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Research Note

A Single Nucleotide Substitution in the αa Gene Confers Oat Pathogenicity to Barley Stripe Mosaic Virus Strain ND18

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A 236-nucleotide region from the αa gene of strain CV42 (pathogenic to oat), when substituted for the homologous region in strain ND18 (nonpathogenic to oat), was shown previously to confer a near wild-type oat pathogenicity to this strain (Weiland and Edwards, 1994, Virology 201: 116-126). The data suggested that six amino acid substitutions in the αa gene were responsible for the differences in oat pathogenicity, and that threonine-724, encoded by CV42, might be a critical amino acid in determining pathogenicity of barley stripe mosaic virus (BSMV) to oat. In the present work, codons specifying T-724, I-764, and N-785 (encoded by CV42 RNA α) were substituted individually and in combination for those coding for P-724, T-764, and K-785 (encoded by ND18 RNA α), respectively, by site-directed mutagenesis. The core K-733, T-734, and K-736 positions (CV42) were substituted for Q-733, S-734, and Q-736 (ND18) as a single block. The results of inoculations with these mutants indicate that the $C^{2261}\rightarrow A^{2261}$ nucleotide substitution (P-724→T-724) by itself is sufficient to enable strain ND18 to infect oat plants, although poorly. Additional substitution of CV42 codons into ND18 RNA α at the remaining five positions altered symptom type, decreased the timing of the appearance of symptoms, and increased the percentage of plants infected per inoculation. Nonetheless, all mutants accumulated to similar levels in inoculated oat protoplasts after a 24-h period. Using a recombinant RNA β from which β-glucuronidase could be expressed, results were obtained suggesting that the multiplication of strain ND18 and the nonpathogenic variants generated in the study was restricted in the inoculated leaf. The data indicate a potential pathway by which pathogenicity to oat evolved in BSMV.

Additional keywords: host range, movement, replication.

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The reaction of a plant host to a viral pathogen is dictated by the genetics of both the host and pathogen, and by environmental factors. In recent years, data have been produced characterizing the genetic determinants of plant viral pathogenesis. Examples of viral pathogenicity determinants that reside in RNA virus structural genes, movement genes, and replication-associated genes (for recent reviews, see Dawson 1992; De Jong and Ahlquist 1991; Fraser 1990), as well as noncoding genomic regions (Petty et al. 1990a), have been reported for a number of cloned viruses. Plant genetic elements that determine resistance to plant viruses are now beginning to be cloned and characterized (Köhm et al. 1993; Whitham et al. 1994).

Barley stripe mosaic virus (BSMV) is the type member of the tripartite plant Hordeiviruses, and is a natural pathogen of cereal crops (reviewed by Jackson et al. 1991). Roles in pathogenesis for the genes encoded by BSMV have been determined using reverse genetics on cloned DNA from which infectious RNA can be transcribed. Whereas RNAs α and γ encode genes essential for the replication of the viral genome, RNA β encodes genes required for movement of the virus in planta and for virus encapsidation (Petty et al. 1990b; see Figure 1A). RNA α encodes a single gene (the αa gene) with a predicted protein product of 130 kDa (Gustafson et al. 1989). The primary amino acid sequence of the αa protein, as predicted from the αa gene nucleotide sequence, indicates that the protein may possess methyltransferase (Rozanov et al. 1992) and nucleic acid helicase (Gorbalenya and Koonin 1989) activities. This is consistent with the required role that the αa gene plays in virus replication.

Recently, we demonstrated that the αa gene also harbors determinants of pathogenicity to oat. Recombinants were generated between the α RNAs of BSMV strains CV42 (pathogenic to oat) and ND18 (nonpathogenic to oat) (Weiland and Edwards 1994). The data indicated that a 236-nucleotide (nt) region of the CV42 αa gene, when substituted for the homologous region in ND18 RNA α, enabled the recombinant ND18 strain to infect oat. Six amino acids encoded in this region differ between the two strains (Fig. 1B), and it was suggested that these differences may determine oat pathogenicity in BSMV. Thus, a recombinant ND18 strain encoding all six amino acids from CV42 possessed near wild-type pathogenicity to oat, whereas a recombinant harboring only the K-733, T-734, and K-736 changes from CV42 was

nonpathogenic to oat. These results revealed the potential importance of the αa protein amino acids T-724, I-764, and N-785 in the pathogenicity of strain CV42 to oat. Although the data using recombinant genomes suggested that the amino acid differences in the αa proteins of ND18 and CV42 were the basis for the differential pathogenicity of these strains to oat, silent nucleotide substitutions were also present in the recombinants used in that study.

