'Candidatus Liberibacter solanacearum' an emerging bacterium associated with vegetative disorders in celery

Gabriela R. Teresani, Edson Bertolini, Ana Alfaro-Fernández, Carmen Martínez, Francisco André Ossamu Tanaka, Elliot W. Kitajima, Montserrat Roselló, Susana Sanjuán, Juan Carlos Ferrándiz, María M. López, Mariano Cambra, María Isabel Font.

First, second, fourth, tenth and eleventh authors: Plant Protection Center. Instituto Valenciano de Investigaciones Agrarias (IVIA). 46113 Moncada, Valencia, Spain; third and twelfth authors: Grupo Virología Vegetal, Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia. 46022 Valencia, Spain; fifth and sixth authors: Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo. 13418-900 Piracicaba, Brazil; seventh author: Servicio de Análisis Agroalimentario, Conselleria de Presidencia y de Agricultura, Pesca, Alimentación y Agua, Generalitat Valenciana. 46460 Silla, Valencia, Spain; eighth and ninth authors: Departamento Técnico, Agrícola Villena Coop. V. 03400 Villena, Alicante, Spain.

*****Corresponding author**: Mariano Cambra; E-mail address:** mcambra@ivia.es

A new symptomatology was observed in celery (*Apium graveolens* L.) in Villena, Spain in 2008. Symptomatology included an abnormal amount of shoots per plant and curled stems. These vegetative disorders were associated with '*Candidatus* Liberibacter solanacearum' and not with phytoplasmas. Samples from plant sap were immobilized on membranes based on the spot procedure and tested using a newly developed real-time PCR assay to detect '*Candidatus* L. solanacearum'. Then, a test kit was developed and validated by intra-laboratory assays with an accuracy of 100 %. Bacterial-like cells with typical morphology of '*Ca*. Liberibacter' were observed using electron microscopy in celery plant tissues. A fifth haplotype of '*Ca*. L. solanacearum', named E, was identified in celery and in carrot after analyzing partial sequences of 16S and 50S rRNA genes.. From our results, celery (Fam. *Apiaceae*) can be listed as a new natural host of this emerging bacterium.

Additional key words: *Apium graveolens,* Spot real-time PCR, detection kit, electron microscopy, haplotypes.

Celery (*Apium graveolens* L., Fam. *Apiaceae*) cultivation is of increasing interest in European countries. In Spain, there are about 1,640 ha of celery crops, mainly in the Mediterranean regions, which account for an annual production of 80,000 t. Celery is affected by fungal, viral and bacterial pathogens causing various well known diseases (36). In 2008 in Villena, Alicante, Spain, celery plants showed symptoms that had not been observed before i.e. abnormal amount of shoots, curling of stems and yellowing (Fig. 1 A, B, C, D). These vegetative disorders were observed in the crop in the three overlapping cultivation cycles (early, medium and late) from March to November, in cvs. Loretta, Monterrey and Imperial of var. dulce (Mill.). Only severe stunting appeared in cv. Brillant of var. rapaceum (Mill). Consequently, there was a relevant yield reduction and economic losses from 2008 to 2009 in Villena, followed by other Spanish celery growing regions. Celery was grown in these areas together with carrots (*Daucus carota,* Fam. *Apiaceae*), being affected by the bacterium '*Candidatus* L. solanacearum' (10, 25).

'*Ca.* L. solanacearum', also named as '*Ca.* L. psyllaurous' (12) is a Gram-negative bacterium. This bacterium cannot be cultured *in vitro* yet. It is restricted to the plant's phloem, transmitted through vegetative propagation and naturally by several psyllid species (23). '*Ca.* L. solanacearum' causes a disease affecting potato (zebra chip)(38). Besides causing disease in potato, '*Ca.* L. solanacearum' can cause serious damage and economic losses in tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), eggplant (*S. melongena*), tamarillo (*S. betaceum*), tomatillo (*Physalis peruviana),* tobacco (*Nicotiana tabacum),* carrot and weeds in the *Solanaceae* family (10, 23).

The bacterium is transmitted in a persistent (transovarial) way by the psyllid *Bactericera cockerelli* (24). It has also been detected in the psyllids *Trioza apicalis*

(25), *B. trigonica* (2), other *Trioza* species and in *Accizia* species (37).

Conventional and real-time PCR methods have been developed to detect and/or identify '*Ca.* L. solanacearum' in plant material and insect vectors, (8, 12, 16, 18, 19, 20, 26, 33, 34, 42). In most bacterial models, real-time PCR has advantages over conventional PCR since it is more sensitive and reliable and performs fast and straight forward (9, 16). The PCR templates require extract preparation and nucleic acid purification which is laborious, time consuming and increases the risk of contamination (32). Nucleic acid purification step can be overcome using direct sample preparation methods such as tissue-print and/or squash or spot immobilization on membranes (9). The main drawback of target immobilization is the small amount of target nucleic acid available on the support. This limitation can be offset by coupling these preparation methods with highly sensitive techniques such as real-time PCR (4).

Four '*Ca.* L. solanacearum' haplotypes (designated A, B, C and D) have been described affecting several crops worldwide. Haplotypes are described from single nucleotide polymorphisms (SNPs) that are inherited as a package in three gene regions, 16S rRNA, 16S/23S intergenic spacer region (ISR) and 50s rRNA (30, 33).

In this paper, the presence of '*Ca.* L. solanacearum' in celery in Spain was reported for the first time. Its symptoms and haplotype status were described herein. A detection method using sample immobilization on membranes and a complete kit for accurate detection of the bacterium based on universal '*Ca.* Liberibacter' primers and a specific probe for real-time PCR were developed and validated, as well.

MATERIALS AND METHODS

Plant material and prevalence of symptoms. About 37 ha of celery crops were annually inspected by technical staff from Agrícola Villena Coop. V. (producers of 20% of fresh celery and carrot in Spain). Inspections were conducted on the field and in the packing house, from 2008 to 2012 at Villena, Alicante, Spain to estimate the percentage of field plants showing symptoms and the number of packing house discards. During this period, 2,655 celery plants were randomly collected from experimental plots to estimate the prevalence of '*Ca.* L. solanacearum'. Symptomatic carrot and celery plants were used as positive control and asymptomatic healthy carrot and celery plants were used as negative control.

In September 2010, at harvest, 502 plants of the celery cvs. Loretta, Imperial and Monterrey from experimental plots were classified into three categories according to the severity of the symptoms. The classification was as follows: i) 174 plants as '+++' with severe symptoms making the celery unmarketable (Fig. 1 A and B), ii) 150 plants as '+' with mild symptoms that could be marketed, and iii) 178 symptomless and marketable plants. Each sample was tested for '*Ca.* L. solanacearum' and phytoplasmas to investigate the etiology of the disorders.

