

Genetic and Biological Characterisation of a *Grapevine virus A* Isolate from the Czech Republic

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Abstract

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An isolate of *Grapevine virus A* (GVA) from the Czech Republic was obtained from the grapevine cultivar Müller Thurgau. Symptoms of GVA – Kober stem grooving disease were not observed in the infected grapevines (which had been grafted onto Kober 5BB rootstock). A partial genomic sequence of the GVA isolate, 1523 nucleotides long, was obtained. The sequence completely covers the genes for both a movement and a coat protein. Compared to the GVA sequences available in databases, the nucleotide identity reached 84%. The amino acid identity in the movement protein reached 88%, and 98% in the coat protein.

Keywords: *Grapevine virus A*; grapevine; movement protein; coat protein; phylogenetic analysis; sequencing

Grapevine virus A (GVA) (FAUQUET *et al.* 2005) is a type member of the genus *Vitivirus*, family *Flexiviridae* (ADAMS *et al.* 2004). It has a single stranded, positive sense genomic RNA. It carries five open reading frames (ORF) encoding: a protein of 194 kDa with replicase motifs (ORF1); a 19 kDa protein with an unknown function, which may be involved in vector transmission (ORF2); a movement protein (MP) of 13 kDa encoded by ORF3; a coat protein (CP) of 21.5 kDa encoded by ORF4; and a 10 kDa nucleic acid-binding protein (ORF5) which affects pathogenesis (MINAFRA *et al.* 1997; GALIAKPAROV *et al.* 2003a, b). GVA is associated with rugose wood disease of grapevine (GARAU *et al.* 1994), showing symptoms on the wood cylinder close to the grafting point between the infected variety and the Kober 5BB rootstock. The virus is found on grapevines worldwide, and has been detected in the Czech

Republic (KOMÍNEK 2008). The objective of this work was to characterise the biological and molecular properties of the Czech isolate of GVA. The two genes (a movement protein gene and a coat protein gene) which have most frequently been studied worldwide were selected for the molecular characterisation.

MATERIAL AND METHODS

Virus source. A 15 year-old grapevine, variety Müller Thurgau, clone 25/7 (Karlštejn Research Station of Viticulture), grafted onto Kober 5BB rootstock, was used as the source plant for the work on the GVA characterisation. The presence of GVA was confirmed by DAS-ELISA, using commercial polyclonal antibodies (Agritest Valenzano, Italy). Symptoms of rugose wood disease on the wood cylinder (typically positioned close to the grafting

point between the infected variety and the Kober 5BB rootstock) were also evaluated.

Primer design. According to GVA sequences available in GenBank (BENSON *et al.* 2007; www.ncbi.nlm.nih.gov), the following primer pairs were designed to amplify two overlapping fragments of the GVA genome:

GVA1F (sense primer) 5' CGTCCAGATTAAGTGTAGACCT 3';

GVA1R (antisense primer) 5' CAGGTACATATTTCGGTCGT 3', product length 958 base pairs (nt 5573-6530 according to GenBank Acc. No. X75433);

GVA2F (sense) 5' CTGATCTGCAGTTTGTGGA 3';

GVA2R (antisense) 5' CACCACACTTACACACATTCAT 3', product length 929 base pairs (nt 6165-7093 Acc. No. X75433).

The two overlapping regions together cover the 3' end region of 19 kDa protein gene (ORF 2), the entire MP (ORF 3) and CP (ORF 4) genes, as well as the 5' beginning of a 10 kDa protein gene (ORF 5).