In order to ascertain whether the amino acids encoded in the 236-nt region of CV42 may be the determinants of oat pathogenicity, we here used site-directed mutagenesis to change the amino acids encoded by the ND18 αa gene so as to reflect the differences between CV42 RNA α and ND18 RNA α in this region. These changes were introduced into the ND18α cDNA clone individually and in combination, and their effects on oat pathogenicity were analyzed (Fig. 2). The suffix "sd" is used to denote those virus variants created by site-directed mutagenesis that have a counterpart variant previously generated by recombination (Weiland and Edwards 1994). Lowercase lettering in mutant nomenclature indicates a residue that is encoded by strain ND18. All cloning, mutagenesis, transcript production, and Northern and Western

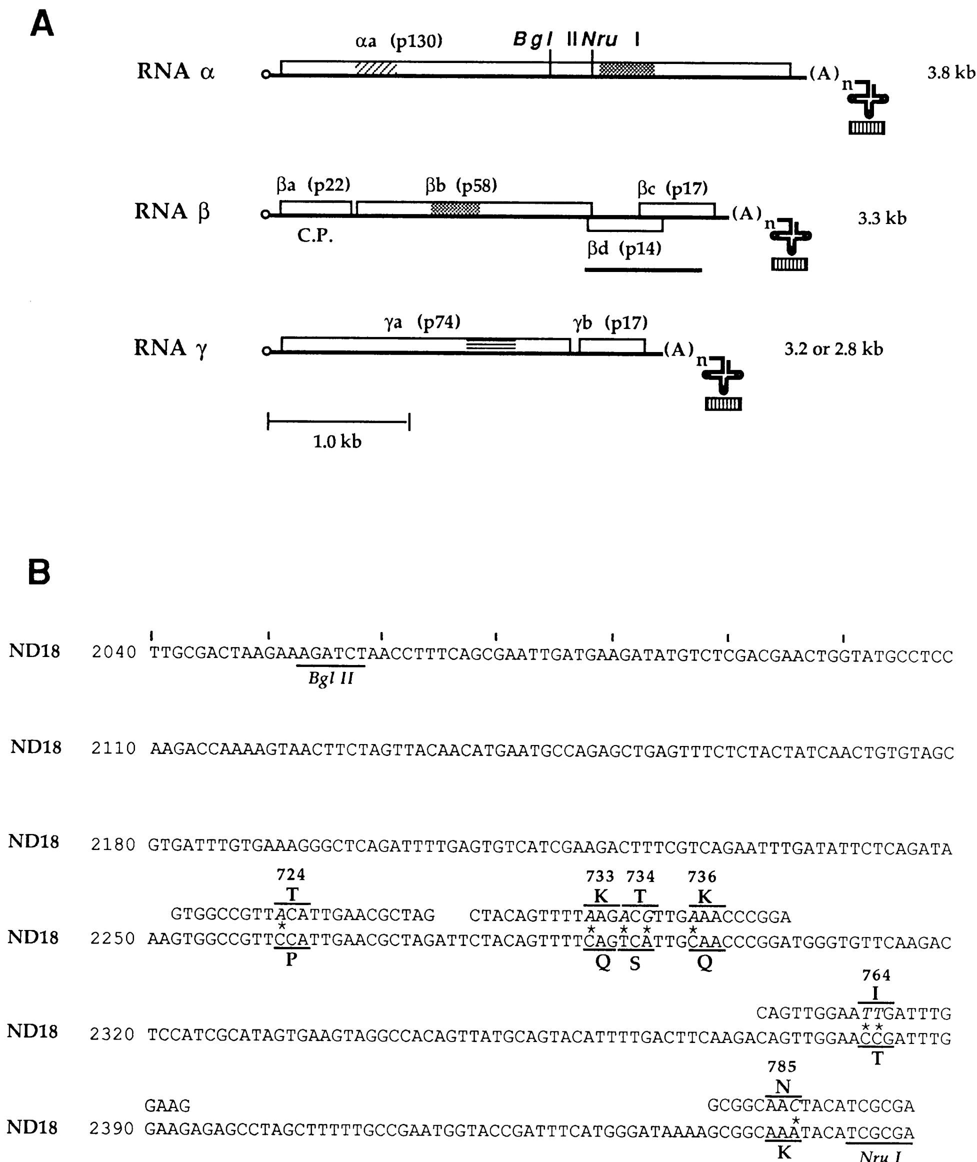


Fig. 1. Genome of barley stripe mosaic virus (BSMV) (**A**) and nucleotide sequence of ND 18 RNA α between the *Bgl*II and *Nru*I sites (**B**). Solid lines represent the genomic RNAs of BSMV with open reading frames depicted as open boxes in **A**. The open circle denotes the 5′-end cap structure and the cloverleaf represents the 3′-end tRNA-like structure. Vertically hatched boxes below the tRNA-like structure and the solid line below RNA β indicate sequences used as probes in Northern blot analysis of inoculated cell extracts. The putative methyltransferase (diagonal hatching), helicase (shading), and RNA polymerase (horizontal hatching) domains are shown within the nonstructural genes, and the viral coat protein (C.P.) is encoded on RNA β. A tandem repeat in the γa gene of ND18 results in the varying size of the γ RNA shown. The relative positions of the *Bgl*II and *Nru*I sites on RNA α between which oat pathogenicity determinants are located are also shown. In **B,** codon triplets of the αa gene between the *Bgl*II and *Nru*I sites that encode different amino acids between ND18 and CV42 are underlined and overlined, respectively. Oligonucleotides used in site-directed mutagenesis are presented above the homologous ND18 sequence, and nucleotide differences that result in predicted amino acid changes in the αa protein are italicized. The relevant amino acids are shown in bold type and their positions in the αa protein are indicated above the amino acid letter.

analysis followed procedures detailed in Weiland and Edwards (1994).

In an effort to assess the ability of the RNA α mutants to replicate in oat protoplasts, capped transcripts of mutant α RNAs were co-inoculated with ND18 transcript RNAs β and γ and protoplasts were harvested 24 h postinoculation for preparation of extracts. At least three independent inoculations were performed for each mutant, and the accumulations of viral RNA and coat protein were assayed by Northern and Western blotting, respectively. With the exception of mutant 18αTKTK-sd, whose reduced accumulation paralleled that of its recombinant counterpart, all other mutants accumulated to levels similar to that of ND18 and CV42 after a 24-h period (not shown), in agreement with previous results (Weiland and Edwards 1994). Thus, the nucleotide substitutions introduced into ND18 RNA α did not appear to significantly alter the ability of these mutants to replicate in oat protoplasts.

Capped transcripts were also used in the inoculation of cv. Rodney oat plants. The results of these inoculations are summarized in Figure 2. Virulence of mutant 18αTKTKIN-sd