Sample preparation. Leaf samples (about 1g/plant) were collected from the middle part of celery and carrot plants into separate plastic bags and stored at 4ºC for up to one week until use. Extracts were prepared using a Homex 6 (Bioreba, Switzerland) homogenizer, grinding the plant material 1:5-10 (w/v) in PBS extraction buffer (NaCl, 8 g/l; $NaH_2PO_4.2H_2O$, 0.4 g/l; $Na_2HPO_4.12H_2O$, 2.7 g/l; pH 7.2). One ml of extract from each plant was stored at -20ºC until use.

Direct sample preparation without DNA purification (spot procedure).

Freshly prepared or frozen plant extracts were immobilized on membranes (32) by spotting 5 µl of crude plant extract onto pieces (approximately 0.5 cm^2) of positively charged nylon membrane (Roche, Mannheim, Germany) or Whatman 3MM filter paper (GE Healthcare Europe GmbH, Freiburg, Germany), in Eppendorf tubes (7). Spotted plant extracts were left to dry for 5 min and then stored at room temperature in the dark until required. The DNA was extracted from each membrane by adding 100 µl of distilled water per tube (3). Each tube was then vortexed and placed on ice. Then, 3 µl from this preparation was used as the template for real-time PCR.

DNA purification. Total DNA was purified from 200 µl of crude plant extract by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol or the CTAB (cetyl trimethyl ammonium bromide) protocol (29). Purified DNA was stored at –20 ºC until use. These DNA templates were considered as the "gold-standard" when comparing the conventional method using purified DNA and the direct methods of sample preparation without DNA purification.

Comparison of sample preparation methods using naturally infected celery plants: DNA extraction *vs.* spot. The 502 plants of the celery cvs. Loretta, Imperial and Monterrey from experimental plots (described above) were tested using two sample preparation methods: DNA extraction (considered as the gold standard technique) (29) and the spot procedure (32). Cohen's Kappa (κ) coefficient and the Bias-adjusted Kappa (BAK) index were used to calculate the coincidence between methods (6). Both sample preparation methods were compared using the same plant extract and the newly developed real-time PCR protocol (see below).

Probe design Nucleotide sequences internal to patented universal '*Ca.* Liberibacter' species forward CaLsppF (5'-GCAGGCCTAACACATGCAAGT-3'), and reverse CaLsppR 5'-(GCACACGTTTCCATGCGTTAT-3') (3) primers were selected to design a '*Ca.* L. solanacearum' specific probe. Alignment of nucleotides based on the 16S rDNA sequences of '*Ca.* L. solanacearum' recovered from GenBank, was performed. Primer Express software (Applied Biosystems, Foster City, CA, USA) was used to design the specific CaLsolP probe. The probe was labeled with fluorescent dyes 5`FAM-AGCGCTTATTTTTAATAGGAGCGGCAGACG-3` TAMRA.

Real-time PCR. Real-time PCR using CaLsppF and CaLsppR primers and the newly designed (CaLsolP) TaqMan probe was carried out using two real-time PCR systems: StepOne Plus (Applied Biosystems) and Light Cycler 480 (Roche). Various concentrations of primers (from 0.3 to 1.0 µM) and probe (from 80 to 300 nM) were used to tested the optimum amplification conditions. The optimum reaction mix consisted of 1 x Path-IDTM qPCR master mix (Ambion, Grand Island, NY, USA), 0.5 µM of each CaLsppF and CaLsppR primers 150 nM of CaLsolP TaqMan probe and 3 µl of purified DNA or DNA from the spotted samples, in a final volume of 12 µl. Positive and negative controls were used in each PCR reaction. The real-time PCR amplification protocol consisted of 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data acquisition and analysis were performed with the thermal cycler's software. The default threshold set by the machine was slightly adjusted above the noise in the linear part of the growth curve. This new real-time assay was compared with the procedure of Li *et al*. (16).

Specificity and sensitivity. One hundred and ten reference '*Ca*. L. solanacearum' DNA samples from 10 different origins, 17 DNA samples of '*Ca*. Liberibacter' species other than '*Ca*. L. solanacearum', 16 strains of bacterial species that affect solanaceous and other crops and 81 unidentified bacterial isolates from celery, carrot and potato microbiota (Table 1), were used to test specificity of the new real-time assay. Sensitivity was tested using serial dilutions of a '*Ca*. L. solanacearum' positive celery extract cv. Loretta (prepared by homogenizing 1:10 (w/v) plant material in PBS buffer pH 7.2) in a healthy celery extract of the same cultivar (negative to '*Ca*. L. solanacearum' by PCR). A sample of each dilution was spotted on Whatman 3MM paper and/or used for DNA purification. Spotted extracts and purified DNA were used to compare four conventional (see below) and two real-time PCR protocols.

Conventional PCR protocols. Four previously described conventional PCR protocols using different primers were compared: OA2 and OI2c (17); Lso TX F and Lso TX R; Lso adk F and Lso adk R (33); LsoF and OI2c (16). Amplifications were performed as described by these authors. Positive and negative controls were used in each PCR reaction.

Intra-laboratory validation of a complete kit. A complete kit (Ref. CaLsol/100; Plant Print Diagnòstics SL, Valencia, Spain) for accurate detection of the bacterium was developed based on the new real-time assay and primers (3). The kit used lyophilized master mix and was based on a direct method of sample preparation. The kit was tested in three different laboratories at Instituto Valenciano de

190 Investigaciones Agrarias (IVIA), using three different real-time PCR systems
191 (StepOne Plus and LightCycler 480 described earlier and SmartCycler-Cepheid). Ten
192 blind samples immobilized on Whatman 3MM filter paper were used: 5 positive for
193 '*Ca.* L. solanacearum' and 5 negative. Template preparation was performed according
194 to the kit manufacturer's instructions. Positive samples were '*Ca.* L. solanacearum'
195 infected extracts from carrot and celery plants. Negative samples were extracts from
196 healthy carrot, celery, potato, *Nicotiana benthamiana* and periwinkle. The
197 amplification conditions for the kit were 4°C higher than those described previously
198 for real-time amplification using fresh (non-lyophilized) master mix.. Six replicate
199 reactions were performed on each sample. Diagnostic parameters (sensitivity,
200 specificity and accuracy) were calculated according to https://www.antonio-
201 olmos.com/parameters/online/calculator.html. Positive or negative results and Ct
202 values were recorded for each sample.
203
204 **Association of '*Ca*. L. solanacearum' and/or phytoplasmas with symptoms.**
205 The 502 plants of celery from experimental plots (described above) were tested for
206 '*Ca*. L. solanacearum' using the real-time PCR kit described in this paper and for
207 phytoplasmas using de procedure of Hren *et al*., (13). Results were compared using
208 multinomial regression analysis ('Program R' www.r-project.org).
209
210 **Electron microscopy.** Leaf midribs from symptomatic plants that had tested
211 real-time PCR positive for '*Ca*. L. solanacearum' from commercial celery fields were
212 prepared for transmission (TEM) and scanning (SEM) electron microscopy. For TEM
213 analyses, midribs were first fixed in 'Karnovisky' solution for 24 h, then fixed with
214 osmium tetroxide in 1% cacodylate buffer for 1 h and contrasted with uranyl acetate

