Characterization of a Phytoplasma Associated with Cabbage Yellows in Iran

M. Salehi, Agricultural Research Center of Fars, Zarghan, Iran; and K. Izadpanah and M. Siampour, Department of Plant Protection, College of Agriculture, Shiraz University, Shiraz, Iran

ABSTRACT

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In 2001, a disease tentatively named Iranian cabbage yellows (ICY) was observed in cabbage fields of Zarghan (Fars Province, Iran). The major symptoms of the disease were yellowing, little leaves, plant stunting, opening of the head, and proliferation of the buds at the base of the stem into a witches'-broom. Among leafhoppers collected in cabbage fields, only *Circulifer haematocephus* transmitted the ICY agent. The disease agent was transmitted by the leafhopper from cabbage to cabbage, cauliflower, rape, and periwinkle, causing phytoplasma-type symptoms in these plants. Polymerase chain reaction (PCR) using phytoplasma-specific primer pair P1/P7 and nested PCR using P1/P7 and R16F2n/R16R2 primer pairs amplified products of expected size (1.8 and 1.2 kb, respectively) from symptomatic cabbage plants. Both restriction fragment length polymorphism (RFLP) of nested PCR products (1.2 kb) and phylogenetic analyses of 16S-23S rDNA spacer region sequence indicated that the ICY phytoplasma had the closest relationship to subgroup A members of the clover proliferation group, including beet leafhopper-transmitted virescence agent, '*Candidatus* Phytoplasma trifolii', Columbia Basin potato purple top phytoplasma, and vinca virescence phytoplasma. Cabbage is reported as a new natural host to the 16SrVI group of phytoplasmas.

Many vegetable crops are subject to infection by phytoplasmas of the aster yellows group (16SrI) (23,30). In cabbage, phytoplasma diseases have been reported from Europe (3,24,34) and the United States (19,21,22). Phytoplasmas belonging to the 16SrI-B group have been found associated with the disease. Several leafhopper species have been reported as vectors of these phytoplasmas (25,26). In the United States, the principal vectors of cabbage yellows phytoplasma are *Macrostelus fasciatus*, *M. quadrilineatus*, *Scaphytopius irroratus*, and *Ceratagallia abrupta* (5,19,23,37).

Cabbage (*Brassica oleracea* var. *capitata*) is an economically important vegetable crop in Iran. In 2001, a disease tentatively named Iranian cabbage yellows (ICY) seriously damaged cabbage crops in Zarghan, Fars Province. Up to 50% infection was observed in certain fields. The present study reports association of a phytoplasma with the ICY disease, vector identification, partial host range determination, and partial characterization of the

disease agent. A preliminary report on the occurrence of the disease has been published (27).

Differentiation of phytoplasmas is largely based on the analysis of 16S rDNA. However, the 16S-23S spacer region (SR) is also a reliable and convenient phylogenetic marker (18,32). Phylogenetic trees derived from SR sequences differentiated phytoplasmas into groups that were in complete agreement with the grouping previously established from full-length analysis of 16S rRNA gene sequences (9,12,15,18,31,33). The SR is less conserved than 16S rRNA gene and could have greater use in differentiation of closely related phytoplasmas than the full-length 16S rRNA gene (32). In the present work, sequence of the SR was used to determine the taxonomic position of the ICY phytoplasma.

MATERIALS AND METHODS

Source of the ICY phytoplasma. A naturally infected cabbage plant with typical yellows symptoms was selected in a severely infected cabbage field in Zarghan (25 km north of Shiraz) and used as the source of the ICY agent in various experiments. The ICY agent was maintained and propagated in both cabbage and a red line of periwinkle (*Catharanthus roseus*) by periodic inoculation using insect vector *Circulifer haematocephus*. Other samples of diseased or symptomless cabbage plants

were collected in Zarghan and used in the polymerase chain reaction (PCR) assay.

