliage, B : yellowing and stunting of the crown of papaya trees.

Table 1. PRSV-P infection on papaya samples from 6 regency of Bali

No.	Sampling sites (village / regency)	Infection percentages*	Symptomps
1	Bangli/Tabanan	73,33, (11/15**)	mottling of leaves, yellowing and stunting of the crown
2	Sempidi/Badung	0 (0/11)	malformation, yellowing of leaves
3	Petiga/Tabanan	0 (0/12)	stunting of the crown
4	Payangan/Tabanan	0 (0/14)	mottling of leaves, stunting of the crown
5	Tohpati/ Klungkung	0 (0/10)	stunting of the crown
6	Jehem/Bangli	0(0/12)	mottling of leaves
7	Sesetan/Denpasar	0 (0/12)	mottling of leaves
8	Kerta/Gianyar	0 (0/15)	yellowing of leaves

* Verification of infection by ELISA
** x/y : (x) sample showed positive infection of (y) samples tested

3.2 Sequence analysis

Infection of PRSV-P was confirmed in leaf samples collected from papaya cultivation areas in Bangli village, Tabanan, Bali, Indonesia by RT-PCR using the PRSV coat protein specific primers (MB12 and MB 11). Amplicons of 905 bp were successfully amplified from infected samples (Figure 2). There is a report from Azad *et al*. (2014) that PRSV diagnosis can be confirmed by molecular diagnosis such as ELISA, immunocapture RT-PCR, RT-PCR and DIBA. RT-PCR method with specific primers can be used to amplify the CP gene and part of NIb gene (Sharma *et al*, 2004; Tuo *et al*, 2014).

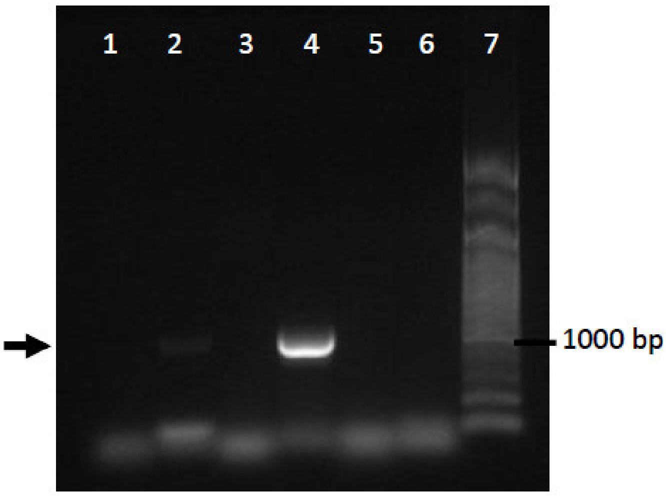

Fig. 2. Amplification of PRSV by RT-PCR using primer pair MB12 and MB 11. The samples in lanes 2,and 4 are leaf tissue collected from Bangli. The samples in lanes 1, 3, 5 and 6 isolated from Petiga, Payangan, Sempidi and Kerta, respectively. Lane 7 is a DNA marker with the 1000 bp marked on the right margin. An arrow on the left margin marks the position of the PRSV amplicon (~905 bp).

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The PCR product was then directly submitted for DNA sequencing and phylogenetic analysis. Result from BLAST and sequence alignments showed that Bangli village isolate (hereafter called Bali isolate) had considerable homology to the China (Accession No. KF 033092.1) isolate (99.45% na-level) (Table 2).

Table 2. Matrix of similarity between PRSV Bali isolate with other PRSV from different geographic location available from GenBank, based on coat protein gene

Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	100.00																		
2	98.44	100.00																	
3	97.29	97.29	100.00																
4	95.04	94.65	97.10	100.00															
5	93.93	93.93	94.95	91.59	100.00														
6	97.37	97.18	97.46	96.62	95.21	100.00													
7	88.64	88.25	91.50	83.52	83.19	92.29	100.00												
8	88.64	88.25	91.50	85.88	85.64	92.29	100.00	100.00											
9	89.31	88.86	91.87	83.12	82.99	92.39	94.55	95.14	100.00										
10	88.91	88.84	90.91	88.98	88.14	91.33	94.00	94.00	93.57	100.00									
11	89.05	88.98	91.38	89.27	88.42	91.80	94.28	94.28	93.86	99.44	100.00								
12	89.40	89.24	92.13	83.46	82.97	92.46	89.01	91.44	88.60	93.57	93.71	100.00							
13	87.42	87.83	89.50	88.47	87.89	89.54	86.19	86.19	86.07	85.77	85.85	85.99	100.00						
14	87.13	87.71	89.60	87.95	87.71	89.63	85.96	85.96	85.66	85.99	86.06	86.05	98.42	100.00					
15	85.73	85.95	88.57	85.84	85.95	88.97	86.30	86.30	85.45	85.31	85.59	86.44	88.46	88.61	100.00				
16	85.15	85.15	85.70	85.15	85.48	86.57	86.03	86.03	85.70	85.48	85.48	86.14	86.68	86.46	86.57	100.00			
17	85.59	85.59	86.14	85.48	85.92	87.01	86.57	86.57	86.24	86.03	86.03	86.57	87.12	86.90	87.12	99.45	100.00		
18	85.59	85.59	86.14	85.70	85.81	87.01	86.68	86.68	86.57	86.24	86.24	86.46	86.79	86.57	86.90	97.93	98.47	100.00	
19	86.03	86.03	86.57	85.92	86.24	87.45	86.90	86.90	86.35	86.46	86.46	87.12	87.45	87.23	87.23	98.03	98.58	98.58	100.00

