GENETIC VARIABILITY OF STOLBUR PHYTOPLASMA IN ANNUAL CROP AND WILD PLANT SPECIES IN SOUTH MORAVIA

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SUMMARY

The genetic diversity of stolbur phytoplasma isolates was assessed by different molecular typing methods in a wide range of plant hosts in a limited geographical area of the Czech Republic. Plants of Apium graveolens, Capsicum annuum, Solanum lycopersicum, Solanum tuberosum, Amaranthus retroflexus, Calystegia sepium, Cirsium arvense, Convolvulus arvensis, and Datura stramonium displaying symptoms of proliferation, discoloration, and malformation of flower were collected in vegetable crop plots in the vicinity of the village of Lednice (south Moravia) and examined for the presence of phytoplasmas. Using 16S rDNA amplification and RFLP assays, stolbur phytoplasma was detected in some of the symptomatic plants. The genotype of the phytoplasma isolates detected was investigated by restriction pattern or sequence analysis of two non-ribosomal house-keeping genes (tuf and secY) and a recently characterized polymorphic gene (vmp1). According to tuf genotyping, all isolates were of the tufAY-b genotype sensu Langer and Maixner (2004), a genotype known to be associated with bindweed (C. arvensis) reservoirs. A finer isolate differentiation was obtained with vmp1 genotyping as four genetic variants carrying different vmp1 RFLP patterns were found, especially in C. arvensis. In addition, preliminary sampling in other south Moravian localities and secY sequence analyses disclosed also the presence of stolbur isolates different from those previously reported in binwedd (C. arvensis) and grapevine.

Key words: stolbur disease, non-ribosomal DNA, vmp1 gene, tuf gene, secY gene, PCR, RFLP.

INTRODUCTION

Phytoplasmas of the stolbur group (subgroup 16SrXII-A) infect a wide range of vegetable crops besides the grapevine. In the Czech Republic, stolbur is a long known disease since local epidemics and vector transmission have been very intensively studied in the years 50 and 60 of the last century (Blattny, 1958; Kosljarova and Bojansky, 1969). Five years ago a major outbreak of stolbur occurred in south Moravia, an important horticultural and viticultural region (Linhartova et al., 2006; Navratil et al., 2008). In 2006 and 2007, symptoms reminiscent of stolbur appeared on several crops and weeds within vegetable crop cultivations in Lednice. Diseased plants were: Solanum lycopersicum showing symptoms of stunting, proliferation, yellow or purplish leaves, and big bud symptoms; Capsicum annuum with yellowing, stunting, and wilting; Solanum tuberosum with proliferation, yellowing, and formation of aerial tubers; and Apium graveolens exhibiting stunting, yellowing and early flowering. In addition, five weed species were displaying stunting, proliferation, yellowing, and flower malformations, i.e. Datura stramonium, Convolvulus arvensis, Cirsium arvense, Calystegia sepium, and Amaranthus retroflexus. Preliminary analyses showed the presence of stolbur phytoplasma in these plants.

The aim of the present study was to investigate the biological diversity of the epidemics by determining the genetic variability of stolbur phytoplasma isolates occurring in a restricted area and to characterize possible relationship between genetic markers and host specificity. For genotyping phytoplasma isolates, non-ribosomal genes were amplified and analysed. These were the vmp1 gene encoding a putative stolbur phytoplasma membrane protein and the secY gene coding for the translocase protein, both described by Cimerman et al. (2008) in addition to the widely used tuf gene encoding the translation elongation factor Tu (Langer and Maixner, 2004).

MATERIAL AND METHODS

Phytoplasma isolates. Sampling was done in late summer and autumn of 2006 and 2007 in south Moravia, in intensive vegetable crops at Lednice and in two vineyards in Perná and Brezí. Total DNAs was extracted using the phytoplasma enrichment procedure by Ahrens and Seemüller (1992) from midribs, leaves, and shoots of the following symptomatic plants: Apium...
gravelens, Capsicum annuum, Solanum lycopersicum, Solanum tuberosum, Vitis vinifera, Amaranthus retroflexus, Calystegia sepium, Cirsium arvense, Convolulus arvensis, Datura stramonium, and Urtica dioica. DNA was extracted also from healthy seedlings of Catharanthus roseus, tomato, and pepper. As a positive control, the stolbur phytoplasma isolate SK927 was used (Navratil et al., 2001). Stolbur reference isolates maintained in periwinkle are listed in Table 1.