Vector search and host range studies. Insects were collected from a cabbage field showing symptoms in Zarghan using an insect collection net and then sorted by their gross morphology. Insects of each group were subjected to nested PCR to identify those carrying phytoplasma. The species positive for phytoplasma by PCR were tested for the ability to transmit the ICY agent. Fertilized females of species positive for phytoplasmas by PCR were individually transferred to infected or healthy cabbage plants for egg deposition and subsequent establishment of inoculative or noninoculative vector colonies. Colonies were regarded as noninoculative if they did not transmit the disease agent and were negative for phytoplasma by PCR. Young adults from inoculative colonies were transferred (10 insects per plant) to 1-month-old healthy cabbage plants. Alternatively, insects collected in infected cabbage fields were placed in groups of 20 in cages containing four pots, each pot with five healthy cabbage plants, to test their transmission ability immediately after field collection. In host range studies, 10 inoculative vector insects per plant were caged on healthy seedlings of alfalfa (*Medicago sativa*), cauliflower (*Brassica oleracea* var. *botrytis*), clover (*Trifolium repens*), periwinkle (*Catharanthus roseus*), rape (*Brassica napus*), and potato (*Solanum tuberosum*). In all experiments, 20 young plants of each species were tested. Inoculation access time was always 2 weeks, after which plants were sprayed with an insecticide and kept under observation in a separate insect-free greenhouse. For back-inoculation, noninoculative vector insects were caged on the tested plants for 10 days and transferred to healthy cabbage seedlings in groups of 10 for 4 weeks.

All plants used in this study were grown from seed, and plastic cages were used to maintain insects on test plants.

DNA extraction and PCR amplification. Total nucleic acid was extracted from half a gram of fresh midrib tissue of diseased or healthy cabbage plants using a phytoplasma enrichment procedure (1). Total nucleic acid was extracted from the leafhoppers using a modified hot cetyltrimethyl ammonium bromide (CTAB)

Corresponding author: K. Izadpanah
E-mail: izadpana@shirazu.ac.ir

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extraction method (8). Insects of each species were ground in batches of three in 1.5-ml Eppendorf tubes containing 600 µl of preheated (65°C) CTAB buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% PVP, 1% 2-mercaptoethanol) and incubated at 65°C for 20 min. Samples were extracted once with an equal volume of chloroform-isoamyl alcohol (24:1). The aqueous phase containing nucleic acid layer was precipitated overnight at -20°C with 0.6 volume of isopropanol. The pellets were washed with 70% ethanol, vacuum dried, suspended in 100 µl of sterile water, and stored at -20°C until use.

In direct PCR, phytoplasma universal primer pair P1/P7 (7,29) was used to amplify an 1,800-bp fragment of the ribosomal operon which includes the 16S rRNA gene, the 16S-23S intergenic spacer region (SR), and a portion of the 5' region of the 23S rRNA gene. To increase the sensitivity of assays, a nested PCR was performed using a 1:40 dilution of P1/P7 primed PCR products as templates and primer pair R16F 2n/R16R2 (11), which yields a 1,200-bp fragment of the 16S rRNA gene.

PCR was performed in 50-µl reaction mixtures containing 100 ng of total nucleic acid, 0.4 µM of each primer, 0.2 µM of each dNTP, and 1× polymerase buffer. The reaction mixtures were overlaid with 30 µl of mineral oil and cycled 35 times with the following parameters: denaturing for 1 min at 94°C (2 min for the first cycle), anneal-

ing for 2 min at 55°C, and primer extension for 3 min (10 min in the final cycle) at 72°C. PCR conditions for the second-round nested PCR were the same except that the annealing temperature was 58°C. Ten microliters of each PCR product were analyzed by electrophoresis in a 1% agarose gel in 1× TBE buffer (67 mM Tris-HCl, 22 mM boric acid, 10 mM EDTA, pH 0.8). DNA bands were stained with ethidium bromide and visualized with a UV transilluminator.

Restriction fragment length polymorphism (RFLP) analysis. For RFLP analysis, 8 µl of the nested PCR products amplified with primer pair R16F2n/R16R2 were digested with restriction enzymes *AluI*, *HaeIII*, *HhaI*, *HinfI*, *HpaII*, *MseI*, *RsaI*, *Sau3AI*, *SalI*, and *TaqI* according to the instructions of the manufacturer (Roche, Mannheim, Germany) at 37°C (65°C for *TaqI*) overnight. The digests were analyzed by electrophoresis through a 5% polyacrylamide gel followed by staining with ethidium bromide and visualization of DNA bands with UV transilluminator.