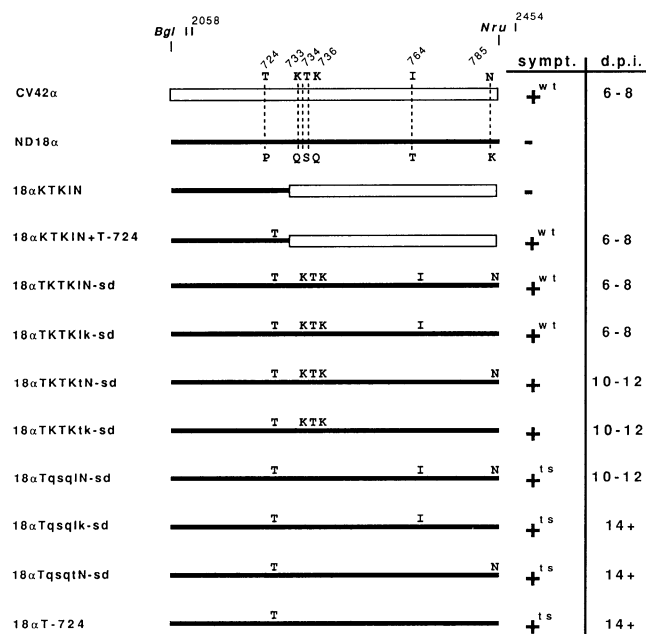


Fig. 2. Diagram of the relative positions of the amino acid changes in the αa proteins encoded by CV42 (open box) and ND18 (black bar). Only the region of the αa protein encoded between the *Bgl*II and the *Nru*I sites is shown. The amino acids are indicated above (CV42) or below (ND18) the depicted region of the αa protein. Mutant 18αKTKIN was described previously and served as a negative control and mutagenesis template in these experiments. Site-directed mutagenesis was used to produce ND18 RNAα mutants that encode either all six amino acids (18αTKTKIN-sd) specified by CV42 RNAα or subsets of these amino acids. Plants (preinoculation) were maintained in a 22°C greenhouse with the daylength extended to 16 h using artificial light. Inoculated plants were incubated in growth chambers at either 22 or 27°C (for temperature sensitivity analysis), both at a 16-h daylength. Eight to ten plants (7 days old) were inoculated with 1 µg of each α, β, and γ RNA in a total volume of 0.3 ml (Weiland and Edwards 1994). Plants inoculated with ND18 RNAs β and γ, and the mutant α RNA developed either no symptoms (–) or symptoms with varying degrees of severity (+). Symptoms were typically a stripe mosaic that ranged in timing of development as shown (d.p.i. = days postinoculation). A subset of the mutants tested failed to produce detectable symptoms when incubated in a growth chamber at 27°C (ts).

was similar to that of recombinant 18αTKTKIN, suggesting that the ability of BSMV to infect oat was determined by those sequences specifying amino acid changes within the 236-nt region. Further evidence of this was afforded by the conversion of the nonpathogenic recombinant 18αKTKIN to an isolate possessing a virulence similar to that of CV42 by a single nucleotide substitution ($C^{2261} \rightarrow A^{2261}$; Fig. 2). This creates a P-724→T-724 amino acid change in the αa protein (isolate 18αKTKIN+T-724). Indeed, the $C^{2261} \rightarrow A^{2261}$ (P-724 →T-724) mutation alone was sufficient to convert strain ND18 from nonpathogenic to pathogenic to oat. Symptoms induced by this mutant (18αT-724) were slow to develop, appearing at 14 days or greater postinoculation (Fig. 2). Moreover, symptoms produced were of a striped nature as opposed to the stripe mosaic pattern generated by other pathogenic mutants. Only those plants that displayed symptoms accumulated BSMV coat protein as detected by Western blot (not shown). No symptoms and no detectable coat protein accumulation were ever observed in plants inoculated with ND18 or recombinant 18αKTKIN (this study; Weiland and Edwards 1994).

Additional mutants were designed to assess the impact on virulence of the remaining amino acids encoded in the 236-nt region of the αa gene that differ between strains CV42 and ND18. Mutant 18αTKTKtk-sd induced the appearance of slowly developing symptoms on oat, in agreement with the symptoms induced by the recombinant 18αTKTK (Weiland and Edwards 1994). Mutants 18αTKTKIk-sd and 18αTKTK tN-sd were constructed in order to ascertain the contribution of I-764 and N-785 to virulence in oat. Mutant 18αTKTKIk-sd induced the production of symptoms on oat that were indistinguishable in rate and type from those induced by 18α TKTKIN-sd. By contrast, symptoms induced by mutant 18αTKTKtN-sd were slow to develop, requiring an average of 10 to 12 days to appear. This suggested that I-764, but not N-785, contributed significantly to virulence in this host.

Finally, mutants 18αTqsqIN-sd, 18αTqsqIk-sd, and 18α TqsqtN-sd were constructed and tested for pathogenicity to oat plants. Previously, the infection of oat by recombinant 18αTQSQIN was shown to be temperature sensitive. Although typical stripe mosaic symptoms were produced on oat inoculated with this recombinant when plants were incubated at 22°C, no symptoms were observed on plants incubated at 27°C (Weiland and Edwards 1994). Site-directed mutant 18α TqsqIN-sd performed similarly to recombinant 18αTQSQIN in both symptom development and temperature sensitivity, again suggesting that the phenotype of the recombinant viruses reflected sequence changes in the αa protein. Symptoms generated by mutants 18αT-724, 18αTqsqIk-sd, and 18α TqsqtN-sd were reduced in rate of onset and severity from those induced by mutant 18αTqsqIN-sd, implying that I-764 and N-785 together were required for increasing the virulence of BSMV to oat. This effect was evident only in the context of the core Q-733, S-734, Q-736 substitution, however, since symptoms produced by infection with mutant 18αTKTKIk-sd were indistinguishable from wild-type (above). Interestingly, mutants 18αT-724, 18αTqsqtN-sd, and 18αTqsqIk-sd displayed an infection frequency of ~10 to 20%, in contrast to the >80% infection frequency characteristic of 18αTqsqIN-sd and other mutants in the study (data not shown). This further indicated that the amino acids I-764 and N-785 could act together to increase the proportion of inoculated plants that be-

