Prevalence of *Tobacco mosaic virus* in Iran and Evolutionary Analyses of the Coat Protein Gene

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The incidence and distribution of *Tobacco mosaic virus* (TMV) and related tobamoviruses was determined using an enzyme-linked immunosorbent assay on 1,926 symptomatic horticultural crops and 107 asymptomatic weed samples collected from 78 highly infected fields in the major horticultural crop-producing areas in 17 provinces throughout Iran. The results were confirmed by host range studies and reverse transcription-polymerase chain reaction. The overall incidence of infection by these viruses in symptomatic plants was 11.3%. The coat protein (CP) gene sequences of a number of isolates were determined and disclosed to be a high identity (up to 100%) among the Iranian isolates. Phylogenetic analysis of all known TMV CP genes showed three clades on the basis of nucleotide sequences with all Iranian isolates distinctly clustered in clade II. Analysis using the complete CP amino acid sequence showed one clade with two subgroups, IA and IB, with Iranian isolates in both subgroups. The nucleotide diversity within each subgroup was very low, but higher between the two clades. No correlation was found between genetic distance and geographical origin or host species of isolation. Statistical analyses suggested a negative selection and demonstrated the occurrence of gene flow from the isolates in other clades to the Iranian population.

**Keywords**: coat protein, gene flow, phylogeny, TMV, Tobamovirus

Iran, with an area of 1.65 million square kilometres, contains several different types of climate. Horticultural crop farming is carried out in most of the geographical regions in Iran, except for the deserts and mountains. The climate of Iran is conducive to the growing of many horticultural crops such as broad beans (*Vicia faba* L.), carrots (*Daucus carota* subsp. *sativus*), varieties of cucurbit plants [including cantaloupe (*Cucumis melo* var. *cantalupensis*), courgettes (*Cucurbita pepo* L. cv. *Zucchini*), cucumber (*Cucumis sativus* L.), melon (*C. melo* L.), pumpkin (*Cucurbita moschata* Duch.), watermelon (*Citrullus lanatus* var. *lanatus*), and winter squash (*Cucurbita maxima* Duch. ex Lam)], eggplant (*Solanum melongena* L.), garlic (*Allium sativum* L.), green beans (*Phaseolus vulgaris* L.), onion (*Allium cepa* L.), pepper (*Capsicum annuum* L.), potato (*Solanum tuberosum* subsp. *tuberosum*), spinach (*Spinacia oleracea* L.) and tomato (*Solanum lycopersicum* L.). Such crop production in the field may be either rain-fed or under irrigation on large scale or small holder commercial farms. Iran is among the main producers of horticultural crops in the world with an approximate production of 1,800,400 tonnes yearly (FAOstat, 2009). Cucurbits and tomato are major horticultural crops in Iran (FAOstat, 2009). Viral diseases of horticultural crops cause important economic impact worldwide. Among all other viral diseases of such crops, diseases caused by tobamoviruses are often the most destructive and difficult to control (Alonso et al., 1989).

On the basis of genome organization and phylogenetic clustering, tobamoviruses are classified into three subgroups (Lartey et al., 1996). Tobamoviruses in subgroup 1, including *Tobacco mosaic virus* (TMV), *Tomato mosaic virus* (ToMV), *Pepper mild mottle virus* (PMMV) and *Tobacco mild green mosaic virus* (TMGMV), are known to infect mostly solanaceous plants. Several tobamoviruses that previously were considered as TMV strains, are now classified as members of new species of the genus *Tobamovirus* on the basis of differences in host range, serological properties and amino acid sequences of encoded virus proteins (Antignus et al., 2001; Wang et al., 1997). TMV is the type member of the genus *Tobamovirus*. The TMV virion is a rigid rod (18 nm × 300 nm) containing a single-stranded, positive-sense RNA (Zaitlin, 1999). TMV can be transmitted by mechanical inoculation, grafting, contact between plants and by seed, but not by any known vector (Broadbent, 1965; Hollings and Huttiniga, 1976). Tobamoviruses collectively have a very wide host range and cause...
serious economic impact and significantly yield losses in many crops such as brassicas, cucurbits, solanaceous crops and different ornamental plants such as chrysanthemums (Chrysanthemum indicum L.), impatients (Impatiens balsamina) and petunia (Petunia × hybrida) (Alexandre et al., 2000; Choi et al., 2009; Kumar et al., 2011; Nassar et al., 2012). Infected plants often show different types of symptoms such as malformations, mosaic and stunting on leaves, flowers, and fruit. TMV has been reported to cause considerable reduction in the tonnage of tomato fruit up to 59% (Cherian and Muniyappa, 1998). Significant yield losses of about 90% due to TMV infection in pepper also have been reported (Chitra et al., 2002). There are many different strains identified for TMV, some of which are capable of overcoming of their hosts resistance (Padgett and Beachy, 1993). Regardless of the importance of field-grown horticultural crops, there is little information available on the incidence and distribution of TMV and its genetic diversity in cultivated crops in Iran. Thus, in this study we determined the incidence of TMV in plants showing symptoms in commercial fields in the main important provinces for horticultural crop cultivation in Iran, and also determined the genetic diversity of the isolates detected in the surveyed regions. We also detected TMV in some weed species growing in and around crop fields which may be potential reservoir hosts for maintaining TMV in these fields. The information obtained is required as the first step toward the search for control strategies of virus diseases in horticultural crops in Iran.