**Phytoplasma detection.** For phytoplasma detection, a total of 94 samples were screened by PCR, using stolbur-specific primers fStol/rStol (Maixner et al., 1995). The same set of samples was simultaneously tested by nested PCR with universal ribosomal primers R16F1/R0 followed by R16F2/R2 (Lee et al., 1995) and subsequent RFLP analysis with AluI, MseI, and RsaI restriction enzymes (MBI Fermentas, Lithuania).

**Typing on non-ribosomal genes.** All stolbur phytoplasma isolates obtained were subjected to genotyping on three non-ribosomal genes: vmp1, secY, and tuf.

The tuf gene was amplified in nested PCR using primers TufIf/r, followed by TufAyIf/r as described by Langer and Maixner (2004). The nested PCR products were digested by HpaII (Schneider et al., 1997).

A fragment of the vmp1 gene was amplified in nested PCR with newly designed primers StolH10F1 (AG-GTTGTAAATCCTTTATGT) and StolH10R1 (GG-GATGGCTTTTCATTATTTGAC), followed by TYPH10F (AAGGTTGATCATCAACAATCAGTC) and TYPH10R (CACCTTCTTTAGGCAACTTC).

PCR conditions were 94°C for 4 min (1 cycle); denaturation at 94°C for 30 sec; annealing at 52°C for 30 sec; and extension at 72°C for 2 min (35 cycles). For inner primer pair annealing the temperature was increased to 62°C, and extension was shortened to 1.5 min. The final PCR products (1.4 kbp) were then digested with RsaI.

A 998 bp long fragment of the secY gene was amplified in nested PCR with newly designed primers PosecF1 (TCTCGTCTTGCTGGCTTCTT) and PosecR1 (ATTAGTAACACTTCTTCTCC), followed by PosecF3 (GGATTGATAGTGCTGCCC) and PosecR3 (GCCCTCTAACGGGTGATTGATTG). PCR conditions were 95°C for 3 min (1 cycle); denaturation at 95°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 1 min (35 cycles). For inner primer pair annealing temperature was increased to 62°C.

All PCR products, as well as RFLP patterns, were analysed by electrophoresis in 1%, and 2.5% agarose gel (MetaPhor® Agarose, USA), respectively, and stained with ethidium bromide.

**Sequencing and phylogenetic analysis of SecY.** Both DNA strands were sequenced using the PosecF3 and PosecR3 nested primers by COGENICS (Grenoble, France) on MegaBACE capillary sequencing instruments. The raw sequence chromatograms were assembled and edited using Phred, Phrap and Consed programs (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998). Multiple sequences alignments were made using the Clustal W program (Higgins et al., 1994). Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007) using maximum parsimony with randomized bootstrapping evaluation of branching validity.

**RESULTS**

**Stolbur phytoplasma detection.** PCR products of the expected size (570 bp for fStol/rStol and 1200 bp for R16F2/R2) were obtained in 49 samples out of 94 analysed (Table 2). No PCR products were obtained from healthy control plants. All R16F2/R2 PCR products amplified gave identical RFLP patterns corresponding to the profile of the stolbur phytoplasma (subgroup 16SrXII-A) (data not shown; see Lee et al., 1998). The presence of stolbur phytoplasma was confirmed in plants showing typical symptoms of infection.

**Genotyping on vmp1 and tuf genes.** The 49 phytoplasma isolates were subjected to genotyping on non-ribosomal genes. A fragment of the vmp1 gene was amplified in nested PCR with primers StolH10F1/R1, followed with TYPH10F/R, and subsequently digested with RsaI to detect genetic variability. Using TYPH10F/R primers PCR products of the same size (about 1450 bp) were obtained from most analysed samples of different host species. All PCR products had a vmp1 gene size larger than the reference isolate stolbur-PO from which the gene was originally cloned (Fig. 1). RFLP

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**Table 1.** Stolbur phytoplasma reference isolates maintained in periwinkle (Catharanthus roseus).

