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Genetic diversity and population structure of beet necrotic yellow vein virus in China

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ABSTRACT

Beet necrotic yellow vein virus (BNYVV) is a serious threat to the sugar beet industry worldwide. However, little information is available regarding the genetic diversity and population structure of BNYVV in China. Here, we analyzed multiple sequences from four genomic regions (*CP*, RNA3, RNA4 and RNA5) of a set of Chinese isolates. Sequence analyses revealed that several isolates were mixed infections of variants with different genotypes and/or different p25 tetrad motifs. In total, 12 distinct p25 tetrads were found in the Chinese BNYVV population, of which four tetrads were newly identified. Phylogenetic analyses based on four genes (*CP*, RNA3-*p25*, RNA4-*p31* and RNA5-*p26*) in isolates from around the world revealed the existence of two to four groups, which mostly corresponded to previously reported phylogenetic groups. Two new subgroups and a new group were identified from the Chinese isolates in *p25* and *p26* trees, respectively. Selection pressure analysis indicated that there was a positive selection pressure on the p25 from the Chinese isolates, but the other three proteins were under a negative selection pressure. There was frequent gene flow between geographically distant populations, which meant that BNYVV populations from different provinces were not geographically differentiated.

1. Introduction

Rhizomania is a major worldwide viral disease of sugar beet (McGrann et al., 2009). In China, it was first reported in the 1970s and is now found in almost all of the sugar beet growing regions of the country (Li et al., 2008). Beet necrotic yellow vein virus (BNYVV) is the causal agent of the disease, which is transmitted by the soil-borne plasmodiophoromycete *Polymyxa betae* Keskin (*P. betae*) (Abe and Tamada, 1986).

BNYVV is a multipartite positive-sense RNA virus which consists of four to five RNA components (Tamada et al., 1989). RNA1 and RNA2 encode the genes responsible for replication, assembly, cell-to-cell movement, RNA silencing suppression and vector transmission. The RNA3-encoded p25 protein is the pathogenic determinant of the virus that controls disease symptoms in sugar beet roots (Tamada et al., 1999) and acts as an avirulence factor in the leaves of some resistant sugar beet lines (Chiba et al., 2008). The RNA4-encoded p31 protein is responsible for vector transmission and root-specific RNA silencing suppression (Rahim et al., 2007). The RNA5-encoding protein, p26, is thought to be an additional pathogenicity factor (Tamada et al., 1989).

BNYVV can be classified into four genotypes (Schirmer et al., 2005; Chiba et al., 2011). A and B types contain four RNAs, and the differences at amino acid positions 62, 103 and 172 of the CP gene allowed us to distinguish between A type ($T_{62}S_{103}L_{172}$) and B type ($S_{62}N_{103}F_{172}$) isolates (Miyanishi et al., 1999). The A type BNYVV has a worldwide distribution (Schirmer et al., 2005), whereas the B type seems to be limited to few areas in Europe (Koenig et al., 2008), Japan (Miyanishi et al., 1999) and China (Li et al., 2008). P and J types possess the fifth RNA (RNA5) and share about 96% sequence identity (Ward et al., 2007). J type isolates possess two deletions at amino acid positions 77 and 227–229 in p26, compared to the P type (Schirmer et al., 2005). P type BNYVV only occurs in France, the United Kingdom and Kazakhstan (Koenig et al., 1997; Koenig and Lennefors, 2000; Ward et al., 2007), and the J type mainly occurs in Japan, China and a single field in Germany (Chiba et al., 2011; Li et al., 2008; Koenig et al., 2008).

The control of rhizomania is exclusively dependent on the cultivation of resistant sugar beet cultivars. The single dominant

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resistant gene *Rz1* has been used in most of the commercial cultivars since its identification (Biancardi et al., 2002). However, resistance-breaking (RB) strains have been found in some *Rz1* cultivars planted areas of the United States and Europe (Schirmer et al., 2005; Liu et al., 2005; Liu and Lewellen, 2007). The amino acids at sites 67–70 of p25 (designated as a tetrad) have been found to be highly variable in worldwide BNYVV isolates (Li et al., 2008; Schirmer et al., 2005; Chiba et al., 2011; Koenig et al., 2008; Mehrvar et al., 2009). Such variations have a pronounced impact on pathogenicity and resistant responses to *Rz1*. For example, the p25 tetrad variations influence its oligomerization and isolate pathogenicity on *Tetragonia expansa* (Klein et al., 2007). The amino acid at site 68 of p25 is required for the induction of the resistant responses in leaves of *Beta vulgaris* plants (Chiba et al., 2008). V_{67} in p25 is the signature of the RB variants of BNYVV from the USA, whereas the wild type p25 contains A_{67} (Acosta-Leal and Rush, 2007). V_{67} in p25 promotes a much higher virus accumulation in the rootlets of mechanically inoculated, partially resistant sugar beet seedlings than A_{67} in p25 does after reverse genetic analysis (Koenig et al., 2009). RB variants containing P_{69} in p25 have been found in some European countries (Bornemann et al., 2014). Amino acids at positions 129, 135 and 179 of p25 are also thought to be associated with RB, as well as the tetrad motif (Acosta-Leal and Rush, 2007; Chiba et al., 2008; Koenig et al., 2009).

