Identification of amino acids of the beet necrotic yellow vein virus p25 protein required for induction of the resistance response in leaves of Beta vulgaris plants

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The RNA3-encoded p25 protein of beet necrotic yellow vein virus (BNYVV) is responsible for the production of rhizomania symptoms of sugar beet roots (Beta vulgaris subsp. vulgaris). Here, it was found that the presence of the p25 protein is also associated with the resistance response in rub-inoculated leaves of sugar beet and wild beet (Beta vulgaris subsp. maritima) plants. The resistance phenotype displayed a range of symptoms from no visible lesions to necrotic or greyish lesions at the inoculation site, and only very low levels of virus and viral RNA accumulated. The susceptible phenotype showed large, bright yellow lesions and developed high levels of virus accumulation. In roots after Polymyxa betae vector inoculation, however, no drastic differences in virus and viral RNA accumulation levels were found between plants with susceptible and resistant phenotypes, except at an early stage of infection. There was a genotype-specific interaction between BNYVV strains and two selected wild beet lines (MR1 and MR2) and sugar beet cultivars. Sequence analysis of natural BNYVV isolates and site-directed mutagenesis of the p25 protein revealed that 3 aa residues at positions 68, 70 and 179 are important in determining the resistance phenotype, and that host-genotype specificity is controlled by single amino acid changes at position 68. The mechanism of the occurrence of resistance-breaking BNYVV strains is discussed.

INTRODUCTION

Hypersensitive resistance is a generalized active response in plants against pathogens, resulting in rapid cell death and restriction of plant growth at the site of infection. Usually, hypersensitive resistance is a highly specific event that depends on a matching specificity between a disease resistance gene (R) in the plant and an avirulence gene (avr) in the pathogen. This genetic model has been interpreted as a receptor–ligand interaction in which an Avr ligand binds directly to a corresponding R protein to initiate a resistance reaction (Flor, 1971; Keen, 1990). However, several avr genes are known to enhance pathogen virulence in susceptible plants (Kjemtrup et al., 2000; Van’t Slot & Knogge, 2002) and therefore an alternative ‘guard’ model has been proposed in which Avr proteins interact with certain host proteins (pathogenicity targets) to promote disease in the absence of their corresponding R proteins (Van der Biezen & Jones, 1998; Dangl & Jones, 2001).

In plant viruses that have very small genomes, different virus proteins, including viral RNA polymerase, coat protein (CP), movement protein or, in potyviruses, genome-linked protein, cytoplasmic inclusion protein, P3 protein and NIa protease, have been identified as Avr determinants (Harrison, 2002; Kang et al., 2005). In one case, an RNA sequence was also shown to act as an Avr factor (Szittya & Burgyan, 2001). Some of these Avr determinants have been reported to function as pathogenic determinants; for instance, turnip crinkle virus CP (Ren et al., 2000; Qu et al., 2003), cucumovirus 2b protein (Li et al., 1999), cauliflower mosaic virus transactivator/viroplasmin protein (Kobayashi & Hohn, 2004) and potyvirus P3 protein (Johansen et al., 2001; Jenner et al., 2003).

Beet necrotic yellow vein virus (BNYVV, genus Benyvirus) causes rhizomania disease of sugar beet (Beta vulgaris subsp. vulgaris) and is transmitted by the soil-inhabiting plasmidophorid Polymyxa betae. The genome of BNYVV consists generally of four, or in some isolates five, distinct positive-stranded RNA components (Tamada, 1999). RNA1 and RNA2 encode ‘house-keeping’ genes involved in viral RNA replication, assembly and cell-to-cell movement. RNA3,
Role of BNYVV p25 as an avirulence determinant

RNA4 and RNA5 are associated with vector-mediated infection and disease development in sugar beet roots. The RNA3-encoded p25 protein is responsible for the induction of rhizomania symptoms of sugar beet roots and severe symptom expression in Chenopodiaceae hosts (Tamada et al., 1989, 1999; Lupin et al., 1992; Klein et al., 2007), whereas RNA4-encoded p31 is involved in efficient vector transmission and root-specific silencing suppression (Rahim et al., 2007). RNA5, which is also associated with symptom severity, has been found in small areas of Europe and in most areas of Asia (Miyanishi et al., 1999; Schirmer et al., 2005; Ward et al., 2007).