RNA isolation, cDNA amplification and sequence analysis. The total RNA was isolated from grapevine phloem tissue, scrapped from dormant canes, using an RNeasy Plant Mini Kit (Qiagen). RT-PCR for the GVA genome amplification was carried-out using a Qiagen OneStep RT-PCR Kit,

in a PTC200 thermocycler (MJ Research). The reverse transcription was done at 45°C for 90 min, followed by a denaturation step at 95°C for 15 min. Subsequently, PCR was done with 40 cycles. Each cycle consisted of denaturation at 94°C for 1 min, followed by annealing at 50°C for 1.5 min, and then extension at 72°C for 2.5 minutes. Cloning and sequencing was carried-out as described earlier (KOMÍNEK *et al.* 2006). The identity of the Czech sequence with other GVA sequences present in the databases was compared, using both the BLASTN 2.2.18 and BLASTP 2.2.18 programmes (ALTSCHUL *et al.* 1997). The phylogenetic analysis was done using the MEGA Program version 4.0 (TAMURA *et al.* 2007), using the Neighbor-Joining method with the default parameters.

RESULTS AND DISCUSSION

The Czech GVA sequences obtained from the overlapping regions were joined, and a sequence 1523 nucleotides long was obtained. The overlapping sequence portions, of all of the clones obtained, were identical; indicating that the source grapevine was probably infected by a single virus isolate.

Four ORFs were detected in the resulting sequence; containing the 3' end of 19 kDa protein gene (ORF 2), whole MP (ORF 3) and CP (ORF 4)

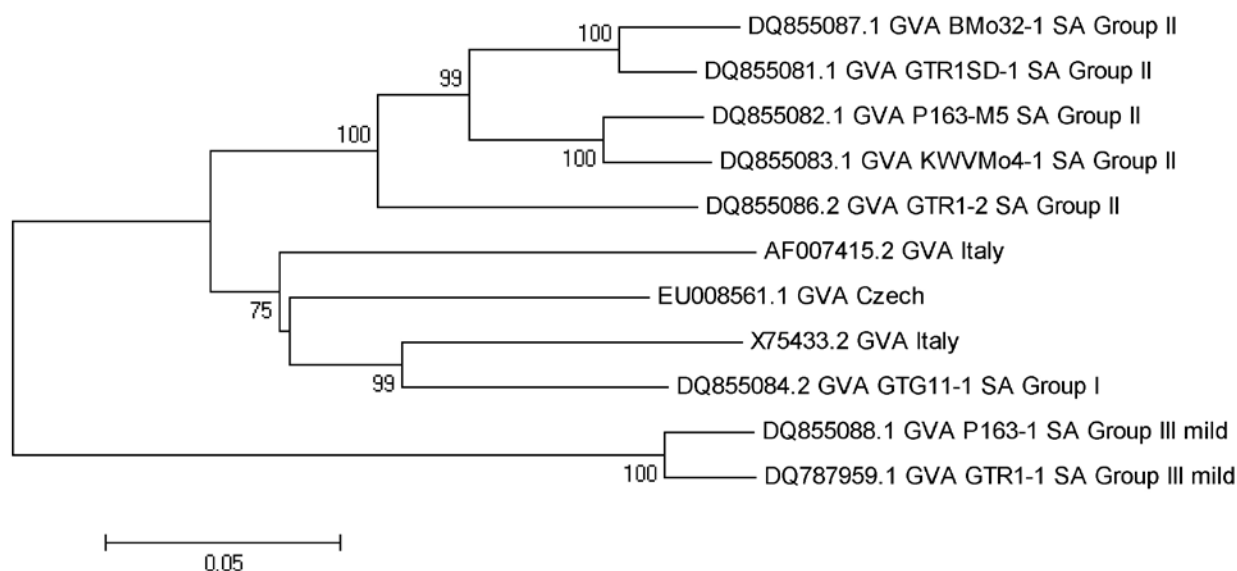


Figure 1. An unrooted phylogenetic tree, based on nucleotide MP-CP sequences – illustrating the position of the Czech GVA isolate, among all other GVA sequences, encompassing whole MP and CP genes. Only bootstrap values greater than 60 (from 500 replicates) are indicated. Accession numbers and geographic origin of the sequences are indicated. SA-GVA isolates from South Africa, groups according to GOSZCZYNSKI and JOOSTE (2003), are indicated