215 0.5% overnight. Samples were then dehydrated using increasing concentrations of
216 acetone (30-100%). Infiltration and embedding was performed in 1:1 acetone (100%):
217 'Spurr' epoxy resin for at least 5 h and then in pure resin 'Spurr' overnight or longer,
218 depending on the infiltration capacity of the samples. Polymerization was carried out
219 at 70°C for three days. The resin blocks were cut into 70 nm sections (using an
220 ultramicrotome with diamond knife), which were placed on copper´s screens,
221 contrasted with uranyl acetate 3% and lead citrate (35). Samples were examined using
222 a JEOL JEM 1011 transmission electron microscope and images were captured using
223 a digital camera.

224 For SEM analyses, midribs were fixed with 'Karnovisky' solution for 24 h,
225 infiltrated with glycerol, immersed in liquid nitrogen and fractured with a scalpel.
226 Fractured pieces were post-fixed in 0.1% OsO_4 for 1 h, dehydrated in acetone, dried at
227 the critical point, gold coated by sputtering and examined in a LEO 435 VP scanning
228 electron microscope (39).

229

230 **Phylogenetic analyses.** '*Ca.* L. solanacearum' PCR products from celery and
231 carrots, amplified with 16S rDNA OA2/OI2c primers (1,168 pb) and with 50S rDNA
232 rplj CL514F/CL514R primers (669pb) (28), were purified using High Pure PCR
233 Product Purification Kit (Roche, Mannheim, Germany) and sequenced**.** Sequences
234 were compared with 23 sequences from other hosts in GenBank (NCBI) . Nucleotide
235 sequences were aligned using Clustalw software implemented in Geneious program.
236 Phylogenetic trees were inferred using MEGA 5.1, and the neighbor-joining algorithm
237 (14), with 1,000 bootstrap replicates.

238 Six '*Ca.* L. solanacearum' sequences from celery and two from carrot based on
239 16S rRNA, 16S/23S ISR and 50S rRNA genes obtained in this work and four

sequences from the previously described haplotypes (A, B, C and D), were aligned within the respective gene regions using Geneious program. The SNPs were visually identified and annotated. A phylogenetic tree was constructed with MEGA 5.1, using the UPGMA algorithm.

RESULTS

Prevalence of symptoms and '*Ca.* L. solanacearum' detection. The prevalence of symptoms in celery cultivars in Villena was: 50% (2008), 60% (2009), 32% (2010), 0.8% (2011) and 0.1% (2012). The cv. Loretta was the most susceptible, showing symptoms in up to 70% of the plants in 2009 and 2010. The cv. Imperial followed by the cv. Monterrey were less susceptible. The number of positive plants for '*Ca*. L. solanacearum' out of the total number of randomly tested plants was 32/52 (62%) in 2009, 252/503 (50%) in 2010, 82/900 (9%) in 2011 and 6/1200 (0.5%) in 2012.

Real-time PCR assay, specificity and sensitivity. The new real-time PCR assay using CaLsolP probe and CaLsppF/CaLsppR primers was specific for '*Ca.* L. solanacearum´ targets with a PCR product of 111 bp. All reference '*Ca.* L. solanacearum' isolates (Table 1) were amplified. No amplification occurred with 17 '*Ca.* Liberibacter' isolates causing citrus huanglongbing (HLB) disease, 16 bacterial species that affect tomato, potato, pepper and other crops, or from 81 microbiota isolates from celery, carrot and potato.

Using purified DNA (from infected celery) only two out of the four conventional PCR protocols detected '*Ca.* L. solanacearum' (Table 2). The protocol

265 of Ravindran *et al.* (33) that uses TX primers was the most sensitive, amplifying down
266 to a 10^{-2} dilution. However, the sensitivity of the real-time PCR assays (i.e. the new
267 assay described in this paper and the assay of Li *et al.* (16)) was higher than with
268 conventional PCR, amplifying down to a 10^{-3} dilution. Using spotted samples, no
269 amplification was obtained with conventional PCR assays and only real-time PCR
270 assays detected '*Ca.* L. solanacearum' down to a 10^{-2} dilution.
271
272 **Comparison of sample preparation methods using naturally infected celery**
273 **plants: DNA extraction *vs.* spot.** Of 502 samples tested using the new real-time PCR
274 assay, 151 samples were positive using both DNA extraction and spot procedure. One
275 hundred samples were positive using only purified DNA and one sample was positive
276 using only spot procedure. The remaining samples were negative independent from
277 the sample preparation method used. The coincidence between methods was 80%.
278 The estimated prevalence (positive by both methods) was 30%. The Cohen's kappa
279 coefficient (κ) was 0.60 ± 0.040 and the Bias-adjusted Kappa (BAK) index was 0.58
280 ± 0.040.
281 The Ct values from spotted samples on nylon or paper membranes were similar
282 (data not shown). In addition, no differences were found between DNeasy or CTAB
283 (data not shown).
284
285 **Intra-laboratory validation of a complete kit.** '*Ca.* L. solanacearum' was
286 detected only in the "blind" positive samples at Ct values ranging from 29.8 ± 2.2 to
287 37.2 ± 1.5 in all three laboratories (data not shown). Similar Ct values were obtained
288 with the different thermal cyclers. The calculated diagnostic parameters (sensitivity
289 and specificity) were 1.0 indicating a 100 % accuracy for the kit.

290

291 **Association of symptoms with '*Ca.* L. solanacearum' and/or phytoplasmas.**
292 The percentage of celery plants positive for '*Ca*. L. solanacearum' in each symptom
293 classification group were: 86% in plants with severe symptoms (+++), 42% in plants
294 with mild symptoms (+) and 21% in symptomless plants (Table 3). Multinomial
295 logistic regression analysis showed that the only significant independent variable (Pr
296 $< 2^{-16}$) was the presence of '*Ca.* L. solanacearum' whereas the presence of
297 phytoplasmas (Pr = 0.84) did not affect the model.