Cloning and sequencing. P1/P7 primed rDNA product from ICY phytoplasma was ligated onto pTZ57R/T vector and cloned into *Escherichia coli* DH5α cells using Inst/A clone PCR product Cloning Kit (Fermentas, Vilnius, Lithuania) according to manufacturer's instructions. Presence of the correct insert was confirmed by restriction endonuclease analysis using *EcoRI* and *PstI* enzymes. Plasmid DNA from cultures of recombinant colonies was puri-

fied using a High Pure Isolation Kit (Roche). Sequencing was performed by SEQ LAB (Goettingen, Germany). M13 forward and reverse primers were used as sequencing primers. The 16S-23S rDNA SR was further analyzed by using primers P3 and P7 (29) as markers. A BLAST search was performed to determine the closest phytoplasma relatives of the ICY phytoplasma strain.

Sequence homology and phylogenetic analyses. The SR sequences of the ICY phytoplasma, 30 other phytoplasmas representing 15 phytoplasma groups (Table 1), and *Acholeplasma laidlawii* as an out-group were aligned using Clustal X 1.81 (IGBMC, Strasbourg, France). A phylogenetic tree was constructed using the neighbor-joining plot option of Clustal X. The SR sequence homology between strains was evaluated after alignments were generated by using homology matrix distance option of DNAMAN program version 4.02 (Lynon Corporation, Quebec, Canada).

RESULTS AND DISCUSSION

Vector identification. Five leafhopper species including *Balclutha saltirella*, *Empoasca decipiens*, *Psammotettix striatus*, *Circulifer haematoceps*, and *Orosius albicinctus* were collected in a yellows-affected cabbage field. Ten samples of each species were assayed for the presence of phytoplasma by nested PCR, using P1/P7 followed by R16F2n/R16R2 primer pair. Six samples of *C. haematoceps* tested

Table 1. Phytoplasmas examined in this study with group designations and GenBank accession numbers of 16S-23S rDNA spacer region sequences

Organism	16S rRNA group/subgroup	GenBank accession no.
Aconitum proliferation (AcP)	16SrI-A	AF510323
Almond witches'-broom (AlmWB) (' <i>Candidatus</i> Phytoplasma phoenicium')	16SrIX-B	AF515636
Apple proliferation (AP) (' <i>Candidatus</i> Phytoplasma mali')	16SrX-C	AJ542541
Ash yellows (AY) (' <i>Candidatus</i> Phytoplasma fraxini')	16SrVII-A	AF092209
Aster yellows (SAY)	16SrI-B	AF222063
Beet leafhopper-transmitted virescence agent (BLTVA)	16SrVI-A	U54987
Bermuda grass white leaf isolate C1 (BGWL-C1)	16SrXIV-A	AJ550984
Bermuda grass white leaf isolate C2 (BGWL-C2)	16SrXIV-A	AJ550985
Brinjal little leaf (BLL)	16SrVI-C	X83431
Iranian cabbage yellows (ICY)	16SrVI	DQ195214
Columbia Basin potato purple top (CBPPT)	16SrVI	AY692279
Clover proliferation (CP) (' <i>Candidatus</i> Phytoplasma trifolii')	16SrVI-A	AY390261
Clover yellow edge (CYE)	16SrIII-B	AF175304
Coconut lethal decline (CLD)	16SrIV-B	U18753
Coconut lethal yellows (LY)	16SrIV-A	AF024639
European stone fruit yellows (Germany) (ESFY)	16SrX-B	U54988
Faba bean phyllody (FBP)	16SrII-C	X83432
<i>Fragaria multicipita</i> multiplier disease (FM)	16SrVI-B	AF036354
Grapevine flavescence doree (FD)	16SrV-A	X76428
Hibiscus witches'-broom (HibWB) (' <i>Candidatus</i> Phytoplasma brasiliense')	16SrXV	AF147708
Lilac witches'-broom (LWB3)	16SrVII-A	AF105317
Lime witches'-broom (LWB) (' <i>Candidatus</i> Phytoplasma aurantifolia')	16SrII-B	U15442
Loofah witches'-broom (LFWB)	16SrVIII	L33764
Mexican periwinkle virescence (MPV)	16SrXIII-A	AF025428
Periwinkle little leaf (PLL)	16SrVI-C	AF228053
Pigeon pea witches'-broom (PPWB)	16SrIX-A	AF025427
Potato witches'-broom (PWB)	16SrVI	AY500818
Rice yellow dwarf (RYD) (' <i>Candidatus</i> Phytoplasma oryzae')	16SrXI	D12581
Stolbur (STOL)	16SrXII	X76427
Vinca virescence (VV)	16SrVI	AY500817
Western X-disease (California) (WX)	16SrIII-A	L04682
Ziziphus witches'-broom (ZWB) (' <i>Candidatus</i> Phytoplasma ziziphi')	16SrV	AY072722

