A single U/C nucleotide substitution changing alanine to valine in the beet necrotic yellow vein virus P25 protein promotes increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings

R. Koenig,1 S. Loss,1 J. Specht,1 M. Varrelmann,2 P. Lüddecke1 and G. Deml1

1Julius Kühn Institut, Bundesforschungsanstalt für Kulturpflanzen, Institut für Epidemiologie und Pathogendiagnostik, Messeweg 11, D-38106 Braunschweig, Germany
2Institut für Zuckerrübenforschung, Abteilung Phytomedizin, Holtenser Landstraße 77, D-37079 Göttingen, Germany

Correspondence
R. Koenig
renate.koenig@jki.bund.de

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Beet necrotic yellow vein virus (BNYVV) A type isolates E12 and S8, originating from areas where resistance-breaking had or had not been observed, respectively, served as starting material for studying the influence of sequence variations in BNYVV RNA 3 on virus accumulation in partially resistant sugar beet varieties. Sub-isolates containing only RNAs 1 and 2 were obtained by serial local lesion passages; biologically active cDNA clones were prepared for RNAs 3 which differed in their coding sequences for P25 aa 67, 68 and 129. Sugar beet seedlings were mechanically inoculated with RNA 1+2/RNA 3 pseudorecombinants. The RNA 1+2 had little influence on virus accumulation in rootlets. E12 RNA 3 coding for V67C68Y129P25, however, enabled a much higher virus accumulation than S8 RNA 3 coding for A67H68H129P25. Mutants revealed that this was due only to the V67 ‘GUU’ codon as opposed to the A67 ‘GCU’ codon.

The Polymyxa betae-transmitted beet necrotic yellow vein virus (BNYVV) is the causal agent of the devastating rhizomania disease. Its genome consists of four, and in some geographical areas five, different RNA species. RNA 1 and 2 contain the genetic information necessary to enable replication, encapsidation, cell-to-cell movement and suppression of RNA silencing in local lesion hosts (Koenig, 2008; Dunoyer et al., 2002). The additional presence of RNA 3, which codes for a 25 kDa protein (P25), is necessary for virus movement in infected sugar beet roots and production of typical rhizomania symptoms. RNA 4 enables efficient vector transmission and suppression of gene silencing in Nicotiana benthamiana roots (Rahim et al., 2007). The severity of symptoms in sugar beet may be enhanced by the additional presence of RNA 5. The three major strain groups of BNYVV, i.e. the A, B and P types, have highly conserved genomes. Nucleotides 199–210 of the P25-coding region, however, show considerable sequence variation, especially in A type BNYVV (Tamada et al., 2003; Meunier et al., 2003; Schirmer et al., 2005; Ward et al., 2007; Kutluk Yilmaz et al., 2007; Li et al., 2008; Koenig et al., 2008). This stretch of nucleotides codes for the P25 aa 67–70, which in the following will be referred to as the ‘tetrad’. Also for the sake of brevity we will refer to the variability of the tetrad rather than its coding sequence, although we do not know whether the effects described below are due to changes at the protein or the nucleic acid level. More than 15 variants of the tetrad have been identified so far, raising the question whether these variations influence the pathogenicity of the virus and may be involved in resistance-breaking phenomena. Klein et al. (2007) studied the influence of P25 sequence variation on its oligomerization and pathogenicity for Tetragonia expansa leaves. Chiba et al. (2008) investigated the influence of mutations in the P25 aa 68, 69 and 70 on the local lesion response in mechanically inoculated leaves of lines of Beta maritima and of the sugar beet varieties Rizor and Monomidori. Acosta-Leal & Rush (2007) studied the P25 amino acid composition in field-grown Rz1 resistance gene-containing sugar beet which did or did not show resistance-breaking. The two amino acids V67 and F135 in P25, as opposed to A67 and D135, correlated with resistance-breaking in the Imperial Valley, California.
USA. Liu & Lewellen (2007) grew sugar beet plantlets in soil samples from all sugar beet growing areas in the US. Considerably increased ELISA values indicated resistance-breaking in the Rz1 gene-containing cultivar Beta 4430 grown in many, though not all, of these soils. At a lower incidence resistance-breaking was also observed with Rz2 and Rz1+ Rz2 resistance gene-containing cultivars. Sequence analysis of the P25-coding region revealed that the main variation occurred in aa 67 and 68. In non-resistance-breaking isolates these two positions were always occupied by ‘AC’, but in resistance-breaking isolates ‘AF’, ‘AL’, ‘SY’, ‘VC’, ‘VL’ as well as ‘AC’ were found.

