Nature of the Greening Bacterium-Like Organism (BLO): Taxonomic Characterization By Use of Cloned DNA Fragments

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ABSTRACT. Using total DNA extracted from phloem tissue of periwinkle plants infected with the Poona (India) strain of the greening bacterium-like organism (BLO), four fragments (Prom, In 2.6, In 1.0 and In 0.6) of the BLO genome were cloned and sequenced. Sequence comparisons of open reading frames of the cloned BLO-DNA with those of other bacteria have shown that Prom-In 2.6 is part of the *nus*G-*rpl*KAJL-*rpo*BC gene cluster, a well-known operon in Eubacteria. The organization of this operon is identical to that in *Escherichia coli* and shows that the greening BLO is a Eubacterium. In 0.1 carries the gene for a bacteriophage type DNA polymerase. No known genes could be identified on In 0.6. Southern and dot hybridizations have shown that at high stringencies, In 2.6 hybridized to DNAs of all Asian strains tested but not to the African strain examined. However, at intermediate strengencies, In 2.6 was able to detect the African strain. In 0.1 hybridized with most of the Asian strains but not with the African strain even at low stringency. In 0.6 reacted only with the Indian (Poona) BLO strain. From these hybridization studies, we infer that Asian and African greening are caused by two different BLO species or subspecies. The 16S rDNA of the greening BLO has been amplified using polymerase chain reaction (PCR), and cloned and sequenced in order to define the taxonomic position of the BLO. The greening BLO has been shown to belong to the α class of the proteobacteria.

Greening disease of citrus is caused by a non-cultured, phloem-restricted bacterium-like organism (BLO) (2) and is transmitted by two insect vectors, the Asian psyllid *Diaphorina citri* (Kuwayama) and the African psyllid *Trioza erytreae* (Del Guercio) (7, 10). Study of the greening BLO has been hampered by the fact that it is not available in culture. In particular, its taxonomic status has remained undetermined. In 1987, we were able to produce, for the first time, monoclonal antibodies against the greening BLO, and these reagents have revealed that several serotypes of the greening BLO occur (3, 4). We have now cloned and sequenced four DNA fragments (Prom, In 2.6, In 1.0, and In 0.6) of the genome of the Indian (Poona) strain of the organism (13). Sequence analysis and hybridization studies with these fragments confirm at the molecular level that the greening BLO is an eubacterium and that Asian and African greening are caused by two different species or subspecies of the BLO (14). We have also used polymerase chain reaction (PCR) to amplify the 16S ribosomal RNA gene (rDNA) of the BLO. Cloning and sequencing of the gene has revealed the phylogenetic relationships of the greening BLO.

MATERIALS AND METHODS

Plant material. Healthy periwinkle and sweet orange seedlings as well as periwinkle and sweet orange plants infected with a strain of the greening-BLO from Nelspruit, South Africa, were grown in a greenhouse at 25 C during the day and 20 C during the night. Those infected with the heat-tolerant Asian strains of the BLO, were grown at 30 C in the day and 25 C at night.

Bacterial strain. *Escherichia coli* dH5(alpha)F' was used as the host for cloning DNA from the Poona strain of the greening BLO; M13mp18 or PUC 18 were the cloning vectors.

Isolation and cloning of nucleic acids from BLO infected plants. DNA was extracted as described in (13) from enzymatically purified phloem tissue (6). DNA cloning was done as described in (13).

Screening recombinants for BLO-DNA inserts. Recombinant vectors containing DNA inserts larger than 0.5

kb were selected and their DNA radiolabeled with (^{32}Pα)dCTP, using the random priming kit of Amersham Corporation (Arlington Heights, IL). The labeled DNAs were used as probes in hybridizations according to Southern (11) with blots of *Hin*dIII-digested DNA from healthy or Poona-BLO infected periwinkle plants.

Extraction of nucleic acids from plants. The CTAB method of Murray and Thompson (8) was used.

Southern hybridization. Four to seven μg of DNA from healthy or infected plants were digested with *Hin*dIII restriction endonuclease, electrophoresed in 0.7% agarose gels, alkali-denatured (0.5N NaOH in 1.5M NaCl for 45min), and transferred to nitrocellulose filter paper according to Southern (11). The filters were then baked at 80 C for 2 hr, prehybridized at 42 C, and hybridized with ^{32}P-labeled DNA. The washings were done as follows: three washes at room temperature for 15 min with 2SSC containing 0.5% SDS followed by two washes at 60 C (high stringency) or at 32 C (intermediate stringency) in 0.1SSC containing 0.1% SDS. The filters were air-dried and exposed to X-ray film for 48 hr with an intensifying screen.