Rhizomania is one of the most economically important diseases of sugar beet and is widely present in sugar beet-growing areas worldwide. Sugar beet production areas have become dependent on resistant sugar beet cultivars for control of the disease. Sugar beet cultivar Rizor was developed as a first-generation BNYVV-resistant crop, and subsequently the sugar beet Holly resistance source (with the Rz1 resistance gene), which has much higher levels of resistance to BNYVV, has been widely exploited in most of the current cultivars (Lewellen et al., 1987; Asher, 1993; Rush, 2003). Recently, however, severe symptoms have been found in resistant cultivars with the Rz1 gene in some areas of the USA and Europe, indicating the emergence of resistance-breaking BNYVV variants (Liu et al., 2005; Acosta-Leal & Rush, 2007; Liu & Lewellen, 2007). It has also been suggested that mutations in the RNA3-encoded p25 protein may be related to such resistance-breaking virus variants. So far, assessment of the pathogenicity of BNYVV strains has mainly been based on symptom expression in the field.

Although the mechanism of resistance to BNYVV remains unknown, the resistance of sugar beet cultivars has been reported to be due to a restriction of virus multiplication and translocation in the roots (Scholten et al., 1994; Tamada et al., 1999). Our previous studies suggested that p25 is involved in this inhibition of virus translocation in the taproots of the resistant cultivar Rizor (Tamada et al., 1999). To simplify studies of this resistance mechanism, various BNYVV isolates were rub-inoculated onto the leaves of sugar beet and wild beet (B. vulgaris subsp. maritima) plants; the results showed that resistance and susceptibility to BNYVV could be assessed based on the phenotype of the inoculated leaves (Tamada, 2007). Considering previous studies that demonstrated the role of RNA3-encoded p25 in the exacerbation of virus-induced symptoms on inoculated leaves (Jupin et al., 1992; Tamada et al., 1999; Klein et al., 2007) and the role of its subcellular localization (Vetter et al., 2004), we assumed that p25 could be involved in the resistance response of the inoculated leaves.

In this study, we present evidence that the BNYVV p25 protein functions as an Avr factor in leaves of resistant B. vulgaris plants and that there is a virus strain–cultivar-specific resistance interaction. In addition, we identified amino acid residues in p25 that control this host-specific resistance.

METHODS

Plant materials, virus isolates and inoculations. Tetraogonia expansa plants were used for virus propagation (Tamada et al., 1989). Sugar beet cultivars Monomidori (susceptible) and Rizor (resistant) and the wild beet B. vulgaris subsp. maritima lines MR0, MR1 and MR2 were used (Tamada, 2007).

BNYVV wild-type (wt) isolates O11 and GW, each containing RNA1–RNA4, were used. The laboratory isolates O11-4 and GW-4, both of which lack RNA3, were obtained from the original O11 and GW isolates by single-lesion transfers on T. expansa leaves as described previously (Tamada et al., 1989). The other six wt BNYVV isolates and their origins are listed in Table 1. Each of the virus isolates was recovered from infested soil as described previously (Miyanishi et al., 1999). The IV4, IP7, USTH and T41 isolates contained RNA1–RNA4, whilst the FP isolate contained the additional RNA5. To eliminate the effect of RNA5, a laboratory isolate lacking RNA5 was selected by single-lesion transfers in T. expansa.

Foliar rub-inoculation and root vector inoculation using P. betae were conducted as described previously (Tamada et al., 1989; Tamada, 2007; Rahim et al., 2007).

Construction of infectious cDNA clones and site-directed mutagenesis. General recombinant DNA techniques were performed using standard procedures (Sambrook et al., 1989). Two full-length RNA3 cDNA clones, pT3O and pT3G, of BNYVV O11 and GW, respectively, were amplified by RT-PCR from purified RNAs of each virus isolate. First-strand cDNA synthesis was conducted using Moloney murine leukemia virus reverse transcriptase (Invitrogen) with an oligo(dT) primer containing a Sall site (italics): 5′-GACCAAGGCTATCGATGTCGACTTTTTTTTTTTTTTTTTT-3′. The resulting negative-strand cDNA was used for PCR, together with a forward primer (5′-GGAATTCATACGACATCAGATAAAATCCATAATCCATAATCCATTA-3′) containing an EcoRI site (italics) and a T7 promoter sequence (underlined) at the 5′ end of RNA3, and a reverse primer (5′-GACCAAGGCTATCGATGTCGAC-3′) containing a Sall site (italics). The resulting PCR fragments were ligated into the EcoRI/Sall sites of pUC19. All point mutations were created by PCR-based overlap-extension mutagenesis. In all cases, the mutant constructs were confirmed by restriction enzyme digestion and sequence analysis.