come infected, although this characteristic was also evident only in the presence of the codons encoding the core Q-733, S-734, Q-736 amino acids. Taken together, the data presented here and in Weiland and Edwards (1994) indicate that, although the $C^{2261} \rightarrow A^{2261}$ (P-724→T-724) substitution is sufficient to confer a mild oat pathogenicity to BSMV strain ND18, no single additional substitution is capable of elevating the virulence to near wild-type. The CV42 RNA α codons encoding K-733 through N-785 appear to be interacting in an unknown manner, resulting in an increase in virulence as a greater number of these codons are substituted into ND18 RNA α. It is plausible that a charge or conformational difference in the αa protein mediated by these changes is responsible for the difference in virulence. The possibility that the nucleotide substitutions themselves are responsible for the change in virulence, manifesting their effect in RNA folding or stability, cannot be ruled out.

With the exception of mutant 18αTKTKtk-sd, all of the mutants described in this work were able to infect oat protoplasts to a similar level (see above). When inoculated to oat plants, however, the mutants exhibited varying degrees of virulence. Because the mutations introduced into the ND18 α RNA may have been detrimental to the intrinsic ability of the virus to infect whole plants, each mutant was inoculated to cv. Black Hulless barley, a host possessing susceptibility to a variety of BSMV strains. All isolates displayed a similar ability to infect Black Hulless barley, and were indistinguishable from infections with wild-type strain ND18. Thus, all mutants described here are fully capable of replicating in, and moving locally and distantly within, an infected plant host.

Although all mutants replicated efficiently in inoculated oat protoplasts and in Black Hulless barley plants, it was relevant to determine whether the mutants that were incapable of systemically infecting oat were nonetheless capable of replication in inoculated oat leaf cells. An engineered tobacco etch virus capable of expressing β-glucuronidase (GUS) has proven very useful in the detailed analysis of the movement and replication of this virus in the infected plant (Dolja et al. 1995 and references therein). Following this approach, we constructed a recombinant RNA β of strain ND18 (18β-Nc/Bg-GUS; Fig. 3) that is capable of expressing GUS in inoculated plants when inoculated with a replication-competent set of RNAs α and γ. The open reading frame encoding β-glucuronidase replaces the triple gene block encoded in this RNA, genes that are required for the systemic infection of cereal plants and for cell-to-cell movement in hosts in which BSMV induces the formation of local lesions (Petty and Jackson 1990a; Petty et al. 1990b).

Transcripts of RNAs 18β-Nc/Bg-GUS and ND18 γ were inoculated to oat in the presence of α RNAs CV42α, ND18α, 18αKTKIN, 18αTKTKIN-sd, 18αKTKIN+T-724, and 18αT-724. Twenty-four hours postinoculation, the inoculated leaves were excised from the plants and were assayed for the presence of GUS. After a 24-h incubation in GUS detection solution, dark pigmented foci were apparent on leaves that were inoculated with transcript mixtures containing CV42α, and mutants 18αT-724, 18αTKTKIN-sd, and 18αKTKIN+T-724 (Fig. 4; Table 1). Blue foci were observed only infrequently on leaves inoculated with RNA mixtures that included ND18α or any nonpathogenic recombinant, and such foci were diffusely pigmented. Since no foci were ever observed after inoculations with ND18 transcript RNAs γ and β-Nc/Bg-GUS alone, the expression of GUS by a contaminating microbe or by transient expression of the β-Nc/Bg-GUS RNA can be ruled out.

The number of foci produced by those mutants and recombinants that were pathogenic to oat varied considerably within and between experiments. Foci varied in appearance from round spots encompassing 10 to 20 cells, to longitudinal streaks that paralleled the leaf veins and spanned a distance from approximately 50 to 100 cells. The greatest proportion of the dye appeared to be present within the mesophyll cells. Whether the presence of blue pigment in these multiple cells is the result of the diffusion or movement of GUS enzyme or GUS product, or the limited cell-to-cell spread of the recombinant virus, remains to be determined. Examination with electron microscopy recently revealed the presence of characteristic BSMV particles in multiple cells located within the blue foci, in support of the latter possibility (J. Weiland and M. Edwards, unpublished). The data in Table 1, nevertheless, display a clear difference (minimally 40-fold) in the number of foci produced by any pathogenic recombinant and any one nonpathogenic recombinant. Thus, the results using the recombinant 18β-Nc/Bg-GUS accurately reflect the oat pathogenicity of each of the RNA α mutants, and provide an additional level of resolution in the analysis of BSMV pathogenicity.