**Materials and Methods**

**Surveys and sample collection.** Samples of horticultural crop species including bean, cabbage (Brassica oleracea var. capitata), cucumber, eggplant, pepper, potato, pumpkin, radish (Raphanus sativus), tomato, watermelon, and zucchini. were collected from April 2009 to May 2010 from open fields in the following 17 provinces (and specified districts) of Iran: Alburz (Karaj), Boushehr (Boushehr), East Azarbaijan (Maragheh), Fras (Darab, Fasa, Jahrum and Larestan), Golestan (Minoodasht), Hamedan (Tuyserkan), Hormozgan (Hajijabad, Kehorestan, Minab, Roudan), Isfahan (Isfahan), Kerman (Jiroft), Kermanshah (Mahidasht), Khuzestan (Dezful), Mazandaran (Noshahr), Qazvin (Qazvin), Qom (Qom), Sistan and Baluchestan (Zabol and Zahedan), Tehran (Tehran, Varamin, Shahriar), and West Azarbaijan (Urmia) (Table 1). Overall, 78 fields were surveyed and 1926 samples (20–30 samples per field) were collected during the growing season. The fields were randomly selected using a predetermined distance criterion, where distance between the fields ranged from 10 to 30 km. A sample consisted of two symptomatic leaves per plant. Viral-like symptoms observed included dwarfing, mosaic, mottling, yellowing, leaf malformation, necrosis of the leaves and stems and vein clearing. In addition, 107 leaf samples were also collected from different asymptomatic weed species belonging to 12 different botanical families within visited horticultural crop fields in the surveyed provinces for the serological detection of TMV and related tobamoviruses (Table 2). Weed samples were collected from around the bases of crops showing symptoms characteristic of infection by TMV.

**Virus testing.** Mature leaf blades were tested in duplicate using double antibody sandwich enzyme-linked immunosorbent assay (DAS–ELISA), with coating antibodies against TMV and related tobamoviruses, as well as alkaline phosphatase conjugates obtained from Agdia (Agdia, Inc., South Bend, IN, USA) (Clark and Adams, 1977).

**Host range.** Leaf samples showing symptoms associated with virus disease and with positive reaction in ELISA that were collected from Alburz, East Azarbaijan, Fars, Golestan and Tehran provinces, which contain the main horticultural crop cultivation regions of Iran, were selected and further evaluated in a host range study. The virus isolates were purified biologically through a single local lesion technique repeated twice on Chenopodium quinoa plants, and then transmitted mechanically to Nicotiana tabacum cv. White Burley for virus propagation according to the published protocol (Adkins et al., 2003). Extracts from the systemically-infected tobacco plants were then used as a source of biologically-purified virus and inoculated mechanically to C. quinoa (family Chenopodiaceae), C. sativus (cucumber; family Cucurbitaceae), Vigna unguiculata cv. Mashhad (cowpea) and Vicia faba (faba bean; family Fabaceae), N. rustica, N. tabacum cv Samsun nn, N. tabacum cv. Virginia, N. tabacum cv. White Burley and S. lycopersicum (tomato), Petunia hybrida (all in the family Solanaceae). (Table 3). Specifically, 1 g of leaf tissue was ground in 2 ml of 0.1 M potassium phosphate buffer, pH 7.5. The extract was rubbed onto leaves of the above herbaceous plants, previously dusted with 300-mesh Carborundum. The experiment was conducted twice using at least three plants for each inoculation. The inoculated plants were then transferred to a greenhouse and maintained at 20 to 30 °C with 12 to 14 h natural lighting for the duration of the test. The final symptom reading was conducted 4 weeks post inoculation, and systemic leaf tissues from each individual plant were collected to confirm the presence of virus by ELISA. The presence of the virus in asymptomatic plants was verified by reverse transcription-polymerase chain reaction (RT-PCR).

**RT-PCR and sequencing analyses.** Total RNA extraction