cDNA plasmids were linearized by Sall and transcribed in vitro in the presence of a cap analogue (New England Biolabs) using a Ribomax Large Scale RNA production system T7 (Promega). For infection experiments, a freshly prepared transcript from each plasmid was mixed with viral RNA partially purified from O11-4 (or GW-4) inoculated T. expansa leaves. The mixture was inoculated onto the leaves of T. expansa. Infected leaves were harvested 8–10 days post-inoculation (p.i.) and used as an inoculum source. The progeny mutant viruses from rub-inoculated leaves or vector-inoculated roots of beet plants were confirmed by sequence analyses as described below.

Nucleotide sequencing and sequence comparisons. Sequences of RNA3 from natural BNYVV isolates were obtained as described previously (Miyanishi et al., 1999). Two specific primers, 3F (5′-AGTTGTTGTTGTCTTCTGATC-3′, nt 408–427) and the reverse primer 3R (5′-CCGTTGAATATCGTGAGTTT-3′, complementary to nt 1249–1268) were used (Saito et al. 1996). PCR products were ligated into a pGEM-T vector (Promega) and transformed into E. coli DH5α (Promega) and transformed into Escherichia coli strain XL1-Blue. The nucleotide sequences of selected clones were determined using a 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).
Biosystems). At least three independent clones for each gene were sequenced. Sequence data were analysed using GENETYX-MAC (SDC).

**Northern blot analysis.** Northern blot analysis from the inoculated leaves and roots of the plants were conducted as described previously (Andika et al., 2005). Total RNA was extracted from 150–300 mg leaf and root tissues. The blot was hybridized with digoxigenin-labelled DNA probes specific for the BNYVV RNA1 (nt 5815–26531), RNA2 (nt 144–711) and RNA3 sequences (nt 444–1104) (Saito et al., 1996). Equal loading was verified by visualization of ethidium bromide-stained 28S rRNA.

**ELISA and tissue immuno-imprinting analyses.** ELISA and tissue immuno-imprinting assay were conducted as described previously (Andika et al., 2005).

### RESULTS

#### Response of beet plants to foliar rub-inoculation and root vector inoculation

Although resistance to BNYVV has generally been estimated by the degree of virus infection in the roots of sugar beet plants, our previous work showed that the resistance response to BNYVV could be evaluated, at least partially, on the basis of the phenotype of the inoculated leaves of infected beet plants after foliar rub-inoculation (Tamada, 2007). Thus, the susceptible phenotype develops bright yellow lesions, whereas the resistant phenotype has no visible lesions or necrotic lesions at the inoculation site. When the two wt BNYVV isolates O11 and GW were rub-inoculated onto the leaves of the susceptible sugar beet cultivar Monomidori and the resistant cultivar Rizor, these two isolates produced bright yellow lesions on inoculated leaves of the Monomidori plants (Fig. 1a). In Rizor, however, O11 produced either no visible lesions or small necrotic lesions on the infection sites, whereas GW produced bright yellow symptoms (Fig. 1a). Thus, the resistance response to BNYVV in the sugar beet cultivars is dependent on the BNYVV isolate.

To examine virus behaviour within the roots of such resistant and susceptible plants and compare it with the foliar response, roots of Monomidori and Rizor plants were inoculated with *P. betae* cultures carrying O11 or GW. At 2–5 weeks after vector inoculation, the levels of virus and viral RNA accumulation in roots were tested by ELISA and Northern blotting, respectively. At 14 days p.i., levels of viral RNA accumulation in Rizor roots infected with O11 were slightly lower than in those infected with GW (Fig. 2a, compare lanes 7–9 with lanes 10–12). In Monomidori, viral RNAs accumulated at much higher levels in roots infected with either O11 or GW (Fig. 2a, compare lanes 1–3 with lanes 4–6). After 3 weeks or more, no clear differences in the accumulation levels of virus and viral RNA in roots were found either between virus isolates or between susceptible and resistant cultivars (data not shown).