Using an infectious cDNA clone of tobacco etch virus capable of expressing β-glucuronidase, Dolja et al. (1995) presented evidence that single infected cells in an inoculated leaf could be detected after staining for GUS. Repeated attempts to visualize single infected cells in any of the BSMV inoculations described here were unsuccessful. The lack of blue foci on leaves inoculated with the nonpathogenic mutants may reflect the inhibition of virus replication in, or movement out of, cells of the intact oat leaf. In the event that efficient virus movement is a prerequisite for the histochemical detection of GUS using this system, a block in the ability of the virus to move cell-to-cell could explain these results. A potential role

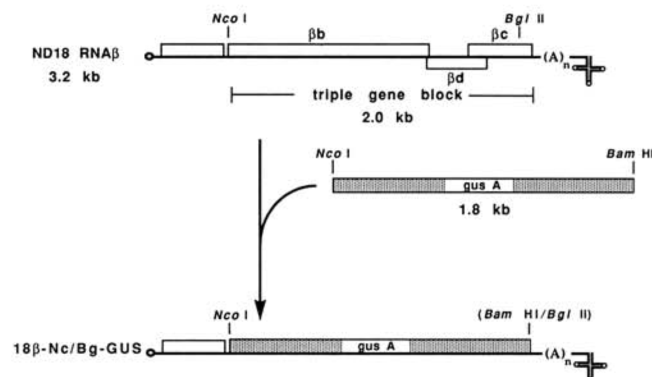
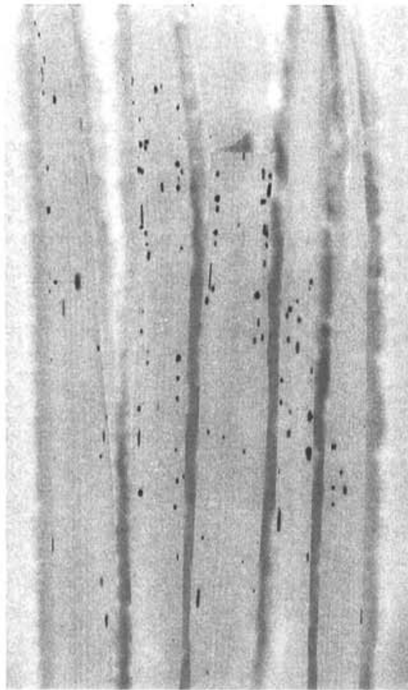
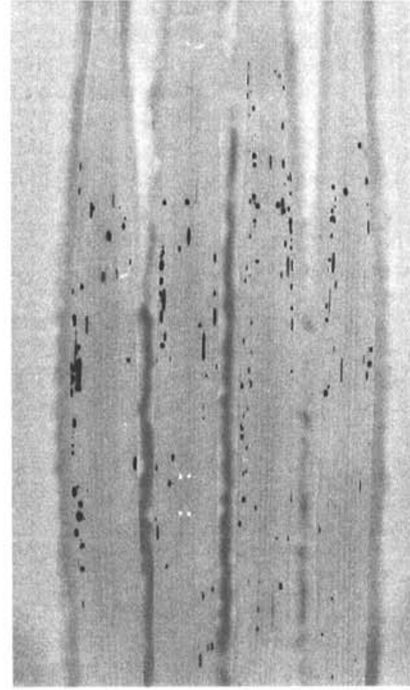
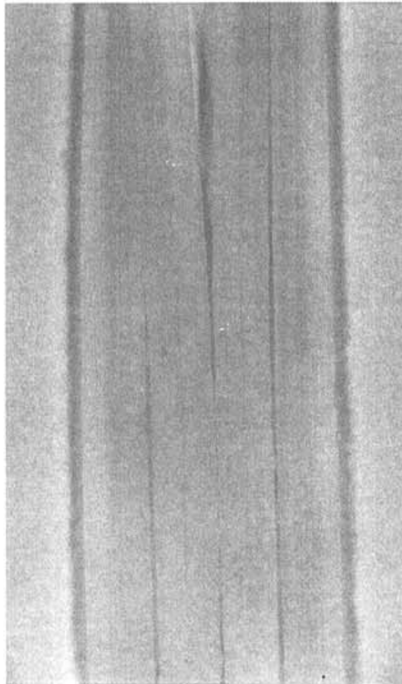