Sequence determination. The cloned DNA fragments were sequenced using the Sequenase™ kit (USB, Cleveland, OH). Subcloning in vector M13mp18 was performed after digestion with several restriction enzymes (*Bgl*II, *Hinc*II, *Hin*dIII, *Pst*I, *Sph*I) (GIBCO-BRL, Gaithersburg, MD) known to cleave within the inserts.

Sequence analysis. The potential translation products, deduced from the nucleotide sequences of the open reading frames (ORFs), were compared to protein sequences contained in GenBank (release 70) via the GenBank server and using the FASTA program (9). Protein sequences showing significant similarity with the ORFs translation products were imported from GenBank. Multiple alignments were performed using CLUSTAL software (5) obtained from EMBL server (European Molecular Biology Laboratories, Heidelberg, FRG). Protein and DNA sequences used for alignments were the following: *rpl*KAJL-*rpo*BC and *sec*E-*nus*G from *E. coli* (accession numbers: J01678, M30610), *rpl*KA from *Serratia marcescens* (X12584), *rpo*B from *Pseudomonas putida* (X15849), *rpl*K from *Streptomyces virginiae* (D10468) and *Thermotoga maritima* (Z11839), *rpl*-KA from *Proteus vulgaris* (X12585), *rpl*JL from *Salmonella typhimurium* (X53072), *rpl*JL from *Synechocystis* sp. (X53178), rpl1 (P04447) and rpl12 from *Bacillus stearothermophilus*, and DNA polymerases I from bacteriophages SPO2 (K02752), T3 (X17255) and T5 (M24354).

Amplification and cloning of the BLO 16S rDNA. The BLO (Poona strain) was first immunocaptured from periwinkle plant extracts by monoclonal antibody (MA) 2D12 (3). For this, PCR-plastic tubes were coated for 4 hr at 37 C with MA 2D12 (10 μg/ml in carbonate buffer pH 9.6). The tubes were rinsed three times with PBS-600 (12). The periwinkle extract (1 g of midribs chopped in 2 ml of PBS-600) was added and kept overnight at 4 C. The tubes were washed three times with PBS 600. DNA amplification was done with the universal primers devised by Weisburg *et al.* (15) for the amplification of procaryotic 16S rDNA. Control tubes contained either water, PBS-600 or healthy periwinkle plant extract. The amplified products were visualized by electrophoresis on 1% agarose gels. The amplified 16S rDNA of the BLO was cloned blunt end in pUC 18 linearized by the restriction enzyme *Sma*I and sequenced as before. Sequence analysis was done as described above and phylogenetic trees were produced using Phylip software.

RESULTS

Molecular cloning of the Poona BLO-DNA. A total of 191 recombinant phages were obtained in the cloning of DNA from phloem tissue infected with the Poona-BLO. Twenty-two of them had inserts larger than 0.5 kb and were screened in Southern hybridizations

against *Hin*dIII restricted DNA preparations from healthy and Poona BLO-infected periwinkle plants. Three recombinants gave a positive hybridization with the DNA from the BLO-infected periwinkle plants, but no hybridization with healthy plant DNA. The three recombinant phages, p3, p10, and p19, had inserts of respectively 2.6, 0.6, and 1.0 kbp. The inserts were called In-2.6, In-0.6, and In-1.0 respectively (13).

Southern hybridizations between Poona-BLO inserts and DNA from plants infected with geographically different BLOs. When In-2.6 was used as probe, it hybridized not only with DNA from Poona-BLO-infected periwinkle or citrus plants but also with DNA from periwinkle or citrus plants infected with all other Asian BLO strains tested, i.e. those from Nakhom Pathom (Thailand), Lipa City (Philippines), Fujian (China), Taiwan, and Bali (Indonesia); in all cases the size of the hybridizing band was the same: 2.6 kbp. Interestingly, no hybridization was observed in the case of the African (Nelspruit) strain of the BLO. In-1.0 hybridized with all Asian BLO strains tested except the Taiwan strain; it did not hybridize with the African strain. DNA polymorphism was observed among the hybridizing bands. With In-0.6, hybridization occurred only with the homologous Poona strain, and not with the other Asian or African strains.