Previously, the wild beet *B. vulgaris subsp. maritima* differential lines MR0, MR1 and MR2 were selected on the basis of phenotype following foliar rub-inoculation; thus, MR0 is a line that is susceptible to BNYVV O11, whilst both MR1 and MR2, which have a different host response, are lines that are resistant to the same O11 virus (Tamada, 2007; Table 1). These three lines were inoculated with O11-carrying *P. betae* cultures. After 8 days to 3 weeks, levels of viral RNA accumulation in roots were analysed by Northern blotting. At 8 days p.i., the accumulation levels of viral RNAs in virus-infected MR2 roots differed among individual plants ranging from high to undetectable (Fig. 2b, lanes 5–8), whilst the MR0 roots had higher levels of RNA accumulation (Fig. 2b, lanes 1–4). However, at 14 days p.i., viral RNAs in MR2 roots accumulated at levels similar to those in MR0 roots (Fig. 2b, lanes 9–16). At 21 days p.i., no differences were found between MR0 and MR2 roots (data not shown). In parallel experiments carried out in MR1 plants, no

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**Table 1.** Origin of the BNYVV isolates used in this study and their phenotypes on *B. vulgaris subsp. maritima* and sugar beet cultivars Monomidori and Rizor

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Location</th>
<th><em>B. vulgaris subsp. maritima</em></th>
<th>Sugar beet cultivars</th>
<th>GenBank accession no. for RNA3</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>MR0</td>
<td>MR1</td>
<td>MR2</td>
</tr>
<tr>
<td>O11</td>
<td>Obihiro, Japan</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S113</td>
<td>Sobetsu, Japan</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>FP*</td>
<td>Pithiviers, France</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>IV4</td>
<td>Veneto, Italy</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>IP7</td>
<td>Puglia, Italy</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>USTH</td>
<td>Texas, USA</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>T41</td>
<td>Tsubetsu, Japan</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>GW</td>
<td>Wallerstadten, Germany</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>

*The natural isolate FP contains RNA5, but a laboratory isolate lacking RNA5 was used here.*
apparent differences in viral RNA accumulation were observed between MR1 and MR0 roots at any time p.i. (data not shown).

Taken together, these data indicate that there were no drastic differences in virus and viral RNA accumulation levels between the roots of beet plants with the resistance and susceptible phenotypes. However, at the early stage of infection, levels of virus accumulation in the roots of resistant plants were slightly lower compared with those in

roots of susceptible plants. Sugar beet cultivar Rizor and wild beet MR2 showed a similar response in foliar rub-inoculation (see below; Tamada, 2007), but roots of the Rizor plants showed higher levels of resistance than those of wild beet MR2 in root vector inoculation.

BNYVV RNA3 is responsible for the resistance and susceptible phenotypes

To confirm the involvement of RNA3 in the resistance and susceptible phenotypes in foliar rub-inoculation, laboratory RNA3-lacking isolates O11-4 and GW-4 were used as inocula. The wt viruses O11 and GW produced bright yellow lesions on inoculated leaves of T. expansa, whereas O11-4 and GW-4 viruses produced faint chlorotic lesions (data not shown; Tamada et al., 1989). However, these RNA3-lacking viruses did not induce clear visible symptoms in MR0 and MR1 plants (Fig. 1b). Therefore, BNYVV infection of these plants was monitored by a tissue immuno-imprinting assay using leaf halves inoculated with O11 or GW or their RNA3-lacking mutants O11-4 and GW-4 (—).

Fig. 1. Symptoms on inoculated leaves of beet plants rub-inoculated with wt BNYVV O11 or GW, or their RNA3-lacking mutant. Symptoms are shown as bright yellow lesions (YS), necrotic lesions (NS), small necrotic lesions (SNS) and no visible lesions (No). The leaves were photographed at 9 days p.i. (a) Symptoms in susceptible sugar beet cultivar Monomidori and resistant cultivar Rizor. (b) Symptoms and tissue immuno-imprinting (TI) for inoculated leaves of MR0 and MR1 plants inoculated with O11 and GW (indicated as RNA3) and their RNA3-lacking mutants O11-4 and GW-4 (—).