<table>
<thead>
<tr>
<th>Stolbur isolate (code)</th>
<th>Original host</th>
<th>Country, year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liban P7 (P7)</td>
<td>Periwinkle</td>
<td>Lebanon, 2001</td>
<td>Verdin et al. (2004)</td>
</tr>
<tr>
<td>Pyrénées Orientales (PO)</td>
<td>Hyalesthes obsoletus</td>
<td>France, 1996</td>
<td>Jarausch et al. (2001)</td>
</tr>
<tr>
<td>VK GGY (GGY)</td>
<td>Grapevine</td>
<td>Germany, 1995</td>
<td>Marcone et al. (1996)</td>
</tr>
<tr>
<td>Stolbur T2_92 (T2_92)</td>
<td>Tomato</td>
<td>Italy, 1996</td>
<td>Minucci and Boccardo (1997)</td>
</tr>
</tbody>
</table>
analysis revealed a variability of the restriction fragments for five different TYPH10/RsaI profiles were identified, which were denoted I, II, III, IV, and V (Fig. 2). The profile distribution among particular host species and localities is shown in Table 2. The most frequent profile was I, as it was detected in seven of eleven host species collected at Lednice. Profiles II, III, and V were found only at Lednice, whereas profile IV was found at Brezí in grapevine and Urtica dioica. Most profiles (I, II, III, V) were identified on vmp1 PCR products from C. arvensis.

To characterize stolbur phytoplasma isolates in more detail, fragments of tuf gene encoding the phytoplasma elongation factor Tu were analysed. PCR using primers Tuf1f/r, followed by TufAy/r, was used to amplify the gene segments. Amplicons ca. 940 bp in size were obtained from all analysed samples (Table 2). The TufAy/HpaII restriction profiles of the isolates included in the study showed no polymorphism (data not shown). All samples yielded to the tuf-b profile.

**Phylogenetic analysis on secY gene.** SecY gene sequences were determined for ten samples from Lednice, Brezi, and Perná as well as for five stolbur reference isolates propagated by grafting in periwinkle plants. All samples gave the expected 998 bp PCR product. Whereas reference isolates gave five different secY sequences, only two types of sequences were obtained from the Czech samples (Fig. 3). The two samples from C. arvensis from Perná and Breží, the samples from C. annuum, D. stramonium, A. graveolens from Lednice and a V. vinifera sample from Perná had the same secY sequence as the reference isolate Stolbur-1925 from German V. vinifera.

**DISCUSSION**

The first epidemic occurrence of potato stolbur in the Czech Republic was recorded in the 1930s on potatoes in south Moravia (Bojnansky, 1958). For long time disease identification was based only on symptomatology and, later, vector transmission was used for epidemiologic studies. As late as 2001 the causal agent of

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**Table 2.** Results of non-ribosomal typing of stolbur phytoplasma isolates.

<table>
<thead>
<tr>
<th>Host species (stolbur phytoplasma positive/tested plants)</th>
<th>Locality</th>
<th>TYPH10F/R tested plants</th>
<th>TYPH10/RsaI profile</th>
<th>TufAy/HpaII unique profile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Apium graveolens (8/9)</td>
<td>Lednice</td>
<td>8</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Capsicum annuum (14/18)</td>
<td>Lednice</td>
<td>14</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Solanum lycopersicum (4/4)</td>
<td>Lednice</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Solanum tuberosum (1/12)</td>
<td>Lednice</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amaranthus retroflexus (1/3)</td>
<td>Lednice</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Calystegia sepium (1/4)</td>
<td>Lednice</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Cirsiump arvense (2/16)</td>
<td>Lednice</td>
<td>2</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Convolvulus arvensis (9/19)</td>
<td>Perná</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Brezí</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Datura stramonium (3/3)</td>
<td>Lednice</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Perná (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vitis vinifera (5/5)</td>
<td>Brezi (3)</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urtica dioica (1/1)</td>
<td>Brezi</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*aBased on analysis of 16S rDNA  
*bCorresponding to the tufAY-b (VK-Type II) by Langer and Maixner (2004).
Fig. 2. RsaI restriction profiles of stolbur phytoplasma DNA amplified using primers TYPH10F/R. Lane M - molecular size marker 100 bp DNA Ladder (Fermentas); profile I: lanes: 1, isolate 2838 from *Convolvulus arvensis*; 2, isolate 2857 from *Cirsium arvense*; 3, isolate 2859 from *C. arvensis*; 7, isolate 2992 from *Capsicum annuum*; 9, isolate 2996 from *C. annuum*; and 12, isolate 3054 from *Apium graveolens*; profile II: lanes: 4, isolate 2900 from *Calystegia sepium*; 5, isolate 2989 from *C. annuum*; 6, isolate 2990 from *C. annuum*; 8, isolate 2995 from *C. annuum*; 11, isolate 3053 from *A. graveolens*; and 14, isolate 3056 from *A. graveolens*; profile III: lane 10, isolate 3002 from *Solanum tuberosum*; profile IV: lanes: 15, isolate 3149 from *Urtica dioica*; 16, isolate 3143 from *Vitis vinifera*; profile V: lane 13, isolate 3055 from *A. graveolens*.