SEQUENCES OF THE CLONED DNA FRAGMENTS

In-0.6. The nucleotide sequence of In-0.6 is 660 bp long and has a G+C content of 38.2 mole%. Two putative ORFs have been detected but no similarities with protein sequences contained in Genbank could be found (data not shown).

Sequence of In-1.0. The nucleotide sequence of In-1.0 was deposited in GenBank under the accession number M94320. It has a G+C content of 46.2 mole% and contains two incomplete ORFs. While no similarity with protein sequences in GenBank was found for the translation product of ORF1, that of ORF2 was found to be similar to the DNA polymerase of *Bacillus subtilis* bacteriophage SPO2 (33% identical amino acids and 38% conservative changes in 206 amino acid overlaps). Similarities between the product of ORF2 and SPO2 DNA polymerase are highly significant since the two proteins are more related to each other than are various bacteriophage DNA polymerases between themselves. Similarities with bacterial DNA polymerases are more limited (data not shown).

Sequence of In-2.6. The nucleotide sequence of In-2.6 was deposited in GenBank under the accession number M94319. It has a G+C content of 39.1 mole% and contains 3 complete ORFs (ORFs 2, 3 and 4) and two incomplete ORFs located respectively at the 5′ end (ORF1) and the 3′ end (ORF5) of the insert (Fig. 1). After searching in GenBank, highly significant similarities were found with bacterial ribosomal proteins L11, L1, L10, L12 and RNA polymerase subunit β, encoded by genes *rpl*K, A, J, L, and *rpo*B respectively. The two incomplete ORFs correspond to the 5′ end of *rpo*B and the

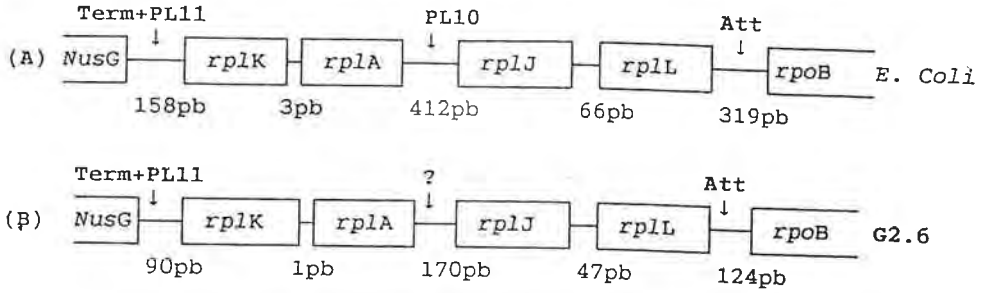

Fig. 1. Organisation of the operon *nus*G- *rpl*KAJL- *rpo*BC in *E. coli* (A) and in the greening BLO (B).

3′ end of *rpl*K, respectively. In the BLO, the genes for these proteins are arranged in the same order as in *E. coli* and other bacterial species (Fig. 1A). This organization is characteristic of the eubacterial kingdom, with the exception of Cyanobacteria, in which the cluster *rpl*KAJL is widely separated from *rpo*BC, a situation also encountered in chloroplasts, archaebacteria and eukaryotes. While the overall gene organization in the greening BLO is highly reminiscent of that in *E. coli*, it may be noticed that the intergenic regions are shorter in the BLO sequence (Fig. 1B). These regions are important in transcriptional and translational regulation.

We have also cloned and sequenced a DNA fragment (Prom, accession number: M94319) upstream of protein L11 to get the missing 5′ end of *rpl*K. This fragment also had an additional ORF, coding for a protein similar to the *nus*G product of *E. coli*. Interestingly, *nus*G is also located upstream of *rpl*KAJL in *E. coli* (Fig. 1A). Thus, the gene order of the *nus*G-*rpl*KAJL-*rpo*BC region is the same in the BLO and in *E. coli*.

Amino acid sequences have been aligned with equivalent sequences from other eubacteria available in GenBank. According to the similarities, the BLO is equally distant to any one of the different bacterial species tested. On the basis of these comparisons, the greening BLO is equally distant to proteobacteria represented by *E. coli*, gram-positive bacteria by *B. stearothermophilus* and cyanobacteria by *Synechocystis* sp.