Fig. 2. Northern blot analysis of total RNA from the roots of beet plants inoculated with BNYVV-carrying P. betae. Each lane shows an individual plant. The rows show detection of BNYVV RNA1, RNA2 and RNA3 and a loading control of ethidium bromide-stained 28S rRNA. (a) Sugar beet plant cultivars Monomidori and Rizor inoculated with O11- or GW-carrying P. betae culture. Samples were collected at 14 days p.i. (b) B. vulgaris subsp. maritima MR0 and MR2 plants inoculated with O11-carrying P. betae culture. Samples were collected at 8 and 14 days p.i.
only GW produced yellow lesions. The presence of O11 RNA3 induced a drastic reduction in the number of infection foci (Fig. 1b). These data indicated that wt BNYVV O11 RNA3 is required for the production of both resistance and susceptible phenotypes.

To examine whether viral components other than RNA3 in BNYVV O11 are involved in induction of the resistance phenotype, we performed reassortment experiments using RNA3 transcripts from the two BNYVV isolates O11 and GW (pT3O and pT3G, respectively; see below). Inoculation tests showed that GW-4-containing pT3O induced the resistance phenotype in MR1 plants, whereas O11-4-containing pT3G produced the susceptible phenotype (data not shown). This result further indicated that the resistance phenotype induced by BNYVV O11 is dependent on either the p25 protein or the RNA3 sequence encoding it.

**Phenotypes of natural BNYVV isolates with different amino acid sequences in the p25 protein**

In addition to the two BNYVV isolates O11 and GW described above, we examined a further six BNYVV isolates (S113, FP, IV4, IP7, USTH and T41) with different amino acid sequences in the p25 protein (see Fig. 4). These isolates were selected as representatives of the many BNYVV isolates collected worldwide. All isolates induced the same bright yellow lesions on inoculated leaves of *T. expansa* as those seen with O11 and GW (data not shown). They were then inoculated onto the leaves of *B. vulgaris* subsp. *maritima* MR0, MR1 and MR2 and sugar beet cultivars Monomidori and Rizor, and their phenotypes were observed. At 8 days p.i., total RNA from the inoculated leaves (except for the FP isolate) was analysed by Northern blotting. The O11 isolate induced the resistance phenotype in MR1 (necrotic lesions) and MR2 (no visible lesions) (Table 1, Fig. 3a). The three isolates S113, FP and IV4 exhibited the susceptible phenotype in MR1 plants, but the resistance phenotype in MR2 plants (Table 1, Fig. 3a). However, S113 and IV4 induced small necrotic lesions in MR2, whereas O11 did not. The other four isolates, IP7, USTH, T41 and GW, showed the susceptible phenotype in both MR1 and MR2 plants (Table 1, Fig. 3a). Northern blot analysis showed that viral RNAs accumulated to very low levels or were below the detection limit in inoculated leaves displaying the resistance phenotype (Fig. 3b). In contrast, high levels of viral RNA accumulation were observed in...
Identification of amino acids that determine the resistance phenotype in MR1 and MR2 plants

To identify which amino acid residues at position 68 in the p25 protein are important for the resistance phenotype determinant, we first constructed four single mutants in which Phe at position 68 of the p25 protein of RNA3 (pT3O) in BNYVV-O11 was replaced by Tyr (F68Y), His (F68H), Cys (F68C) or Ala (F68A) (Table 2). Transcripts from cDNAs were co-inoculated with RNA of O11-4 (RNA3-lacking virus) into *T. expansa* leaves, in which all virus mutants produced yellow spot lesions (data not shown). As shown in Fig. 5(a), the F68Y and F68H mutations induced the resistance phenotype in MR2 plants and the susceptible phenotype in MR1 plants. Inoculation of MR2 plants with F68H resulted in large numbers of necrotic lesions, and accumulation levels of viral RNAs were higher than those inoculated with the wt virus (pT3O) or the mutant virus F68Y (Fig. 5b). The other two single mutants (F68C and F68A) failed to show any resistance phenotype (Table 2). Levels of viral RNA accumulation in leaves with the resistance phenotype were lower than in the susceptible phenotype (Fig. 5b), except for the F68C mutant, which bypassed the resistance mechanism and accumulated more than the control (Fig. 5b, compare RNA1 and RNA2 accumulation for F68C and O11-4). In leaves showing the resistance phenotype, it is noteworthy that levels of RNA3 accumulation were very low or below the detection limits (Fig. 5b). The presence of RNA1 and RNA2 in such plants probably arises from preferential multiplication of RNA3-lacking mutants in the inoculated leaves, as shown with O11-4 and demonstrated earlier (Fig. 1b). Taken together, these data indicate that single amino acid residues at position 68 in the p25 protein control the degree of resistance in MR1 and MR2 plants; thus, the p25 protein with Phe-68 may act as a stronger elicitor than p25 proteins with Tyr-68 or His-68.