positive (data not shown). Other insect species as well as *C. haematoceps* from noninoculative colonies tested negative. Each species was further evaluated as vectors using groups of 20 leafhoppers collected in the field and directly caged on healthy cabbage plants. Among these leafhoppers, only *C. haematoceps* was able to transmit the ICY phytoplasma to healthy cabbage, and 16 out of 20 cabbage plants exposed to this leafhopper developed symptoms of ICY. Similar results were obtained when noninoculative *C. haematoceps* leafhoppers fed first on yellows-affected cabbage plants were transferred to healthy cabbage seedlings.

Host range and symptomatology. Field symptoms of ICY consisted of plant yellowing, little leaves, plant stunting, opening of the head, and proliferation of buds at the base of the stem into a witches'-broom (Figs. 1 and 2). Similar symptoms were obtained under greenhouse conditions by vector inoculation of cabbage seedlings (Fig. 3).

Of the plant species tested, cauliflower, periwinkle, and rape were infected at the rate of 90, 70, and 80%, respectively, by the ICY phytoplasma using the leafhopper vector. In periwinkle, ICY phytoplasma caused floral virescence, phyllody, proliferation, small leaves, yellowing, terminal witches'-broom, and stunting. Proliferation of white heads into shoots with small leaves and shortened internodes were obvious symptoms of infection on cauliflower. Inoculation of rape resulted in severe witches'-broom through proliferation of crown buds and severe stunting (Fig. 4). The infection of cauliflower, periwinkle, and rape with ICY phytoplasma was confirmed by back-inoculation to cabbage. Under these conditions alfalfa, clover, and potato were not infected.

PCR amplification. With universal primer pair P1/P7, DNA fragments of approximately 1.8 kb were amplified from 12 of 15 diseased cabbage plants (data not shown). A 1.2-kb product was obtained from all 15 samples by nested PCR. PCR products were not obtained from asymptomatic control plants collected in the field (Fig. 5).

RFLP analysis. The R16F2n/R16R2-nested PCR product (1.2 kb) of ICY phytoplasma was subjected to RFLP analysis (Fig. 6). Comparison of the RFLP pattern with published RFLP patterns of other phytoplasmas (2,13,22,35,36) indicated that ICY phytoplasma was similar to members of clover proliferation (CP), 16SrVI, phytoplasma group. Based on *AluI*, *RsaI*, *HpaII*, *MseI*, *Sau3AI*, and *HhaI* analyses, the ICY phytoplasma was indistinguishable from several isolates of alfalfa witches'-broom, clover proliferation, and potato witches'-broom phytoplasmas, members of 16S rVI-A subgroup reported from Canada (19,33). Comparison of RFLP patterns of *AluI*, *HaeIII*, *HhaI*,

Fig. 1. Little leaf, chlorosis, and stunting in a yellows-affected cabbage field in Zarghan.

Fig. 2. Opening of the head and proliferation of basal stem buds in a cabbage plant naturally infected with phytoplasma (left) compared with a healthy cabbage plant (right).

Fig. 3. Proliferation of buds at the base of the stem into shoots with small yellow leaves, shortened internodes, and witches'-broom in two experimentally infected cabbage plants.

Fig. 4. Severe crown proliferation of rape experimentally infected with Iranian cabbage yellows phytoplasma (arrow).

Fig. 5. Electrophoresis pattern of 16S rDNA fragment (1.2 kb) amplified by nested polymerase chain reaction from 12 Iranian cabbage yellows-affected cabbage plants (lanes 1-12). H, healthy plant; M, DNA ladder.

Fig. 6. Restriction fragment length polymorphism (RFLP) profiles in 5% polyacrylamide gel of 16S rDNA amplified by nested polymerase chain reaction from the Iranian cabbage yellows phytoplasma. M, DNA ladder (fragment sizes from top to bottom: 1,114, 900, 692, 501, 489, 404, 320, 242, 190, 147, 124, 110 bp).