298

299 **Electron microscopy observations.** Bacteria-like organisms (BLOs) were
300 observed using SEM in the phloem sieve tubes of celery samples that had tested real-
301 time PCR positive for '*Ca.* L. solanacearum' (Fig. 2 A and B). The BLOs were
302 neither observed in the associated companion or mesophyll cells, nor in symptomless
303 celery plants testing negative for '*Ca.* L. solanacearum'. Using TEM, observed BLO
304 cells were pleomorphic and surrounded by an electron dense cell wall separate from
305 the cytoplasmic membrane, which was slightly rippled, wrinkled or uneven (Fig. 2 C
306 and D). The BLO cells were triple-layered membrane (i.e. outer cell wall membrane
307 and the inner cytoplasmic) suggesting the presence of '*Ca.* Liberibacter' cells (Fig. 2
308 D) rather than phytoplasma cells which do not have a cell wall.

309

310 **Phylogenetic analyses**. Based on bootstrap consensus phylogenetic trees of the 16S
311 and 50S rRNA genes, celery strains grouped into two for 16S rRNA (data not shown).
312 The first cluster contained the Spanish celery isolates collected in 2007, 2009 and
313 2010, the carrot isolates from Finland and Spain, and one Spanish isolate recovered
314 from *B. trigonica*. The second cluster contained the celery and carrot isolates

315 collected in Spain in 2011. These clusters belonged to a broad cluster which was
316 separated from another cluster that grouped all the tomato isolates from USA. For 50S
317 rDNA, celery isolates were grouped into the same clusters as for 16S rDNA (data not
318 shown). Table 4 is based on a previous report (31) with the addition of descriptions of
319 SNPs for the new haplotype numbered as 5 and named E. Today, this haplotype is
320 represented by isolates from celery and carrot plants grown in Spain in 2011. A
321 cladogram (Fig. 3) on the 16S rRNA gene shows the divergence of the different
322 haplotypes described up to now. GenBank accession numbers were KF737346
323 (Celery, Spain, 16S rDNA) and KF737348 (Carrot, Spain, 16S rDNA).

324 ## DISCUSSION

325

326 Species of '*Ca*. Liberibacter' are emerging plant pathogens associated with
327 economically important diseases (5, 23) but their causes are still not clear. Although,
328 '*Ca.* L. solanacearum' is only pathogenic to *Solanaceae* (18), it has also been
329 associated with diseases in other botanical families such as *Apiaceae* in different
330 geographical areas (23). The emergence of the disease in celery crops may be linked
331 to the proximity of infected carrots with '*Ca.* L. solanacearum', to the presence of *B.*
332 *trigonica* populations (1, 2, 41) and probably to the existence of other psyllid vector
333 species. Infection of carrots with '*Ca.* L. solanacearum' in different geographical
334 areas (i.e. Finland, France, Spain, and Sweden) suggests that carrot seeds might be the
335 first source of inoculum in these countries, despite seed transmission of '*Ca.* L.
336 solanacearum' has not been reported yet (2). The bacterium '*Candidatus* L.
337 solanacearum' was consistently detected in symptomatic celery plants using
338 conventional PCR and confirmed by sequencing. Phytoplasmas belonging to Aster

339 yellows and Stolbur groups and *Celery mosaic virus* (11) were sporadically detected
340 (data not shown).

341 Liberibacters such as '*Ca.* L. americanus' and '*Ca.* L. solanacearum' have been
342 described to be heat sensitive (21, 27); thus temperature may be an important factor in
343 the epidemiology of the disease. During the study period, the prevalence of symptoms
344 in celery decreased markedly from >30% plants in 2008-2010 to <1% plants in 2011-
345 2012. These figures corresponded to a decrease in '*Ca.* L. solanacearum' detection
346 from 62-50% in 2009-2010 to 9-0.5% in 2011-2012. The summer of 2008 was the
347 coldest within the 2008-2012 period, showing only a few peaks above 30ºC. These
348 temperatures probably favored the multiplication of '*Ca.* L. solanacearum' in plants
349 as well as in psyllid vectors. In the following years, temperatures above 30ºC were
350 frequent. These high temperatures may have resulted in a decrease in the prevalence
351 of the bacterium. The fact that '*Ca.* L. solanacearum' was detected in greenhouse
352 plants grown at 15-25ºC but not in the same plants after one month at about 30ºC
353 (data not shown), provides additional evidence that the bacterium is heat sensitive.

354 The designed TaqMan probe resulted specific to '*Ca.* L. solanacearum'. The
355 new real-time PCR protocol and the one previously described (16) showed similar
356 sensitivity; being higher than the most sensitive conventional PCR protocol (33).
357 Using DNA extraction, the detection level was the 10^{-4} dilution of the celery extract.
358 Detection levels decreased to 10^{-2} dilution when spots of crude plant extracts were
359 used as template.

360 The use of friendly direct methods of sample preparation prior real-time PCR is
361 highly recommended for large-scale use (9). In this case, we used a spot procedure as
362 in other validated methods (4, 6, 32, 40) for '*Ca.* L. solanacearum' detection. The
363 sensitivity and specificity of the conventional preparation of celery samples based on

DNA extraction was equal to 1.0 and it was considered as a gold standard, independent from the false positives inherent to high sensitivity (22). Nevertheless, spot's sensitivity (0.60) and specificity (0.99) guarantee a low number of false positives. The calculated Cohen's kappa coefficient and the BAK index showed a moderate concordance between methods as defined by Landis and Koch (15). The agreement between spotted samples and the gold standard, the simplicity in the preparation and the considerably lower cost compared with DNA purification, confirm the appropriateness of the spot method for detection of '*Ca*. L. solanacearum' and for large-scale screening purposes.

The efficacy of a commercially available complete kit was demonstrated through intra-laboratory performance studies, showing an excellent sensitivity and specificity. The recommended kit's real-time protocol differs from the protocol that uses fresh master mix in four degrees (64ºC instead of 60ºC) for the amplification reaction. In fact, in previous assays lyophilization was shown to alter the specificity of the TaqMan probes.