Hybridization between In-2.6 and DNA of the African strain of the BLO. We have shown that In-2.6 hybridizes not only with DNA from the homologous Poona-BLO strain, but also with the DNA of all six Asian strains tested. In contrast, no hybridization was obtained with the single African strain available (Nelspruit strain). Because In-2.6 is now known to harbor genes for conserved proteins (ribosomal proteins), lack of hybridization with DNA of the African strain was surprising. Therefore we carried out the hybridizations not only at high stringency, as previously done, but also at lower stringency. As shown in Fig. 2, right panel, at high stringency only the Asian strain gave a hybridization signal, as reported earlier, with a band of the expected size, i.e. 2.6 kbp (track 2). At intermediate stringency, hybridization is now also seen with the African strain but the hybridizing fragment is smaller (2.1 kbp) (Fig. 2, left panel, track 3). No hybridization was obtained with *E. coli* DNA (track 4) nor with that of healthy periwinkle plants (track 1). From these results it can be calculated that there are at least 30% mismatches between the Poona-BLO gene cluster and its counterpart in the African strain. As a comparison, there are 26% mismatches between the *rpl*KA nucleotide

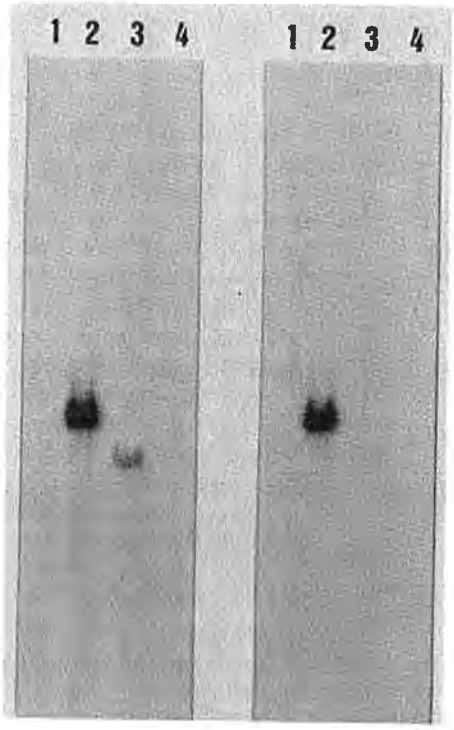

Fig. 2. Southern blot hybridizations between In-2.6 and *Hin*dIII restricted DNA from: healthy (track 1), Poona-BLO-infected periwinkle plants (track 2), Nelspruit-BLO infected periwinkle plants (track 3) and *E. coli* (track 4) at high (right panel) and moderate (left panel) stringencies.

sequences of *S. marcescens* and *P. vulgaris*, two bacteria belonging to the same phylogenetic cluster but to different genera.

Amplification, cloning and sequencing of the 16S rDNA of the greening BLO. Fig. 3 shows the result of DNA amplification with universal primers for the amplification of 16S rDNA. No DNA is amplified when water (track T1) or PBS-600 (Track T2) is present in the PCR tubes. However, a DNA band, around 1500 nucleotides long is observed in the amplification from both healthy (track PS) and infected (track PI) periwinkle extracts, in spite of the immunocapture step done with MA 2D12 to capture the BLO. Analysis of the amplified products was done by digestion with restriction enzyme *BclI*. As shown on Fig. 4, the DNA amplified from healthy periwinkle plants is hydrolyzed into two fragments of 1250 and 250 bp each while, in addition to these fragments, the

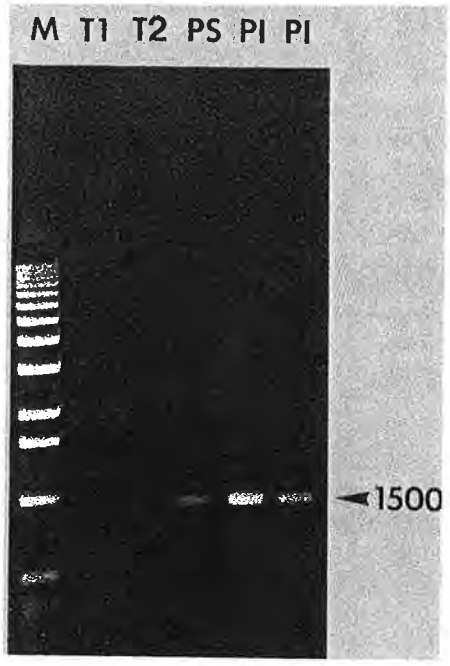

Fig. 3. Electrophoretic analysis of PCR amplified products with universal primers for 16S rDNA from: T1: water, T2: PBS 600, PS: healthy periwinkle extract, PI: Poona-BLO-infected periwinkle extract, M: size markers.