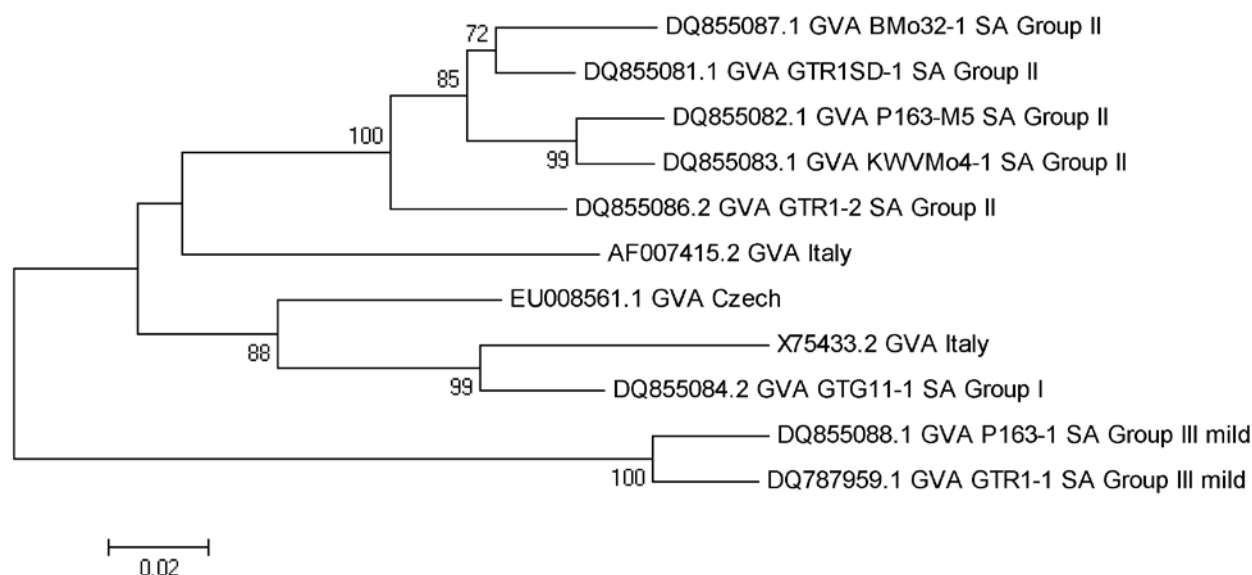


Figure 2. An unrooted phylogenetic tree, based on amino acid MP sequences – illustrating the position of the Czech GVA MP sequence, among other complete GVA MP sequences. Only bootstrap values greater than 60 (from 500 replicates) are indicated. Geographic origin of the sequences is indicated. SA-GVA isolates from South Africa, groups according to GOSZCZYNSKI and JOOSTE (2003), are indicated

genes, and the 5' beginning of a 10 kDa protein gene (ORF 5).

The 3' end of 19 kDa protein gene of the Czech sequence was 140 nucleotides (nt) long, corresponding to 46 amino acids (aa). The total length of this gene is 534 nt, corresponding to 178 aa, according to MINAFRA *et al.* (1997).

The MP gene sequence was 837 nt long, corresponding to 279 aa.

The CP gene was 597 nt and 199 aa long. Both genes were the same size as had been published by MINAFRA *et al.* (1997).

The 5' portion of a 10kDa protein gene contains 85 nt, corresponding to 28 aa. The total length of this gene is 273 nt, corresponding to 91 aa (MINAFRA *et al.* 1997).

The Czech GVA sequence is available in GenBank, Acc. No. EU008561.

Comparing the whole length of the Czech sequence, eleven sequences of the same length were available in databases. Three of them originated from Italy: X75433 (MINAFRA *et al.* 1997), AF007415 (RUBINSON *et al.* 1997), and AY244516 (GALIAKPAROV *et al.* 1999). Sequence AY244516 was removed from our analyses because it is, in fact, derived from the same source as is AF007415. Another eight sequences originated from South Africa (GOSZCZYNSKI & JOOSTE 2003). These

authors divided their GVA sequences into three groups, on the basis of the phylogenetic analysis. Only the sequence DQ855084 (representing Group I) was used for analysis, together with five sequences from Group II (DQ855081, DQ855082, DQ855083, DQ855086, and DQ855087), as well as two sequences (DQ855088 and DQ787959) from Group III (causing mild symptoms on *Nicotiana benthamiana*).