In carrot plants, mixed infections of '*Ca.* L. solanacearum' and phytoplasmas are frequently detected (1) and it was not possible to associate either pathogen with the symptoms observed. In celery plants mixed infections were less frequent and in multinomial logistic regression analysis the presence of '*Ca.* L. solanacearum' was the only significant independent variable. The presence or absence of phytoplasmas not affected the regression analyses. In (14%) of the samples showing severe symptoms (+++), '*Ca.* L. solanacearum' was not detected either by real-time PCR or by conventional PCR. These results were not considered as false positive because visual inspection of symptoms is not specific. In fact, phytoplasmas, that cause similar symptoms, were detected in these plants. In addition, only typical '*Ca*. Liberibacter'-

like cells with an electron dense layer cell wall with a triple-layered ultrastructure (5) were observed in microscopic examinations by TEM and SEM (Fig. 2).

Several haplotypes have been described in '*Ca*. L. solanacearum'. Haplotype A has been found from Central to North America whereas haplotype B has been found in Mexico, North America and New Zealand. Both haplotypes are present in solanaceous crops and are transmitted by the vector *B. cockerelli* (30). Haplotype C is present in Finland and was first described in carrot in association with the carrot psyllid *T. apicalis* (30). Haplotype D was described in mainland Spain and the Canary Islands, associated with carrot and *B. trigonica* (23, 31). The new haplotype E, was characterized by five nucleotide changes in 16S rDNA, seven in the ISR-23S and five in the 50S rDNA regions. The spatial and temporal coexistence of the same haplotypes in carrot and celery suggests the natural transmission between both plant species.

B. cockerelli, the vector of the zebra chip disease, has never been detected in Spain. Haplotypes D and E detected in carrot and celery plants could be associated with their natural spread by *B. trigonica* and maybe by other psyllid species. The almost permanent presence of '*Ca*. L. solanacearum' along the year in carrot and celery crops, even in symptomless plants, could also contribute to the spread of the bacterium.

In conclusion, we have demonstrated that the bacterium '*Ca*. L. solanacearum' is directly associated with the vegetative disorders found in celery in Spain. The developed real-time PCR detection system and the newly discovered haplotype of this bacterium will allow increasing the knowledge on the biology and epidemiology of '*Ca*. L. solanacearum' and developing appropriate control strategies.

414 **ACKNOWLEDGMENTS**

415

416 This work was supported by grants from Generalidad Valenciana,
417 MAGRAMA (National Reference Laboratories), INIA (RTA2011-00142), Agrícola
418 Villena Coop. V. from Spain and FP7-ERANET EUPHRESCO (266505/PHYLIB).
419 We thank Prof. J.M. Bové for the kind supply of the '*Ca*. Liberibacter' isolates
420 maintained at INRA-Bordeaux-France, M. Colomer from Plant Print Diagnòstics S.L.
421 for preparation of kit prototypes, Dr. C. Jeffries and V. Mulholland from SASA,
422 Edinburgh-UK and Dr. M. Cambra-López for the critical reading of the manuscript
423 and excellent suggestions and to P. Bartolomé from Agrícola Villena Coop. V. for
424 assistance in sample collection.. Dr. E. Bertolini is recipient of an INIA-CCAA 2011-
425 2016 contract from Ministerio de Ciencia e Innovación, Spain and G.R. Teresani is
426 recipient of PhD grant 2010-2014 from Coordenação de Aperfeiçoamento de Pessoal
427 de Nível Superior (CAPES), Ministério da Educação, Brazil.

428

LITERATURE CITED

1. Alfaro-Fernández, A., Cebrián, M.C., Villaescusa, F.J., Hermoso de Mendoza, A., Ferrándiz, J.C., Sanjuán, S., and Font, M.I. 2012b. First report of '*Candidatus* Liberibacter solanacearum' in carrots in mainland Spain. Plant Dis. 96: 582.
2. Alfaro-Fernández, A., Siverio, F., Cebrián, M.C., Villaescusa, F.J., and Font, M.I. 2012a. '*Candidatus* Liberibacter solanacearum' associated with *Bactericera trigonica*-affected carrots in the Canary Islands. Plant Dis. 96: 581.
3. Bertolini, E., Cambra, M., Serra, P., López, M.M., Lopes, S., Duran-Vila, N., Ayres, J., and Bové, J.M. September 2010. Direct procedures for specific detection of '*Candidatus* Liberibacter' spp. using immobilized targets and real-time PCR and detection kit. Spanish patent 201001157.
4. Bertolini, E., Moreno, A., Capote, N., Olmos, A., De Luis, A., Vidal, E., Pérez-Panadés, J., and Cambra, M. 2008. Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real-time PCR. Eur. J. Plant Pathol. 120: 177-188.
5. Bové, J.M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. Plant Pathol. 88: 7-37.
6. Byrt, T., Bishop, J., Carlin, J.B. 1993. Bias, prevalence and kappa. Journal of Clinical Epidemiology 46, 423-429.
7. Capote, N., Bertolini, E., Olmos, A., Vidal, E., Martinez, M.C., and Cambra, M. 2009. Direct sample preparation methods for the detection of *Plum pox virus* by real-time RT-PCR. Int. Microbiol. 12: 1-6.
8. Crosslin, J.M., Munyaneza, J.E., Brown, J.K., and Liefting, L.W. 2010. Potato zebra chip disease: a phytopathological tale. Online. Plant Health Progress.

454 doi:10.1094/PHP-2010-0317-01-RV.

455 9. De Boer, S.H., and López, M.M. 2012. New grower-friendly methods for plant
456 pathogen monitoring. Annu. Rev. Phytopathol. 50: 197-218.

457

458 10. EPPO. 2013. Data sheets on pests recommended for regulation. *'Candidatus*
459 Liberibacter solanacearum'. Bulletin OEPP/EPPO Bulletin 43: 197-201.

460 11. Font, M.I., Cebrián, M.C., Villaescusa, F.J., Córdoba, M.C., Ferrándiz, J.C.,
461 Sanjuán, S., Alfaro-Fernández, A., Hermoso de Mendoza, A., and Jordá, C. 2010.
462 First report of phytoplasmas disease in celery and parsnip in Spain. Petria 20:
463 260-261.

464 12. Hansen, A.K., Trumble, J.T., Stouthamer, R., and Paine, T.D. 2008. A new
465 huanglongbing species, "*Candidatus* Liberibacter psyllaurous," found to infect
466 tomato and potato, is vectored by the psyllid *Bactericera cockerelli* (Sulc). Appl.
467 Environ. Microbiol. 74: 5862–5865.

468 13. Hren, M., Boben, J., Rotter, A., Kralj, P., Gruden, K., and Ravnikar, M. 2007.
469 Real-time PCR detection systems for Flavescence dorée and Bois noir
470 phytoplasmas in grapevine: comparison with conventional PCR detection and
471 application in diagnostics. Plant Pathol. 56: 785–796.