The partially contradictory results obtained by different authors might be due to mutations in other genome areas or additional effects exerted by other factors, e.g. the vector Polymyxa betae or other synergistically acting pathogens. In various areas, different Polymyxa strains may be present that might differ in their transmission efficiency for the virus, regardless of its P25 amino acid composition. Alternatively, differences in the tetrad might influence the transmission of the virus by its vector. In the present study we investigated whether the composition of the tetrad alone can influence BNYVV accumulation in roots of partially resistant sugar beets that were mechanically inoculated with pseudorecombinant virus in the absence of P. betae.

The E12 and S8 isolates of BNYVV served as our starting material. They came from areas in Spain and in Sweden where resistance-breaking had and had not been observed, respectively (personal communications by J. Ayala, Asociación para la Investigación de la Mejora del Cultivo de la Remolacha Azucarera, Valladolid, Spain, and K. Lindsten, Swedish University of Agricultural Sciences, Uppsala, Sweden, who kindly provided infected plants). The almost complete nucleotide sequences of RNAs 1 and 2 of these isolates lacking only the 5’ and 3’ terminal primer regions were determined from overlapping PCR products. These first nearly complete European A type RNA 1 and 2 sequences to be described were closely related to one another (99.9% identity in both RNAs), but more distantly to those of other BNYVV types (Fig. 1). The relationships among RNAs 1 of various BNYVV types are closer than those among their RNAs 2 (Fig. 1). RNAs 3 proved to be heterogeneous. S8 contained one full-length RNA 3 plus a mutant lacking ~300 internal nucleotides. E12 contained a mixture of three different full-length RNAs 3, i.e. RNA 3 E12-1, E12-2 and E12-3. Table 1 shows the differentiating P25 amino acids in E12 and S8 RNAs 3.

Full-length cDNAs of the S8 and E12-1 RNAs 3 (Table 1) were cloned into the pe35Stu_pa vector (kindly provided by Professor E. Maiss, Institute of Plant Diseases and Plant Protection, Hannover, Germany) in which they are under the control of an enhanced 35S promoter and a 3’ polyadenylation signal for the generation of in vivo transcripts. For the production of mutants in which the coding sequences for the P25 tetrads were exchanged the SpeI, Hpal and NrlI sites in the RNA 3 cDNA sequences were used. The correctness of insertions and modifications was verified by sequencing. Subisolates of S8 and E12 containing only their RNAs 1+2 were obtained by nine successive single-lesion passages on Chenopodium quinoa. During such passages, BNYVV isolates tend to lose their RNAs 3 and 4 (Koenig et al., 1986); this process was followed by PCR.

RNAs 1+2 in the S8 and E12 subisolates and cDNA clones of the various wild-type or mutated RNAs 3 (Table 1) were used to produce pseudorecombinants in two passages in C. quinoa. For the first passage 120 local lesions of a subisolate

![Fig. 1. Trees showing the average percentages of nucleotide sequence identities between RNAs 1 (a) or RNAs 2 (b) of the European A types E12 and S8 and other BNYVV types. Calculations are based on the unweighted pair group mean average (UPGMA) method (Sneath & Sokal, 1973) used by the DNAMAN software. EU, Europe; Jap, Japan; Kas, Kazakhstan.](image)

<p>| Table 1. Amino acid tetrad in positions 67–70 (bold) and other differentiating amino acids in the RNA 3-encoded P25 of isolates E12 and S8 and in two artificial mutants derived from the E12-1 and the S8 wild-types |</p>
<table>
<thead>
<tr>
<th>Origin and designation of RNA 3</th>
<th>P25 tetrad and differentiating amino acids</th>
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<tr>
<td><strong>Wild-types</strong></td>
<td></td>
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<tr>
<td>E12-1*</td>
<td>T22 V67C68H69G70 Y129D135</td>
</tr>
<tr>
<td>E12-2</td>
<td>T22 V67C68H69G70 H129D135</td>
</tr>
<tr>
<td>E12-3</td>
<td>M22 A67C68H69G70 H129D135</td>
</tr>
<tr>
<td>S8*</td>
<td>T22 A67C68H69G70 H129D135</td>
</tr>
<tr>
<td><strong>Artificial mutants</strong></td>
<td></td>
</tr>
<tr>
<td>E12-1-ACHG*</td>
<td>T22 A67C68H69G70 Y129D135</td>
</tr>
<tr>
<td>S8-VCHG*</td>
<td>T22 V67C68H69G70 Y129D135</td>
</tr>
</tbody>
</table>