When compared on the nucleotide level, the Czech GVA sequence had an identity of about 84% compared to most of the GVA isolates, with the exception of the mild GVA isolates (DQ855088 and DQ787959) from South Africa (GOSZCZYNSKI & JOOSTE 2003), which only have a 77% identity.

On the amino acid level, different identities were observed when comparing the movement protein and the coat protein. In the movement protein, the identity of a Czech sequence (compared to available sequences) reached 87% to 88%. Higher levels of identity were obtained in the coat protein, reaching 94% to 98% in most of the GVA sequences analysed. A lesser identity level was obtained in the coat protein sequences from mild GVA isolates (GOSZCZYNSKI & JOOSTE 2003) which only reached an 84% identity.

Phylogenetic analysis of the nucleotide sequence showed a further interesting result – the Czech

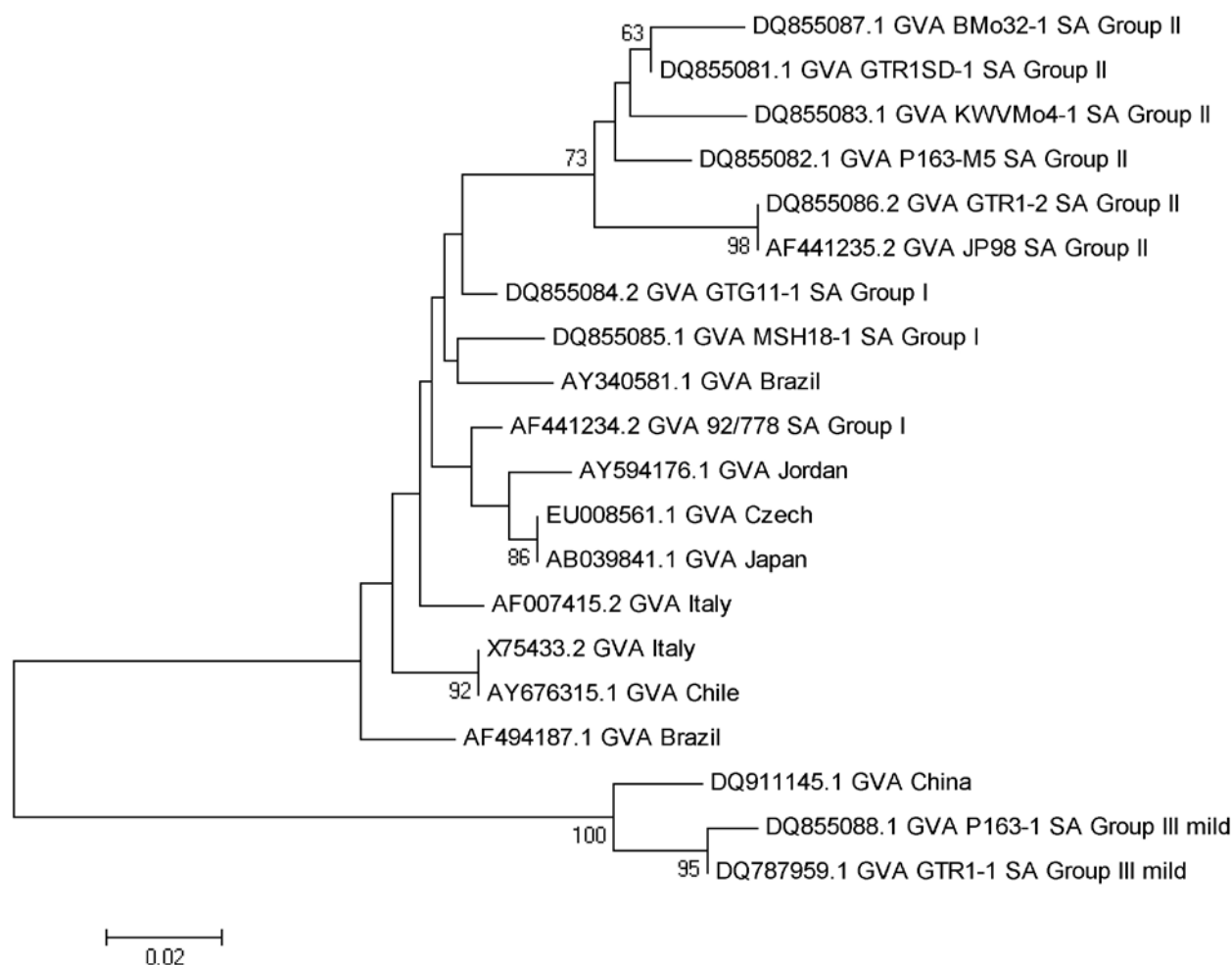


Figure 3. An unrooted phylogenetic tree based on amino acid CP sequences – illustrating the position of the Czech GVA CP sequence, among other complete GVA CP sequences. Only bootstrap values greater than 60 (from 500 replicates) are indicated. Geographic origin of the sequences is indicated. SA-GVA isolates from South Africa, groups according to GOSZCZYNSKI and JOOSTE (2003), are indicated

sequence does not match exactly any of the three GVA groups described by GOSZCZYNSKI and JOOSTE (2003); forming what is probably a new GVA subgroup (close to a Group I) together with the sequence AF007415 from Italy (Figure 1).

To further analyse the relationship between the Czech GVA sequence and those already published worldwide, a phylogenetic analysis of the amino acid sequences was performed; it was divided into separate analyses of the movement protein and the coat protein. Results are given in Figure 2 (MP) and Figure 3 (CP).

All three groups, according to GOSZCZYNSKI and JOOSTE (2003), are clearly visible in both phylogenetic trees.

Analysis of the movement protein showed the same location of the Czech GVA sequence as was found on the nucleotide level.

The coat protein has been more widely studied than the movement protein, giving us the possibility to use a greater number of sequences from databases. It is clear that seven sequences form a separate subgroup outside the nearest group (Group I), which may correspond to the putative Group IV, according to MUROLO *et al.* (2008). These separated sequences originated from Italy (two sequences), Brazil, Chile, Jordan, Japan and the Czech Republic (this present work).