472 14. Kumar, S., Tamura, K., and Nei, M. 2004. MEGA3: Integrated software for
473 molecular evolutionary genetics analysis and sequence alignment. Brief.
474 Bioinform. 5: 150-163.

475 15. Landis, J.R., and Koch, G.G. 1977. The measurement of observer agreement for
476 categorical data. Biometrics 33: 159-174.

477 16. Li, W., Abad, J.A., French-Monar, R.D., Rascoe, J., Wen, A., Gudmestad, N.C.,
478 Secor, G.A., Lee, I.M., Duan, Y., and Levy, L. 2009. Multiplex real-time PCR for

479 detection, identification and quantification of '*Candidatus* Liberibacter
480 solanacearum' in potato plants with zebra chip. J. Microbiol. Methods 78: 59-65.
481 17. Liefting, L.W., Sutherland, P.W., Ward, L.I., Paice, K.L., Weir, B.S., and Clover,
482 G.R.G. 2009a. A new "*Candidatus* Liberibacter" species associated with diseases
483 of solanaceous crops. Plant Dis. 93: 208-214.
484 18. Liefting, L.W., Weir, B.S., Pennycook, S.R., and Clover, G.R.G. 2009b.
485 "*Candidatus* Liberibacter solanacearum", a liberibacter associated with plants in
486 the family Solanaceae. Int. J. Syst. Evol. Microbiol. 59: 2274-2276.
487 19. Lin, H., Doddapaneni, H., Munyaneza, J.E., Civerolo, E., Sengoda, V.G.,
488 Buchman, J.L., and Stenger, D.C. 2009. Molecular characterization and
489 phylogenetic analyses of 16S rRNA from a new species of "*Candidatus*
490 Liberibacter" associated with zebra chip disease of potato (*Solanum tuberosum*
491 L.) and the potato psyllid (*Bactericera cockerelli* Sulc). J. Plant Pathol. 91: 215-
492 219.
493 20. Lin, H., Glynn, J., Islam, M.S., Wen, A., and Gudmestad, N.C. 2011a. Multilocus
494 sequencing typing markers for genotyping and population genetic analysis of
495 '*Candidatus* Liberibacter solanacearum', p. 32-35. *In* Workneh F, Rashed A,
496 Rush CM (ed), Proc. 11th Annual Zebra Chip Reporting Session, San Antonio,
497 TX.
498 21. Lopes, S.A., Frare, G.F., Bertolini, E., Cambra, M., Fernandez, N.G., Ayres, A.J.,
499 Marin, D.R., and Bové, J.M. 2009. Liberibacters associated with citrus
500 huanglongbing in Brazil: '*Candidatus* Liberibacter asiaticus' is heat tolerant,
501 '*Candidatus* Liberibacter americanus' is heat sensitive. Plant Dis. 93: 257-262.

502 22. López, M.M., Llop, P., Olmos, A., Marco-Noales, E., Cambra, M., and Bertolini,
503 E. 2009. Are molecular tools solving the challenges posed by detection of plant
504 pathogenic bacteria and viruses? Curr. Issues Mol. Biol. 11: 13-46.
505 23. Munyaneza, J.E. 2012. Zebra chip disease of potato: biology, epidemiology and
506 management. Am. J. Pot. Res. 89: 329-350.
507 24. Munyaneza, J.E., Crosslin, J.M., and Upton, J.E. 2007. Association of
508 *Bactericera cockerelli* (Homoptera:Psyllidae) with "zebra chip", a new potato
509 disease in southwestern United States and Mexico. J. Econ. Entomol. 100: 656-
510 663.
511 25. Munyaneza, J.E., Fisher, T.W., Sengoda, V.G., and Garczynski, S.F. 2010a. First
512 report of "*Candidatus* Liberibacter solanacearum" associated with psyllid-
513 affected carrots in Europe. Plant Dis. 94: 639.
514 26. Munyaneza, J.E., Fisher, T.W., Sengoda, V.G., Garczynski, S.F., Nissinen, A.,
515 and Lemmetty, A. 2010b. Association of "*Candidatus* Liberibacter
516 solanacearum" with the psyllid *Trioza apicalis* (hemiptera:Triozidae) in Europe.
517 J. Econ. Entomol. 103: 1060-1070.
518 27. Munyaneza, J.E., Sengoda, V.G., Buchman, J.L., and Fisher, T.W. 2012. Effects
519 of temperature on "*Candidatus* Liberibacter solanacearum" and zebra chip potato
520 disease symptom development. Plant Dis. 96: 18-23.
521 28. Munyaneza, J.E., Sengoda, V.G., Crossling, J.M., Rosa-Lozano, G., and Sanchez,
522 A. 2009. First report of '*Candidatus* Liberibacter psyllaurous' in potato tubers
523 with zebra chip disease in Mexico. Plant Dis. 93: 552.
524 29. Murray, M.G., and Thompson, W.F. 1980. Rapid isolation of high molecular
525 weight plant DNA. Nucleic Acids Res. 8: 4321-4325.
526 30. Nelson, W.R., Fisher, T.W., and Munyaneza, J.E. 2011. Haplotypes of

527 "*Candidatus* Liberibacter solanacearum" suggest long-standing separation. Eur. J.
528 Plant Pathol. 130: 5-12.
529 31. Nelson, W.R., Sengoda, V.G., Alfaro-Fernández, A., Font, M.I., Crossling, J.M.,
530 and Munyaneza, J.E. 2012. A new haplotype of "*Candidatus* Liberibacter
531 solanacearum" identified in the Mediterranean region. 135: 633-639.
532 32. Olmos, A., Dasi, M.A., Candresse, T., and Cambra, M. 1996. Print-capture PCR:
533 a simple and highly sensitive method for the detection of *Plum pox virus* (PPV) in
534 plant tissues. Nucleic Acids Res. 24: 2192-2193.
535 33. Ravindran, A., Levy, J., Pierson, E., and Gross, D.C**.** 2011a. Development of
536 primers for improved PCR detection of the potato zebra chip pathogen,
537 "*Candidatus* Liberibacter solanacearum". Plant Dis. 95: 1542-1546.
538 34. Ravindran, A., Levy, J., Pierson, E., and Gross, D.C. 2011b. LAMP PCR lights
539 the way for a simple, fast method for detection of Lso in infected potatoes and
540 psyllids, p. 79–82. *In* Workneh F, Rashed A, Rush CM (ed), Proc. 11th Annual
541 Zebra Chip Reporting Session, San Antonio, TX.
542 35. Reynolds, E.S. 1963. Use of lead citrate at high pH as an electronopaque stain in
543 electron microscopy. J. Cell Biol. 17: 208-213.
544 36. Schneider, R.W**.** 2013. Diseases of celery (*Apium graveolens* L. var. *dulce* (Mill.)
545 Pers.). APS Press. St. Paul, MN. USA.
546 37. Scott, I., Berry, N., Walker, G., Pitman, A., Workman, P., and Wright, P. 2009.
547 Psyllid, Liberibacter, and phytoplasma science research programme update.
548 Horticulture New Zealand, Plant and Food Research. doi:10.1094/PHP-2010-
549 0317-01-RV.
550 38. Secor, G.A., Rivera-Varas, V., Abad, J.A., Lee, I.M., Clover, G.R.G, Liefting,
551 L.W., Li, X., and De Boer, S.H. 2009. Association of '*Candidatus* Liberibacter