Notes: 1: JN831646.1 Papaya ringspot virus AS, 2: JN831644.1 Papaya ringspot virus AS, 3: S89893.1 Papaya ringspot virus Australian, 4: KC345609.1 Papaya ringspot virus France, 5: DQ374153.1 Papaya ringspot virus Brazil, 6: AF319499 Papaya ringspot virus Mexico, 7: X97251.1 Papaya ringspot virus Taiwan, 8: X78557.1 Papaya ringspot virus Taiwan, 9: AB369277.1 Papaya ringspot virus Korea, 10: AB583220.1 Papaya ringspot virus Japan, 11: AB583218.1 Papaya ringspot virus Japan, 12: AY010722.1 Papaya ringspot virus THAILAND, 13:DQ666639.1 Papaya ringspot virus India, 14: DQ666641.1 Papaya ringspot virus India, 15: AB583209.1 Papaya ringspot virus Myanmar, 16: Papaya ringspot virus Bali isolate, 17: KF033092.1 Papaya ringspot virus China, 18: KF033089.1 Papaya ringspot virus China, 19: KF033078.1 Papaya ringspot virus China

Results of phylogenetic analysis using Maximum Parsimony approaches with 1000 bootstrap replications indicates that Bali isolate is a clade with the China isolates (Accession No. KF 033092.1, KF 033089.1, KF 033078.1) with 100% bootstrap support (BS). It was shown that Bali isolate of PRSV is clustered together with the PRSV isolate from China, whereas the other cluster divided into three sub clusters. Isolates from Asian (THAILAND: Accession No. AY010722.1; Taiwan: Accession No. X97251.1, X78557.1; Korea: Accession No. AB369277.1; Japan: Accession No. AB583220.1, AB583218.1) to be in one sub cluster, except Indian isolates (Accession No. DQ666639.1, DQ666641.1) are in a sub cluster with isolates of Myanmar (Accession No. AB583209.1) (Fig 3). Similar results were found in previous studies. Research on the phylogeny based on CP gene sequences of 93 PRSV isolates from different geographical locations has been done by Gonsalves *et al* (2008) to create a phylogeny tree using neighbor-joining method. The results of these studies showed that sequence of Sri Lanka and India isolates are close genetic relationship when compared to isolates from other countries. Whereas, based on phylogenetic analysis of PRSV isolates from Thailand and Taiwan, showed that Asian isolates to be in one cluster, except India isolates are in a cluster with American isolates (Lu *et al*, 2008). In this research, the high homology of PRSV Bali isolate with China isolate likely caused by PRSV Bali isolate originating from China via infected plants or papaya fruit imported from China.

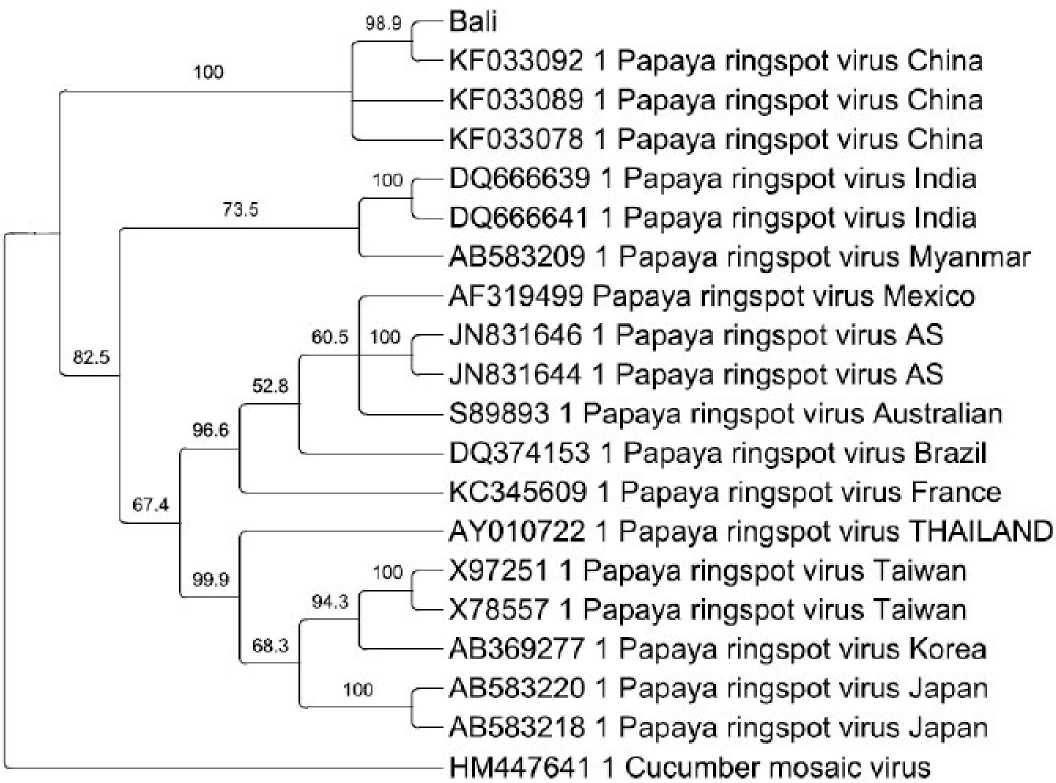

Fig 3. Phylogenetic relationship between PRSV Bali isolate with other PRSV from different geographic location available from GenBank, based on coat protein gene

4. Conclusion

Based on the disease symptoms, RT-PCR detection and molecular characteristics, it can be concluded that the PRSV as causing agent of ringspot disease on papaya cultivation in Tabanan, Bali, Indonesia. PRSV Bali isolate showed a similar characteristics with China isolate. This is the first report of PRSV in Bali.

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