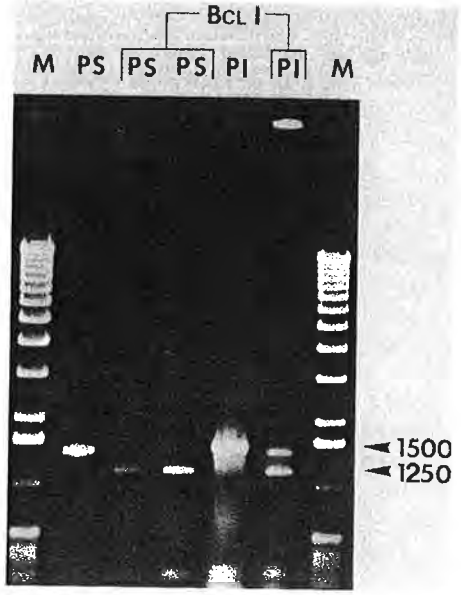

Fig. 4. Electrophoretic analysis of amplified DNA after *BclI* restriction enzyme digestion. PS: healthy periwinkle extract, PI: Poona-BLO-infected periwinkle extract. Tracks 2, 5: untreated DNA; Tracks 3, 4, 6: DNA digested with *BclI*; M: size markers.

DNA band of 1500 bp is still present in the amplification from GO-infected periwinkle extracts. The DNA amplified from both healthy and BLO-infected periwinkle extracts and sensitive to *BclI* digestion has been partially sequenced and shown to be chloroplastic 16S rDNA (results not shown). The DNA band present in infected periwinkle extracts only and not digested by *BclI* was thought to be the 16S rDNA of the greening BLO; it has been cloned and sequenced. Sequence comparison of the putative 16S rDNA of the greening BLO with the 16S rDNAs of other bacteria has revealed that it was indeed a 16S rDNA of bacterial origin. A 30 mer long oligonucleotide, specific for the amplified DNA, was synthesized and used in dot blot hybridizations against DNA extracted from healthy or greening-infected periwinkle plants (13). Fig. 5 shows that the oligonucleotide hybridized with the DNA extracted from greening-infected periwinkle or citrus plants, but not with that extracted from healthy periwinkle or citrus plants.