Fig. 3. Evolutionary relationships of secY genetic loci for Czech and reference stolbur isolates. Reference isolates are denoted STOLBUR and Czech field samples names begin by CZ31. The evolutionary history was inferred using the Maximum Parsimony method. The trees are drawn to scale with branch lengths calculated using the average pathway method and represent the number of nucleotide changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 841 positions in the final dataset for and phylogenetic analyses were all conducted in MEGA4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. SecY sequence of *Candidatus Phytoplasma australiense* was chosen as an outgroup. Bar length represents 5 nucleotide changes.
stolbur disease was identified by PCR/RFLP analysis of 16S rDNA gene (Navratil et al., 2001).

The highly conserved 16S rDNA gene, commonly used for detection of stolbur phytoplasma, is not suitable for detailed study of genetic diversity and phylogenetic relationships among isolates and for correlating isolates with host range and geographic distribution. Thus, for obtaining new information on the molecular variability of stolbur phytoplasma isolates from annual crop and wild plant species from south Moravia we have chosen the more variable non-ribosomal vmp1, tuf, and secY genes.

The analyzed HpaII restriction profiles of tuf gene of the studied isolates showed no polymorphism. All samples from vegetable crops and weeds, revealed the same profile corresponding to the tuf-b type (VK Type II) described by Langer and Maixner (2004). These authors characterized three tuf type phytoplasma isolates in Germany and studied the relationships between tuf type phytoplasma and its host. They showed the stolbur phytoplasma type tuf-b as being the most widespread and associated with C. arvensis as its main wild host reservoir. Our results are in agreement with these findings as we detected this genotype in bindweed, but also in U. dioica from Brezi, which is supposed to host only the tuf-a (VK Type I) genotype (Langer and Maixner, 2004). The second house-keeping gene used for the genetic characterization of the stolbur isolates, confirmed that most of the isolates were of the same secY genotype with the exception of three grapevine and the nettle samples from Brezi.

While size variations of vmp1 were observed by Pacifico et al. (2007, 2009) in French and Italian bois noir phytoplasma isolates, all amplicons obtained from Czech samples were of the same size. In fact, as compared with our samples, a higher variability in restriction sites leading to 12 RFLP patterns was detected in French and Italian grapevine and in Italian C. arvensis isolates. However, the samples analyzed by Pacifico et al. (2007, 2009) were geographically distant whereas our set of isolates originated from a single location. It must be pointed out that we found three new RFLP patterns which were not detected in western Europe (Pacifico et al., 2009)

A situation characterized by a high genetic diversity of stolbur isolates was found in an abundantly weeded vegetable plantation at Lednice in an area of about 10 ha. Stolbur infection was confirmed in nine symptomatic plant species and a pronounced polymorphism (four of five Czech profiles) was detected. Nonetheless, no association of a RFLP pattern with a particular host could be established among the set of phytoplasmas studied.

This work represents the first complex study of stolbur phytoplasma variability among isolates from a single location, whereas other investigations have described genetic variability among isolates of different geographical origin. (Langer and Maixner, 2004; Pacifico et al., 2007). The vmp1 gene polymorphism presently observed, confirms the high level of discrimination provided by this non-ribosomal gene for the molecular characterization of stolbur phytoplasma isolates.

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