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"*CANDIDATUS* LIBERIBACTER ASIATICUS", ASSOCIATED WITH CITRUS HUANGLONGBING, INFECTS POLLEN, SEED COAT AND ENDOSPERM OF PUMMELO IN CHINA

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SUMMARY

"*Candidatus* Liberibacter asiaticus" (Las), one of the three known bacterial species associated with citrus Huanglongbing (HLB), is unevenly distributed in bark tissue, leaf midrib, roots, and different floral parts of infected citrus trees. In this study, 10 petal, 12 stamen, 12 pistil, 3 pollen, 20 seed hull, 20 seed coat and 20 endosperm samples were collected from HLB-infected Shatianyou pummelo trees [= *Citrus maxima* (Burm.) Merr. cv. Shatian Yu] in China and analyzed for the presence of Las. The bacterium was detected by conventional PCR using the Las-specific primer pair OI1/OI2c. Amplification products of the expected size (*ca.* 1100 bp) were obtained from 9 petal (90%), 11 stamen (91.7%), 12 pistil (100%), 2 pollen (66.7%), 18 seed coat (90%), 15 endosperm (75%) samples, but not from any of the seed hull samples. The sequence of a representative amplicon matched that of Las. To our knowledge, this is the first report of the presence of Las in pollen, seed coat and endosperm of pummelo in China.

Key words: *Candidatus* Liberibacter asiaticus, Huanglongbing, pollen, seed coat, endosperm.

Huanglongbing (HLB) or greening, one of the most destructive diseases of citrus, occurs in many parts of the world including Asia, Africa, Oceania, South America, and North America (Bové, 2006). Its causal agents are non-culturable plant pathogenic bacteria belonging to three different species, i.e. "*Candidatus* Liberibacter asiaticus" (Las), "*Ca*. L. africanus" (Laf) and "*Ca*. L. americanus" (Lam) (Murray and Schleifer, 1994; Texeira *et al*., 2005).

HLB was first reported from southern China in 1919 (Reinking, 1919), since then it has spread to 11 southern provinces of the country (Deng *et al*., 2008; Lou, 2008). Repeated surveys have shown that Las is the causal agent of HLB in China (Deng *et al*., 2008; Lou, 2008; Ding *et al*., 2009), and is transmitted naturally by the Asian citrus psyllid *Diaphorina citri*, and experimentally by grafting and dodder (Halbert and Manjunath, 2004). Las can infect most cultivars of citrus and citrus relatives within the family *Rutaceae* (Halbert and Manjunath, 2004), and is unevenly distributed in the bark tissue, leaf midrib, roots, and different floral parts of infected trees (Tatineni *et al*., 2008). Recently, it was found that also the seed coat and seed of sweet orange and grapefruit can be colonized by Las (Hilf, 2011). Now we report that Las is present in the pollen, seed coat and endosperm of "Shatianyou" pummelo [= *Citrus maxima* (Burm.) Merr. cv. Shatian Yu].

In the autumn of 2011 in an orchard of the Chinese province of Guangxi, we observed a few HLB-infected 'Shatianyou' pummelo trees with mature fruits and characteristic blotchy mottle symptoms that blossomed abnormally. One hundred twenty flower samples and 200 seed samples were collected from five of these trees. Flowers were divided into 10 petal, 12 stamen, 12 pistil and 3 pollen samples, whereas seeds were divided into 20 hull, 20 coat and 20 endosperm samples (Table 1). Flower and seed samples from healthy 'Shatianyou' pummelo plants grown in a screenhouse were used as negative controls, whilst positive controls consisted of 10 symptomatic leaf samples from the five selected pummelos (Table 1). DNA was recovered fom each individual sample with a modified DNA extraction method using Cetyltrimethylammonium bromide (CTAB) (Lin and Walker, 1997). Precipitated DNA was re-dissolved in 50 µl of 1X TE buffer and analyzed on 1.2% agarose gels and adjusted to suitable concentration for further usage.

The primer pair OI1/OI2c, constructed on the 16S rDNA sequence (Jagoueix *et al*., 1994), was used for detection of Las by conventional PCR with an automated thermocycler (ABI Veriti™ Thermal Cycler, USA). The volume of the reaction mixture was 25 µl, containing 1X PCR buffer, 1.5 mM Mg^{2+}, 0.2 mM dNTPs, 0.4 µM of each primer, 1 µl of sample DNA, 1 U of TaKaRa *Taq* HS polymerase (Takara, Japan). The reaction program was: a first step at 94°C for 5 min followed by 35 cycles of 94°C for 45 sec, 68°C for 45 sc, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were