In the case of p25 proteins with Tyr-68 as mentioned above, the S113 and FP isolates induced the resistance phenotype in MR2 plants, but GW and T41 isolates did not. From a comparison of amino acid sequences of the p25 proteins of these isolates (Fig. 4), we particularly noticed amino acid changes at position 179 [Asn (N) and Asp (D)] and at position 70 [Gly (G) and Arg (R)] (Fig. 4). To examine the effect of these amino acid residues, we produced single or double amino acid substitutions in a pT3G (wt GW) background. Infection with the double mutant (R70G/D179N) produced the resistance phenotype in MR2 plants, whilst the single mutant (R70G) produced the susceptible phenotype (Table 2), indicating that Asn-179 is involved in the resistance phenotype.

To confirm further the effect of amino acid residues at positions 68, 70 and 179, we then produced double or triple amino acid substitutions in a pT3G background. As shown in Table 2, infection with a triple mutant (Y68F/R70G/D179N) resulted in the resistance phenotype in both MR1 and MR2 plants, whereas double mutants (Y68F/
R70G and Y68F/D179N) produced the susceptible phenotype in either MR1 or MR2. This result further indicated that both Gly-70 and Asn-179 are needed to produce the resistance phenotype.

Taken together with the results from the natural isolates, we concluded that, in addition to amino acid residues (either Phe or Tyr) at position 68, 2 aa residues, Gly and Asn, at positions 70 and 179, respectively, are important in determining the resistance phenotype. A Phe at position 68 is a crucial amino acid residue for the resistance determinant in MR1 plants. However, in the case of the S113 isolate (with Tyr-68), Arg and Val are at positions 69 and 70, respectively (Fig. 4), suggesting that either or both amino acids may be involved in producing the resistance phenotype, although this was not confirmed in this study.

**DISCUSSION**

Our previous and present studies demonstrate that resistance and susceptibility to BNYVV can be assessed, at least partially, based on phenotypes produced after foliar rub-inoculation (Tamada, 2007). The resistant plants displayed a range of symptoms from no visible lesions to necrotic lesions, and only very low levels of virus accumulated, whereas the susceptible plants showed bright yellow lesions and developed high levels of virus accumulation. We also found that these phenotypes were determined by the RNA3-encoded p25 protein and that there was a virus strain–cultivar-specific resistance interaction. In addition, the 2 aa residues 68 and 70 within the p25 tetrad sequence (Schirmer et al., 2005; Klein et al., 2007) and residue 179 were identified as key factors in determining the resistance response, and single amino acid residues at position 68 were found to control the host-genotype specificity. In our preliminary analyses using Western blotting, the p25 protein was consistently detected in leaves showing the susceptible phenotype, but not in leaves with the resistance phenotype (data not shown). It appeared to be difficult to detect the p25 protein in resistant plants in which BNYVV replication had been strongly suppressed. Thus, it remains unclear how p25 expression levels relate to the resistance response, although Klein et al. (2007) demonstrated that foliar responses were independent of the p25 expression level and were mainly due to the tetrad sequence variation.