552 solanacearum' with zebra chip disease of potato established by graft and psyllid
553 transmission, electron microscopy, and PCR. Plant Dis. 93: 574-583.
554 39. Tanaka, F.A.O., Coletta-Filho, H.D., Alves, K.C.S., Spinelli, M.O., Machado,
555 M.A., and Kitajima, E.W. 2006. Detection of the "*Candidatus* Liberibacter
556 americanus" in phloem vessels of experimentally infected *Cataranthus roseus* by
557 scanning electron microscopy. Fitopatol. Bras. 32: 519.
558 40. Vidal, E., Yokomi, R,K., Moreno, A., Bertolini, E., and Cambra, M. 2012.
559 Calculation of diagnostic parameters of advanced serological and molecular
560 tissue-print methods for detection of *Citrus tristeza virus*: a model for other plan
561 pathogens. Phytopathology 102: 114-121.
562 41. Villaescusa, F.J., Sanjuán, S., Cebrián, M.C., Alfaro-Fernández, A., Font, M.I.,
563 Ferrándiz, J.C., and Hermoso de Mendoza, A. 2011. Prospección de posible
564 vectores (Hemiptera:Cicadellidae, Aphididae y Psylloideae) de patógenos en apio
565 y zanahoria. Bol. San. Veg. Plagas. 37: 163-171.
566 42. Wen, A., Lin, H., and Gudmestad, N.C. 2011. Development of PCR assay using
567 SSR primers for detection and genotyping of "*Candidatus* Liberibacter
568 solanacearum", p. 74-78. *In* Workneh F, Rashed A, Rush CM (ed), Proc.11th
569 Annual Zebra Chip Reporting Session, San Antonio, TX.
570

571 TABLE 1. '*Candidatus* Liberibacter solanacearum' samples, reference '*Ca.* Liberibacter' species, other bacterial strains that affect solanaceous
572 plants and microbiota from celery, potato and carrot used in specificity assays.
573

Bacterial isolates and strains	**Host – year**	**Number**	**Origin (reference)**	**Real-time PCR CaLsppF/CaLsppR**
'*Candidatus* Liberibacter solanacearum'	*Apium graveolens – 2010*	10	Villena-Spain (IVIA[a]-A10.1-10)	+
'*Ca.* Liberibacter solanacearum'	*A. graveolens – 2011*	10	Villena-Spain (IVIA[a]-A11.1-10)	+
'*Ca.* Liberibacter solanacearum'	*A. graveolens – 2012*	15	Villena-Spain (IVIA[a]-A12.1-15)	+
'*Ca.* Liberibacter solanacearum'	*Bactericera trigonica – 2010*	10	Canary Islands-Spain (ICIA[b]-Bt10.1-10)	+
'*Ca.* Liberibacter solanacearum'	*B. trigonica – 2011*	10	Canary Islands-Spain (IVIA[a]-Bt11.1-10)	+
'*Ca.* Liberibacter solanacearum'	*B. nigricornis – 2012*	10	La Rioja-Spain (IVIA[a]-Bn 12.1-10)	+
'*Ca.* Liberibacter solanacearum'	*Daucus carota – 2010*	15	Villena-Spain (IVIA[a]-Z10.1-15)	+
'*Ca.* Liberibacter solanacearum'	*D. carota – 2010*	10	Canary Islands-Spain (ICIA-Z10.1-10)	+
'*Ca.* Liberibacter solanacearum'	*D. carota – 2011*	10	Villena-Spain (IVIA[a]-Z11.1-10)	+
'*Ca.* Liberibacter solanacearum'	*D. carota – 2012*	10	Canary Islands-Spain (ICIA[b]-Z12.1-10)	+
'Ca. Liberibacter americanus'	*Catharanthus roseus*	1	Brazil*	-
'Ca. Liberibacter americanus'	*Citrus* sp.	1	Brazil*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Brazil	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	China*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	Philippines*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Philippines (PH 70)*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	Florida-USA*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	India (Poona 70)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	India (Poona 70)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Indonesia-Bali*	-

'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Malaysia (95-1)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Mauritius Island (95-11)*	-
'Ca. Liberibacter africanus'	*Citrus* sp.	1	Reunion Island-France (USA-7)*	-
'Ca. Liberibacter africanus'	*C. roseus*	1	South Africa (AFS 84)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Taiwan (LK 70)*	-
'Ca. Liberibacter asiaticus'	*C. roseus*	1	Thailand (TH 88)*	-
'Ca. Liberibacter asiaticus'	*Citrus* sp.	1	Vietnam (95-35)*	-
Agrobacterium tumefaciens	*Prunus* sp.	1	USA (C 58)	-
Clavibacter michiganensis subsp. *michiganensis*	*Solanum lycopersicum*	1	Spain (IVIA 873-6)	-
C. michiganensis subsp. *sepedonicus*	*S. tuberosum*	1	USA (NCPPB 2140)	-
Dickeya sp.	*S. tuberosum*	1	Spain (IVIA[a] 1374-13)	-
Dickeya sp.	*S. tuberosum*	1	Spain (IVIA[a] 2688-1-2)	-
Pectobacterium atrosepticum	*S. tuberosum*	1	United Kingdom (SCRI 1001)	-
Pectobacterium carotovorum	*S. tuberosum*	1	United Kingdom (SCRI 194)	-
Pseudomonas cichorii	*Lactuca sativa*	1	Spain (IVIA[a] 593)	-
Pseudomonas corrugata	*S. lycopersicum*	1	UK (NCPPB 2445)	-
Pseudomonas mediterranea	*S. lycopersicum*	1	Spain (IVIA[a] 592-4-4)	-
Pseudomonas syringae pv. *syringae*	*Pyrus communis*	1	Spain (IVIA[a] 773-1)	-
Pseudomonas syringae pv. *tomato*	*S. lycopersicum*	1	Spain (IVIA[a] 1001-1a)	-
Ralstonia solanacearum	*S. tuberosum*	1	Spain (IVIA[a] 1602)	-
Rhodococcus fascians	*Dahlia sp.*	1	France (CFBP 41)	-
Rhodococcus sp.	*Nicotiana tabaccum*	1	Spain (IVIA[a] 4264)	-
Xanthomonas vesicatoria	*S. lycopersicum*	1	Spain (IVIA[a] 3617)	-
Microbiota strains	*S. tuberosum*	50	Spain (IVIA[a] 1F to 50F)	-
Microbiota strains	*D. carota*	21	Spain (IVIA[a] 3925-1 to 3925-21)	-
Microbiota strains	*A. graveolens*	10	Spain (IVIA[a] AM-1 to AM-10)	-