Transmission via *P. betae* enables the virus population to survive for decades in contaminated soils (Abe and Tamada, 1986), which provide ideal settings for the long-term evolution of the virus. In addition, planting resistant cultivar promotes the adaptive evolution of the virus, and consequently, the emergence of RB variants. Phylogenetic analyses have revealed a complex pattern for the Chinese BNYVV isolates, suggesting that China might be one of the origins of BNYVV (Li et al., 2008; Chiba et al., 2011). Like other countries, economic sugar beet production in China is strongly dependent on large scale cultivation of resistant sugar beet cultivars to control the disease. However, the high risk of the emergence of RB variants was not a concern in the past. In addition, a previous study with several soil borne populations has shown considerable within-isolate tetrad diversity in other countries, but no correlation between virus type and tetrad diversity (Bornemann and Varrelmann, 2013). In contrast, little information is available regarding the within-isolate genetic diversity of individual BNYVV isolate in China.

In this study, four genomic regions (*CP*, RNA3, RNA4 and RNA5) from 15 Chinese isolates were sequenced. At least nine clones were sequenced from each genomic region of each isolate in order to analyze within-isolate diversities. These data, together with data for previously reported Chinese isolates, were analyzed to obtain information on the sequence variation, phylogeny, selection pressure and population genetics of BNYVV in China. The results obtained in this study will improve our knowledge of BNYVV genetic diversity in China, which will lead to a better understanding of the epidemiology of the virus. This information is a prerequisite to designing appropriate disease management strategies.

2. Materials and methods

2.1. Virus isolates

The isolates used in this study were derived from sugar beet and soil samples that had been collected from 11 locations throughout the main sugar beet growing provinces (or autonomous regions) in China between 2010 and 2014 (Table 1). Two sugar beet samples with rhizomania symptoms were collected from Wuwei (Gansu Province) and Yili (the Xinjiang Uygur Autonomous Region), respectively. Thirteen soil samples were randomly collected from sugar beet fields at Hohhot, Baotou and Shangdu in the Inner Mongolia Autonomous Region, Harbin, Shuangyashan and Qiqihar in Heilongjiang Province, Zhangye in Gansu Province, Shihezi in the Xinjiang Uygur Autonomous Region and Datong in Shanxi Province. The soil-born isolates were isolated from the bait plants (susceptible *cv*. TY-309) grown in the respective soil samples for four to six weeks. One isolate represents virus sequences derived from a single sample. Nucleotide sequences from 23 isolates from China and 67 isolates from other countries were retrieved from GenBank (accession numbers in Supplementary Table S1).

2.2. Characterization of BNYVV isolates

Root tissues (0.1–0.2 g) from either sugar beet plants or bait plants were collected in 2-ml microfuge tubes, which contained a sterile stainless steel grinding ball, and then directly processed or stored at −80 °C. During RNA extraction, the root tissue was first powdered by immersing the tubes containing the sample in liquid nitrogen and then immediately shaking them at 30 frequencies/s for 2 min in a MM 400 grinder (Retsch, Haan, Germany). Then total RNA was extracted as described previously (Guo et al., 2005). The primers used for cDNA synthesis and amplification of the *CP* gene, full-length RNA3, RNA4 and RNA5 are listed in Supplementary Table S2. For first strand cDNA synthesis, 3 μl of total RNA was added to 0.5-ml tubes, which were then chilled for a short period of time. Then, 27 μl of the following reaction mixture was added: 10.5 μl of RNase-free sterile water, 6 μl of 5× RT Buffer (Promega, WI, USA.), 6 μl of 2.5 mM dNTPs (Takara, Dalian, China), 3 μl of 18TR, 0.5 μl of recombinant RNase inhibitor (Takara) and 1 μl of M-MLV reverse transcriptase (Promega). The reactions were incubated at 37 °C for 1–2 h. The PCR reaction mix consists of 16.75 μl of sterile water, 2.5 μl of 10× *Taq* Buffer (Tiangen, Beijing, China), 2 μl of 2.5 mM dNTPs (Takara), 0.5 μl of 10 μM forward and reverse primers that were specific for each genomic region, 0.25 μl of *Taq* DNA Polymerase (Tiangen), and 2.5 μl of cDNA product. The PCR profiles for all primer sets consisted of an initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 54 °C for 30 s, and extending at 72 °C for 50 s (*CP*) or 1 min 30 s (RNA3, RNA4 and RNA5) and a final extension for 10 min using a Mastercycler 5333 Thermocycler (Eppendorf, Hamburg, Germany). The PCR products were cleaned using a DNA gel extraction kit (Axygen, CA, USA), ligated into the pMD19-T vector (Takara) and transformed into the *Escherichia coli* MC1022. The individual clones were sequenced commercially (Invitrogen, Beijing, China). We tested the *Taq* DNA polymerase to insure the accuracy of the results. As the largest length sequenced in this study was 1.7 kb (full-length BNYVV RNA3), a known plasmid clone containing a full-length BNYVV RNA3 cDNA sequence was used as a PCR template. We carried out the experiment using the same conditions as above. Ten clones were randomly chosen and sequenced. The sequences of all ten clones were identical to the template sequence, which indicated that the *Taq* DNA polymerase could insure the accuracy of the sequences.

2.3. Sequence analyses

The basic processing of the cDNA sequences, such as assembling, correction and alignment was performed using DNAMAN 7 (Lynnon, CA, USA). Multiple sequence alignments were performed by CLUSTALX 2.0 using the default settings (Larkin et al., 2007). The number of haplotypes (h) and haplotype diversity (Hd) determined for each gene within the individual isolates using DnaSP 5 (Librado and Rozas, 2009). The numbers of haplotypes were identified by the nucleotide variations within at least one site of the nucleotide sequences from the four genes analyzed in the different isolates. Clones sharing 100% nucleotide identity were considered