An interesting result is that the GVA sequence of the coat protein from Japan was 100% identical (on the amino acid level) with the Czech GVA sequence. The Japanese GVA sequence is only present in the database, and was not published as a scientific communication; therefore, we do not have any further information concerning the biological properties of the Japanese GVA isolate.

In order to have more conclusive results from the observed sequence diversity, the biological properties of GVA from the Czech Republic should be determined. For this reason, more than 100 grapevines cv. Müller Thurgau infected with GVA (as confirmed by DAS-ELISA) were removed to check for symptoms close to the point of grafting. No symptoms were observed in these plants, although a GVA-susceptible rootstock (Kober 5BB) was used in all grapevines from the Karlštejn Research Station.

Similarly, no symptoms of GVA (such as from rugose wood disease) have been reported in grapevines in the Czech Republic (HOLLEINOVÁ, personal communication), even though Kober 5BB is a frequently used rootstock in the Czech Republic. This rootstock is also used as an indicator for the presence of GVA, because it reacts to a GVA infection by changes in the woody cylinder – pits and grooves are visible close to the point of grafting – a disease called Kober stem grooving (GARAU *et al.* 1994; CHEVALIER *et al.* 1995) that is part of the “rugose wood” disease complex.

We can conclude that the Czech isolate of GVA does not cause symptoms in the grapevine cultivar Müller Thurgau when grafted upon Kober 5BB rootstock.

Analysis of its nucleotide and amino acid sequence shows its divergence from other GVA isolates occurring in countries (such as South Africa) with high levels of GVA pathogenicity on grapevines.

This current work describes the first sequence of GVA from the Czech Republic. A broader survey for GVA variability, analysing more sequences from the Czech Republic, is not needed until new data concerning its occurrence and pathogenicity within our country can be obtained.

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