*Only RNA 3 cDNA clones encoding these P25 sequences were used in pathogenicity tests.
containing only RNA 1+2 were ground in a mortar with 2.8 ml of 50 mM Na/K phosphate buffer pH 7.2; 400 µl of an aqueous solution containing ~400 µg individual RNA 3 cDNA plasmids were then added. The mixtures were rubbed on 40 leaves of C. quinoa plants in the eight to ten leaf stage. After 10–14 days bright yellow and pale green lesions were readily distinguished on these leaves. Only the bright yellow lesions were used to prepare the inoculum for the second C. quinoa passage, because only they contain the plasmid-derived RNA 3 (Tamada et al., 1989). After about 10 days many yellow lesions appeared on the leaves. Only leaf areas densely covered with local lesions were used to prepare the inoculum for sugar beet seedling inoculations, which in earlier studies in our laboratory were successful only with highly concentrated, freshly prepared sap preparations. After dilution with three volumes of 50 mM phosphate buffer pH 7.2, the sap expressed from these local lesions was used immediately for sugar beet inoculation. An aliquot of the sap was stored for later analyses (see below). Inoculations were done essentially as described previously (Koenig & Stein, 1990). Eight-day-old beet seedlings (20 at a time) were placed in a centrifuge tube (diameter 2.2 cm) together with 90 mg carborundum and 4 ml diluted sap. The tubes were vortexed twice for 30 s with a 60 s interval at a medium speed (Vortex Genie, Scientific Industries; speed setting 3). The inoculated plantlets were left for a further 5 min in the inoculation mix and were then washed in 1 l of 0.05 % Ferty 3 Grün nutrient solution (Planta Düngemittel). For hydroculture the plantlets were grown in the same solution in dark plastic boxes (~34 × 12 × 9 cm). The boxes were covered with plastic lids containing 39 holes into which the inoculated seedlings of the two cultivars (see below) were placed in an alternate arrangement using rings of rubber foam. One hole in each box was not used for plantlets, but for refilling the boxes with nutrient solution every 3–4 days. For each pseudorecombinant five hydroculture boxes were used and the average ELISA readings were determined for the plantlets harvested after about 18–21 days from each of these five boxes. The roots of each variety and box (19 plantlets each) were homogenized together in 30 times their weight of ELISA sample buffer (v/w).

Experiments were done with two partially resistant sugar beet cultivars: cultivar A contained Rz1 and Rz2 resistance genes, whereas cultivar B contained only Rz1. Virus accumulation occurred in both cultivars, and in all experimental variants (Fig. 2) it was higher in cultivar B, which is in line with field observations. In both cultivars the VCHG tetrad-encoding E12-1 RNA 3 (Table 1), which originated from an area where resistance-breaking had been observed, enabled a much higher virus accumulation than the AHHG tetrad-encoding S8 RNA 3 which came from a non-resistance-breaking area (Fig. 2, first four columns; additional experiments, results not shown). The same phenomenon was seen when the inoculum contained RNAs 1+2 from S8 rather than E12 (result not shown). Tests with ELISA, and in some experiments also with RNA 3-specific qPCR, showed that this enhanced virus accumulation was not due to a possibly higher virus concentration in the VCHG P25-encoding inoculum compared with the AHGH P25-encoding inoculum. Virus concentrations were either almost identical for the four pseudorecombinants shown in Fig. 2 or, in some experiments, slightly lower for the VCHG P25-encoding ones, possibly because the latter tended to form fewer local lesions on C. quinoa leaves than the AHGH- and ACHG-encoding pseudorecombinants. Despite this apparently somewhat lower pathogenicity of the VCHG-encoding pseudorecombinants for C. quinoa leaves, they always accumulated to higher levels in beet roots. Inspection of the sequencing electropherograms, obtained for PCR products encompassing the entire P25-coding regions of all four pseudorecombinants in both beet varieties (Fig. 2), gave no indications that any mutations had occurred during the two virus passages in C. quinoa and the 3 weeks