In contrast to foliar rub-inoculation, in root vector inoculation, no apparent differences in virus accumulation levels in roots were found between plants having resistant and susceptible phenotypes. We observed a lower accumulation of the virus in resistant plants when compared with the susceptible plants, but only at the early stage of infection (1–2 weeks, depending on the plant line or cultivar) (Fig. 2). This may be explained by the assumption that resistance in the leaf and root operates by a similar mechanism; perhaps most of the roots are infected with viruliferous primary zoospores of *P. betae*. However, by 2 weeks or later, newly developing roots are continuously infected with the secondary zoospores produced, unlike artificial leaf inoculation, so that virus accumulation in roots may easily reach high or maximum levels, even if the

### Table 2. Constructs of BNYVV RNA3-encoded p25 mutants and their phenotypes on inoculated leaves of *B. vulgaris* subsp. *maritima* MR0, MR1 and MR2

<table>
<thead>
<tr>
<th>p25 clone (mutation)*</th>
<th>Amino acid substitution at position:</th>
<th>Phenotype in <em>B. vulgaris</em> subsp. <em>maritima</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>68</td>
<td>70</td>
</tr>
<tr>
<td>pT3O (wt O11)</td>
<td>F</td>
<td>G</td>
</tr>
<tr>
<td>pT3O (F68Y)</td>
<td>F→Y</td>
<td>G</td>
</tr>
<tr>
<td>pT3O (F68H)</td>
<td>F→H</td>
<td>G</td>
</tr>
<tr>
<td>pT3O (F68C)</td>
<td>F→C</td>
<td>G</td>
</tr>
<tr>
<td>pT3G (wt GW)</td>
<td>Y</td>
<td>R</td>
</tr>
<tr>
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<td>Y</td>
<td>R→G</td>
</tr>
<tr>
<td>pT3G (R70G/D179N)</td>
<td>Y→F</td>
<td>R→G</td>
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resistance response has occurred in the roots. Under natural conditions, however, the resistance of sugar beet cultivars is known to be due to restriction of virus multiplication and translocation in taproots rather than in rootlets (Scholten et al., 1994; Tamada et al., 1999). Taken together, we consider that resistance in the root and leaf operate by a similar mechanism to some extent, but that resistance appearing in leaves is remarkably stronger than that appearing in roots by vector infection. In this regard, it is noteworthy that RNA silencing-mediated resistance to BNYVV is less effective in roots than in leaves (Andika et al., 2005, 2006).

The sugar beet cultivar Rizor used in this study was developed as a first-generation BNYVV-resistant cultivar (Asher, 1993). BNYVV resistance is known to be widespread in wild beet B. vulgaris subsp. maritima (Whitney, 1989; Geyl et al., 1995), from which the three differential lines MR0, MR1 and MR2 were selected by foliar rub-inoculation (Tamada, 2007). The infection phenotype in MR2 plants was similar to that in Rizor plants (Table 1, Fig. 3). However, a resistance response such as that seen in the Rizor plants has not been observed in other resistant material such as Holly1-4 plants (containing the Rz1 gene), which have much higher levels of resistance to BNYVV (Scholten et al., 1994): thus, BNYVV induced bright yellow lesions on the inoculated leaves of Holly1-4 plants, independent of the identity of the BNYVV isolates, although yellow lesions in these plants were much smaller in size than those in susceptible beet plants (T. Tamada, unpublished results). These observations suggest that the resistance mechanism to BNYVV in the Rizor source may differ from that in the Holly source. Alternatively, in Holly1-4 plants, BNYVV resistance mechanisms in the roots may be different from those in the leaves as described above.

Although such a resistance mechanism to BNYVV in roots represents a higher degree of complexity, by using the wild beet B. vulgaris subsp. maritima differential lines selected by Tamada (2007) we have assessed pathogenic differences between natural BNYVV isolates and site-directed mutant viruses. Consequently, the different responses of various BNYVV isolates were found to be due to the amino acid residues at position 68 in the p25 protein, with p25 proteins harbouiring Phe-68 acting as a stronger elicitor than p25 proteins with Tyr-68 or His-68. For example, MR1 plants are resistant to isolate O11 with Phe-68, but not to isolate S113 with Tyr-68 or isolate IV4 with His-68. In MR2 plants, O11 (with Phe-68) usually induced no symptoms, but IV4 (with His-68) virus or the pT3O mutant containing His-68 induced larger necrotic symptoms in which the p25 elicitor seemed to be less active. In contrast, isolates IP7 (with Leu-68) or USTH (with Cys-68) showed bright yellow susceptible phenotypes in MR1 and MR2 plants. Possibly, differences in the nature of the amino acid residue at position 68 may affect the accessibility of host factors that specifically recognize p25 or act on p25 oligomerization, as stated previously (Klein et al., 2007). These results also suggest the possibility that single amino acid changes (Phe, Tyr or His→Leu or Cys) at position 68, or amino acid changes at position 70 (Gly→Arg) or 179 (Asn→Asp), may result in the occurrence of resistance-breaking virus strains.