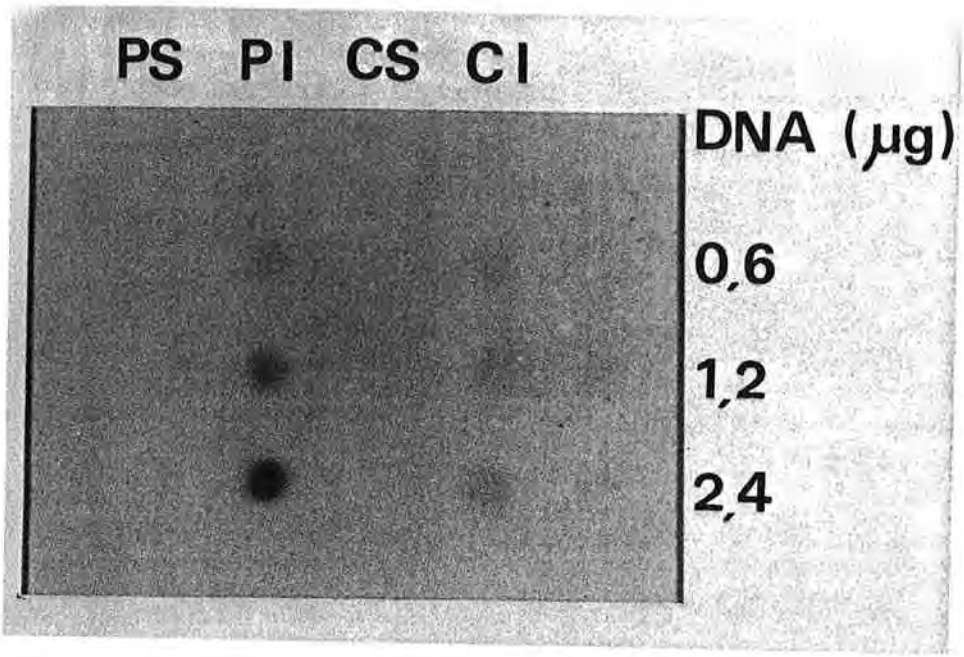

Fig. 5. Dot hybridization of DNA extracted from healthy (PS, CS) or BLO-infected (PI, CI) periwinkle (PS, PI) or citrus (CS, CI) plants with an oligonucleotide deduced from the sequence of the 16S rDNA.

This indicates that the amplified DNA is indeed the 16S rDNA of the greening BLO. Phylogenetic analysis with this 16S rDNA sequence has shown that the greening BLO was an (Alpha) proteobacterium and that in this group the BLO represents a new genus since its 16S rDNA has only 87% sequence homology with that of the most closely related bacterium of this group.

DISCUSSION

We have digested, with restriction endonuclease *Hin*dIII, the total phloem DNA of periwinkle plants infected with the Poona (India) strain of the greening BLO and cloned the restriction fragments in the replicative form (RF) of phage M13mp18. By differential hybridizations involving the DNAs from healthy and Poona-BLO infected periwinkle plants, we have selected three recombinant phages containing DNA inserts, In-2.6, In-1.0, and In-006 corresponding to fragments of the Poona-BLO genome.

The three inserts do not cross-hybridize and have different specificities towards various strains of the greening BLO. In-0.6, the smallest insert, hybridizes only with DNA from plants infected with the homologous Poona strain, i.e. the strain used to produce the recombinant DNA; it does not recognize the other strains tested. No known genes could be identified in this DNA fragment. In-1.0 give positive hybridization signals with most Asian BLO strains tested, but not with the South African strain. It contains an ORF coding for a DNA polymerase resembling that of bacteriophage SPO2 of *B. subtilis*. This ORF has a higher G+C content (46%) than the three other BLO-DNA fragments (ca. 39%). This observation may indicate that the polymerase DNA fragment is of foreign origin. In addition this sequence is not present in the Taiwan and African strains tested and consequently may not be essential. The specificities of probes In-0.6 and In-1.0 were identical whether hybridizations were performed at high or intermediate stringencies.

Fragment In-2.6 and the 5′ overlapping Prom fragment hybridized with

all Asian strains tested and also with the African strain when conditions of lower stringency was used for hybridization. These fragments harbor the *nus*G-*rpl*KAJL-*rpo*B gene cluster, and the organization of this region of the genome is typical of eubacteria. However, comparisons of deduced protein sequences with their counterparts in other bacterial species, failed to discover a specific relationship of the BLO to a known bacterial species. From these results we conclude that the differences between Asian and African BLO strains are greater than those between different Asian strains. This conclusion confirms earlier observations based on the behaviour of the greening BLO towards temperature (1).

In order to determine the phylogenetic position of the greening BLO, the sequence of its 16s rDNA has been compared to those of other bacteria. This has shown that the greening BLO belongs to the α proteobacteria, one of the major groups of gram negative bacteria in which plant symbionts and uncultured human bacteria are present. In addition many representatives of this group have arthropod vectors and live in close association with eucaryotic cells. This is precisely the case of the greening organism which has psyllid vectors and invades the salivary glands of the vector. In conclusion, and even though the greening BLO has not been cultured, it has been possible, by studying DNA fragments of the organism, to demonstrate at the molecular level that it is a gram negative bacterium and to determine precisely its phylogenetic position. This will provide new perspectives for studying and culturing the BLO.

ACKNOWLEDGEMENTS

We are grateful to the following persons who have supplied some of the greening infected plants used in this work: Prof. C. Ke and R. J. Wu from Fujian Academy of Agricultural Sciences, Fuzhou, China; Dr. C. Gonzales and Dr. R. Olfato, Agricuture Research Center, Lipa City, Philippines; Drs. M. Promintara, P. Patrakosol, and S. Tontyaporn, Agricultural University, Bangkok, Thailand; Dr. A. Muharam, Agricultural Research Center, Malang, Indonesia. We also thank IRFA-CIRAD for financial support of the work.

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