HinfI, *HpaII*, *MseI*, *RsaI*, *Sau3AI*, *TaqI*, and *ThaI* enzymes showed that the ICY phytoplasma and a clover proliferation phytoplasma (22) are distinguishable only by *Sau3AI*.

Sequence homology and phylogenetic analyses. A BLAST search showed that the SR sequence of ICY phytoplasma (Acc. no. DQ195214) shared closest homology with SR sequences from members of the clover proliferation, 16SrVI, group. Among members of CP group, ICY had maximum SR sequence homology (99.6%) with beet leafhopper transmitted virescence agent (BLTVA) (16SrVI-A), '*Candidatus* Phytoplasma trifolii', Columbia Basin potato purple top (CBPPT) phytoplasma, and vinca virescence (VV) phytoplasma and less homology (97.6%) with phytoplasma from *Fragaria multicapita* (16SrVI-B) (FM). SR sequence homology between ICY and other selected members of CP group including brinjal little leaf (BLL), periwinkle little leaf (PLL) (16SrVI-C), and potato witches'-broom (PWB) phytoplasmas was 98.7, 98.7, and 99.2%, respectively. In this analysis, percent homology of the ICY phytoplasma SR sequence with that of aster yellows (SAY), a non-16SrVI related phytoplasma, was 69.7%.

Phylogenetic analysis of SR sequences of 30 phytoplasma strains and *Acholeplasma laidlawii* as the outgroup yielded a tree (Fig. 7) whose branching order was in general agreement with previous findings (22,30). In this analysis, ICY clustered closely with other known members of the 16SrVI group. Tree branching patterns indicated that the ICY phytoplasma was evolutionarily closest to the BLTVA, '*Ca. Phytoplasma trifolii*', and PWB (16SrVI-A) phytoplasmas.

Disease symptoms, insect transmission, and a positive reaction in PCR showed that ICY in the Fars Province of Iran was of phytoplasmal etiology. On the basis of molecular analyses, this phytoplasma is related to the clover proliferation group, 16SrVI, and most closely related to BLTVA, '*Ca. Phytoplasma trifolii*', and PWB strains, the 16SrVI-A subgroup phytoplasmas (13), as well as to VV and CBPPT strains. ICY phytoplasma was not transmissible to alfalfa, clover, and potato, hosts of alfalfa witches'-broom agent in Canada, '*Ca. Phytoplasma trifolii*', and agents of CBPPT and PWB, respectively. This is supporting evidence that in spite of the similarity of RFLP patterns and more than 99% sequence homology, ICY phytoplasma is distinct from these phytoplasmas. In other countries, an aster yellows-related phytoplasma (16SrI-B) is associated with cabbage yellows (20,23), but no reports were found of cabbage as a host for the 16SrVI phytoplasma group. Previous studies have also indicated that different phytoplasmas can cause apparently identical symptoms in the same host. Examples

Fig. 7. Phylogram of 16S-23S rDNA spacer region (SR) sequences of 30 phytoplasmas and *Acholeplasma laidlawii* (D13260) as the outgroup and the position of Iranian cabbage yellows (ICY) phytoplasma (arrow). Bar, 1 nucleotide substitution per 10 nucleotides. See Table 1 for abbreviations.

are grapevine yellows, big bud of tomato, and disorders of periwinkle (20).

C. haematoceps has been previously reported as the vector of several plant pathogens including *Beet curly top virus* (14,17), *Spiroplasma citri* (4,16), and sesame phyllody phytoplasma (28). *C. haematoceps* is reported here for the first time as a natural vector of a clover proliferation-related phytoplasma. At least two members of 16SrVI group (BLTVA and CBPPT phytoplasmas) are transmitted by *C. tenellus* (6,10). *C. haematoceps* and the related species *C. tenellus* appear to behave similarly in relation to plant pathogens; therefore, transmission of ICY phytoplasma by *C. haematoceps* also confirms relatedness of this phytoplasma to the clover proliferation group and the close relationship with 16SrIV-A subgroup. In addition to periwinkle, cauliflower and rape are experimental hosts of the ICY phytoplasma. However, the extent of natural infection of cauliflower and rape by ICY phytoplasma remains to be determined.

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