Indeed, sequence analyses of BNYVV isolates collected worldwide revealed that there were sequence variations in p25, in which a tetrad amino acid motif was identified as a variable region (Fig. 4; Schirmer et al., 2005). Interestingly, strong-positive selection pressure has been exerted on the p25 gene, but not on the CP gene (Schirmer et al., 2005; T. Tamada, unpublished data). It is thus suggested that the large-scale or continuous planting of sugar beet cultivars with the first Rizor-resistant source and subsequent Rz1-resistant source in past years (Asher, 1993; Rush, 2003) may have caused significant selection pressure on the virus, leading to partial or total breakdown of resistance as observed in other virus–host interactions (Harrison, 2002). This raises the possibility that amino acid or sequence changes other than those identified in this study may be associated with the emergence of virus isolates breaking the Rz1-mediated resistance (Liu et al., 2005; Acosta-Leal & Rush, 2007; Liu

Fig. 5. Phenotypes and viral RNA accumulation in inoculated leaves of B. vulgaris subsp. maritima lines MR1 and MR2 following rub-inoculation with the BNYVV RNA3-encoded p25 mutants shown in Table 2. See Fig. 3 for phenotype abbreviations. (a) Phenotypes induced by the mutant viruses on inoculated leaves at 9 days p.i. (b) Northern blot analysis of total RNA from the inoculated leaves shown in (a). The three BNYVV RNAs and a loading control of ethidium bromide-stained 28S rRNA are shown. The phenotype of a mock-inoculated control leaf or an O11-4-inoculated leaf is indicated by a dash.
& Lewellen, 2007). We are currently conducting further research on resistance mechanisms in roots.

It is not clear how BNYVV p25 is involved in the resistance response. Our observations that p25-lacking or -defective mutant virus can replicate and move from cell to cell in the leaves of either susceptible or resistant plants, and that, in incompatible interactions, tiny lesions are often observed at the inoculation site, suggest that p25 is involved in suppression (inhibition) of cell-to-cell movement rather than virus replication. It is thus likely that the virus can replicate in primary infected cells, but that its movement to adjacent cells is blocked or its accumulation is suppressed. There are many studies showing that viral Avr factors are involved in suppression of cell-to-cell movement in hosts (Harrison, 2002; Kang et al., 2005), but BNYVV is a unique virus in the sense that p25, encoded by one of the extra genomic RNAs and separate from the viral major genes encoding replicase, protease, movement protein and CP, has a dual function as a virulence and Avr determinant.

The BNYVV–host interactions are rather similar to interactions that occur between fungal or bacterial pathogens and their hosts. In particular, the behaviour of the p25 protein may be comparable to that of members of the bacterial AvrBs3 protein family of Xanthomonas (Lahaye & Bonas, 2001). Thus, both proteins are recognized inside the plant cell, and they contain a functional nuclear localization signal (NLS) and acidic transcriptional activation domain (Van den Ackerveken et al., 1996; Vetter et al., 2004; Klein et al., 2007). The NLS in both proteins interacts with pepper and rice importin-α (Szurek et al., 2001; Vetter et al., 2004), suggesting that both the AvrBs3 protein and the p25 protein can recruit the host’s nuclear import machinery and that their targets are in the nucleus (Lahaye & Bonas, 2001). The copy number of the 34 aa repeat unit of the AvrBs3 protein determines the specificity of recognition by the Bs3 resistance gene (Herbers et al., 1992), whereas in p25 protein it is determined by single amino acid changes (Table 2) that influence known properties of p25 (Klein et al., 2007). Some members of the AvrBs3 family are involved in disease symptom formation (Swarup et al., 1992; Yang et al., 1996; Bai et al., 2000), and in those cases, the protein displays a virulence phenotype in one host, but not in other hosts. In contrast, p25 is responsible for the induction of rhizomania disease (Tamada et al., 1999) and clearly functions at the cultivar-specific level. Finally, the p25 protein is dispensable for the basic virus life cycle in foliar rub-inoculated plants, and thus the BNYVV pathosystem will be useful for further studies of the molecular basis of plant–pathogen interactions.

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REFERENCES