![Fig. 2. Average ELISA readings obtained with the rootlets of sugar beet seedlings of cultivar A (Rz1 + Rz2-mediated resistance) and B (only Rz1-mediated resistance) after mechanical inoculation with pseudorecombinant BNYVV containing RNA 1 + 2 of the E12 isolate and either wild-type RNA 3 of the S8 or E12 isolates or the RNA 3 mutant S8-VCHG or E12-1-ACHG (Table 1), respectively. Average of two experiments involving 190 plantlets of each variant in ten hydroculture boxes each. Bars indicate standard error. The experiments were laid out as a complete randomized block design and data were evaluated by means of the SAS 9.1 software (SAS Institute). Averages labelled with different lower-case letters indicate a significant difference between treatments (multiple comparison for the effect of cultivar virus, P<0.05, Tukey test). In a total of seven experiments (four of them only with wild-type RNAs 3) the absolute values differed somewhat, probably due to seasonal variations, differences in the duration of hydroculture etc.; in all experiments, however, the VCHG-encoding pseudorecombinant(s) enabled a much higher virus accumulation than the AHGH- and ACHG-encoding ones.](http://vir.sgmjournals.org)
of multiplication in beets. In order to exclude contaminations with residual plasmid DNA, controls without reverse transcriptase were included in RT-PCRs.

The S8- and E12-1-encoded P25s differ not only in the first two amino acid positions of the tetrad which are occupied by ‘AH’ in S8 and ‘VC’ in E12-1, but also in position 129 where S8 has ‘H’ and E12-1 has ‘Y’ (Table 1). In order to find out which of the three amino acids (or their coding sequences) is responsible for the greatly increased BNYVV accumulation in partially resistant beets, we prepared the RNA 3 mutants S8-VCHG and E12-1-ACHG (Table 1, Fig. 2). S8-VCHG RNA 3, which differs from E12-1 RNA 3 only in position 129 where it has ‘H’ rather than ‘Y’, enabled the same increased BNYVV accumulation in both partially resistant sugar beet cultivars as E12-1 RNA 3 (Fig. 2), indicating that it is not the amino acid in position 129 (‘H’ or ‘Y’) which influences BNYVV accumulation, but rather one or both amino acids in positions 67 and 68 (or their coding sequences). With mutant E12-1-ACHG, which differs from wild-type E12-1 RNA 3 only in having ‘A’ rather than ‘V’ in position 67, the level of virus accumulation fell to that of the S8 RNA 3 coding for an AHHG tetrad (Fig. 2, last two and first two columns) indicating that it must be the ‘V’ in position 67 (or its coding sequence) which is responsible for the increased BNYVV accumulation rather than the amino acid in position 68 (either ‘H’ or ‘C’). The codon for ‘V’ (valine) in the tetrad is ‘GUU’, whereas that for ‘A’ (alanine) is ‘GCU’. The increased BNYVV accumulation in roots of mechanically inoculated, partially resistant beets was, therefore, due to a single nucleotide exchange in position 200 of the P25-coding region.

Our results are in line with field observations reported by Acosta-Leal & Rush (2007), who identified a ‘V’ at position 67 in Rz1 resistance gene-containing beets showing resistance-breaking, but an ‘A’ in those which did not. In position 68 these authors detected either a ‘C’ or an ‘L’ which did not correlate with resistance-breaking. The amino acid in position 68 can, however, influence the type of local lesions formed in beet leaves (Chiba et al., 2008). It remains to be shown whether the ‘E’ in position 135 found by Acosta-Leal & Rush (2007) in plants showing resistance-breaking also contributes to an increased virus accumulation in beet roots. Liu & Lewellen (2007) also found virus with an ‘A’ in position 67 of P25 among the resistance-breakers. This may indicate that mutations in other genome areas or additional factors such as differences in vector efficiency or the presence of other viruses producing rhizomania-like symptoms, e.g. beet black scorch virus (Weiland et al., 2006), could be involved in resistance-breaking phenomena.

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References


Influence of RNA 3 variations on BNYVV accumulation