574 *From INRA-Bordeaux, France, provided by Prof. J.M. Bové

575 [a)] IVIA - Instituto Valenciano de Investigaciones Agrarias

576 [b)] ICIA - Instituto Canário de Investigaciones Agrarias

577 [c)] NCPPB - National Collection of Plant Pathogenic Bacteria

578 [d)] SCRI - Scottish Crop Research Institute

579

580

581

582

583

584

585

586

587

588

589

590 TABLE 2. Comparison of the sensitivity of conventional and real-time PCR-based protocols using purified DNA and the spot procedure.

	PCR protocol	DNA extracted from crude extract serial dilutions							Direct spot from crude extract serial dilutions						
		1	**10**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**1**	**10**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
Conventional	Liefting *et al.*, 2009	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	Li *et al.*, 2009 (Lsof/OI2c)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ravindran *et al.* 2011(adk)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ravindran *et al.2011* (TX)	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Real-time	Li *et al.*, 2009 (Lsof/HLBr)	+(22,8)*	+(25,7)*	+(28,4)*	+(33,7)*	+(34,5)*	-	-	+(28,8)*	+(32,5)*	+(34,5)*	-	-	-	-
	This paper	+(23,6)*	+(26,5)*	+(28,3)*	+(31,6)*	+(32,5)*	-	-	+(28,8)*	+(28,2)*	+(34,0)*	-	-	-	-

591 * Positive sample (Ct)

592 TABLE 3. Association of symptom intensity in celery plants grown in experimental
593 plots and detection of '*Ca.* Liberibacter solanacearum' and/or phytoplasmas by real-
594 time PCR

Symptoms	Real-time PCR			
	'*Ca.* L. solanacearum'		Phytoplasmas	
	Positive	Negative	Positive	Negative
+++[a]	150	24	28	146
+[b]	63	87	25	125
-[c]	38	140	26	152
Total	251	251	79	365

595 [a] Celery plants with severe symptoms (unmarketable)

596 [b] Celery plants with mild symptoms (marketable)

597 [c] Celery plants with no symptoms

598

599 TABLE 4**.** SNP differences between haplotypes. The reference sequence for the 16S
600 and 23S rRNA genes is EU 812559.1 and for 50S rRNA gene is EU 834131.1.
601 Nucleotide numbers count from the beginning of the reference sequence, haplotypes
602 A, B, C and D as previously described by Nelson *et al*. (32)

Description	**Gene region**	**Haplotypes**				
		A	**B**	**C**	**D**	**E**
116 C>T	**16S rRNA**	C	C	C	T	C
151 A>G		A	A	A	A	G
212 T>G		T	C	T	T	T
581 T>C		T	C	T	T	T
959 C>T		C	C	C	C	T
1039 A>G		A	A	G	G	A
1073 G>A		G	G	G	A	G
1620 A>G	**ISR-23S**	A	A	A	A	G
1632 G>A		A	A	A	A	G
1648 G>A		G	G	G	G	A
1742 A>G		A	A	A	G	A
1748 C>T		C	C	C	T	C
1858_1859insG		-	G	G	-	-
1859_1860insT		-	T	-	-	-
1860_1861delT		T	T	T	-	T
1873 A>G		A	A	A	A	G
1920 T>C		T	T	C	T	T
583 G>C	**50S rRNA**	G	G	C	G	G
622 A>G		A	A	A	G	A
640 C>T		C	C	T	C	C
669 G>C		G	C	G	G	G
689 C>T		C	C	C	T	T
691 G>T		G	T	T	G	G
700 A>G		A	A	A	G	A
712 G>T		G	T	G	G	G
722 G>A		G	G	G	G	A
749 C>A		C	C	C	A	C
780_781insA		-	-	A	A	A
785 G>A		G	A	G	G	G
849 T>C		T	T	T	C	C
909 T>C		T	C	C	C	C
920 T>C		T	C	C	C	T
922_923insTGT		-	-	TGT	-	-
955 G>T		G	G	T	G	G
987 T>G		T	G	G	G	G
993 A>G		A	A	G	A	A
1041 G>A		G	A	A	G	G
1049 A>G		A	G	A	A	A
1068 C>T		C	C	C	C	C
1107 G>A		G	A	G	G	G
1110_1111insC		-	-	C	-	-
1122 G>A		G	A	A	A	A
1143 G>A		G	A	G	G	G

603

FIGURE CAPTIONS

FIG. 1. Severe symptoms in celery plants associated with '*Candidatus* Liberibacter solanacearum'. Abnormal amount of shoots and curling of stems (left) compared with a symptomless plant (right) (A). Proliferation, abnormal amount of shoots (B). Curling of stems (left) compared with a normal stem (right) (C). Mild symptoms in marketable plants (D).

FIG. 2. Scanning (SEM) and transmission (TEM) electron microscopy photomicrographs of celery tissue samples. Presence of bacteria-like organisms (BLOs) (white arrows) in the phloem sieve tubes analyzed by SEM (A and B). TEM studies shows BLO (black arrows) individual cells pleomorphic in shape and surrounded by an electron dense cell wall separate from the cytoplasmic membrane, slightly rippled and wrinkled (C and D). Cells showing a triple-layered ultrastructure of both the outer cell wall membrane and the inner cytoplasmic membrane (between two black arrows) suggesting the presence of '*Ca*. Liberibacter'-like cells (D). CW-cell wall.

FIG. 3. Cladogram of '*Candidatus* Liberibacter solanacearum' haplotypes on the 16S rRNA gene segment (1011 bp) with '*Candidatus* Liberibacter asiaticus' as the outgroup using the UPGMA algorithm. GenBank accession number and origin are indicated.