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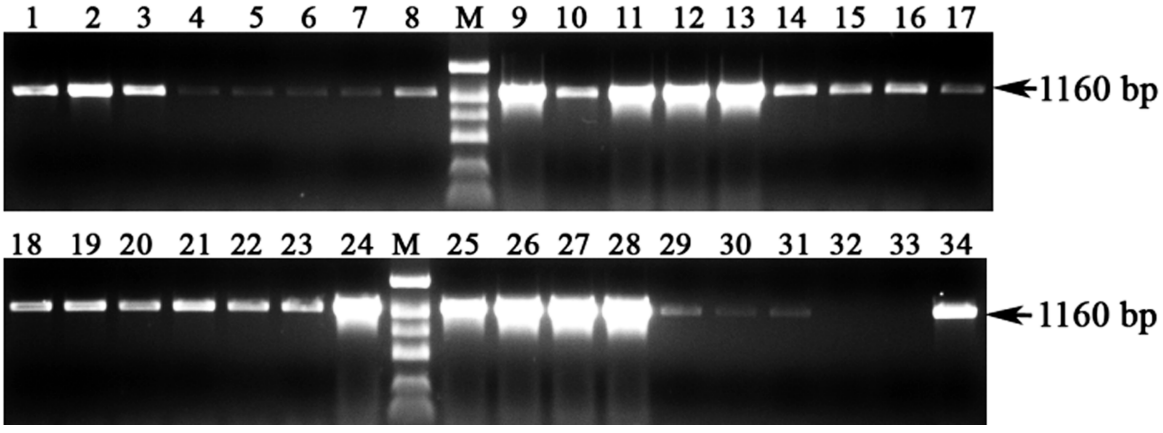

Fig. 1. Agarose gel electrophoresis of the DNAs amplified with primer pair OI1/OI2c specific for "*Candidatus* Liberibacter asiaticus". Lane M, 250 bp molecular ladder (Generay, China); lanes 1 to 8, samples from endosperm; lanes 9 to 17, seed coats; lanes 18 to 21, petals; lanes 22 to 25, stamens; lanes 26 to 29, pistils; lanes 30 to 32, pollen; lane 33, negative control; lane 34, positive control. Samples 1 to 32 were collected from Huanglongbing-infected 'Shatianyou' pummelo trees.

analyzed by electrophoresis on 1.2% agarose gels containing ethidium bromide.

A PCR amplification product with estimated size of *ca.*1100 bp was collected from the agarose gel, purified with a QIAquick gel extraction kit (Qiagen, USA) and directly sequenced three times in both directions. The obtained sequence was deposited in GenBank (accession No. JN211032) and compared with similar sequences from database using the BLASTn network service available at NCBI.

Electrophoresis of PCR products showed that the *ca.* 1100 bp bands attributed to Las were consistently obtained from most of analyzed samples (Fig. 1). Detection level in petal, stamen, pistil, pollen, seed coat, and endosperm samples were 90% (9 out of 10 samples), 91.7% (11 out of 12 samples), 100% (12 out of 12 samples), 66.7% (2 out of 3 samples), 90% (18 out of 20 samples), and 75% (15 out of 20 samples), respectively (Table 1). However, none of the seed hulls was positive. A product of the same size (*ca.*1100 bp) was also amplified from the 10 leaf samples used as positive controls. There was no amplification from negative controls.

A 1073 bp nucleotide sequence was obtained from the directly sequenced amplicon. This sequence shared a virtually complete identity (99.9%, 1 divergent base in 1073) with each of the 16S rDNA sequences of several Las isolates available in GenBank database, confirming the Las nature of the pathogen detected in this study. To our knowledge, this is the first report of the presence of Las in pollen, seed coat and endosperm of pummelo in China.

Recent studies have claimed that seeds from HLB-infected citrus fruits do not transmit the disease (Albrecht and Bowman, 2009; Hartung *et al.*, 2010; van Vuuren and Cook, 2011). However, since the presence of the HLB agent in the tested seeds was not investigated, the conclusion reached does not seem to rest on convincing evidence. By contrast, the high incidence of Las presence in pollen and endosperm ascertained in this study, suggests that there is a potential risk of Las spreading through pollen and seeds of HLB-infected pummelo trees.

Table 1. Detection of "*Candidatus* Liberibacter asiaticus" from different parts of Huanglongbing-infected 'Shatianyou' pummelo trees by conventional polymerase chain reaction.

	No. tested positive/No. of samples tested							
Sources	Leaves	Petals	Stamens	Pistils	Pollen	Seed hulls	Seed coat	Endosperm
Tree 1	2/2	3/4	3/4	4/4	1/1	0/5	5/5	5/5
Tree 2	2/2	4/4	4/4	4/4	1/1	-	-	-
Tree 3	2/2	2/2	4/4	4/4	0/1	0/5	5/5	5/5
Tree 4	2/2	-[a]	-	-	-	0/5	5/5	4/5
Tree 5	2/2	-	-	-	-	0/5	3/5	1/5
Total	10/10	9/10	11/12	12/12	2/3	0/20	18/20	15/20

[a] Samples not available from the specific source.

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