Fig. 3. Barley stripe mosaic virus (BSMV) RNA β (ND18) and the restriction sites used in the construction of recombinant 18β-Nc/Bg-GUS. For the construction of pβNc/Bg-GUS, plasmid pND18β was digested with *Nco*I and *Bgl*II. The 1.8-kb *Nco*I-*Bam*HI fragment of pRTL2-4G (kindly provided by W. G. Dougherty), encoding the β-glucuronidase structural gene, was gel-purified by standard methods (Sambrook et al. 1989) and was ligated into the digested pND18β DNA. The resulting plasmid replaces the primary initiation codon (AUG) of the BSMV βb gene (Petty and Jackson 1990b) with that of the β-glucuronidase gene.







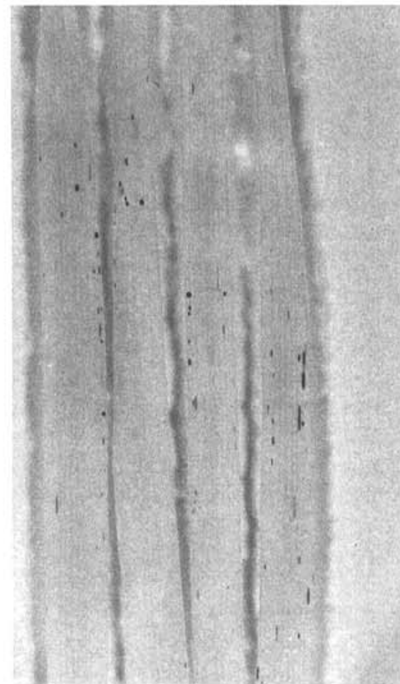


Fig. 4. Histochemical detection of GUS in leaves inoculated with barley stripe mosaic virus (BSMV) ND18 transcripts γ and 18β-Nc/Bg-GUS, along with RNA α transcripts 42α, 18α, 18αKTKIN+T-724, and 18αT-724. Inoculated leaves harvested at 24 h postinoculation were gently abraded on one side with a suspension of Carborundum in water. Leaves were then placed in a solution containing 100 mM sodium phosphate (pH 7.0), 10 mM Na_2EDTA, 0.5 mM potassium ferrocyanate, 0.1% TRITON X-100 and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) and incubated at 37°C without agitation for 24 h (modified from Stomp 1992). The buffer was then replaced with several exchanges of methanol for tissue clearing. An occasional infection focus was observed in inoculations that included 18α. The phenotype produced by inoculations with 18αKTKIN in this assay was similar to that produced by inoculations that included 18α.

Table 1. Quantitation of infection foci on inoculated oat leaves stained for the presence of GUS

	Average number of foci per inoculated leaf[a]		
	Experiment 1	Experiment 2	Experiment 3
CV42α	32.5	13.1	7.8
ND18α	0.4	0.2	0.2
18αTKTKIN-sd	11.8	59.4	...
18αKTKIN	0.2	0.3	...
18αKTKIN+T-724	40.7	17.0	...
18αT-724	31.7	10.7	...

[a] Averages from five to six inoculated leaves per mutant per experiment sampled at 24 h postinoculation.

in virus movement for the αa gene (Weiland and Edwards 1994) and the analogous gene in cucumber mosaic virus (Gal-On et al. 1994) has been postulated previously. The interpretation of the results using the 18α-Nc/Bg-GUS recombinant, however, awaits further data, since the potential for artifacts to occur exists when using the GUS system (Baulcombe et al. 1995). Alternatively, the nonpathogenic mutants may not be able to replicate efficiently in cells of the inoculated leaf. Possible resistance mechanisms affecting virus replication include active systems that are induced or constitutively expressed by the host plant, or those that represent an effective absence of host-factors required by the viral replicase for its function. The molecular bases of virus resistance mechanisms that affect virus replication are just now beginning to be elucidated (Köhm et al. 1993; Lindbo et al. 1993).

The data presented in this work suggest that amino acids encoded in the αa protein are determinants of BSMV pathogenicity to oat. Since a single nucleotide substitution was able to render the nonpathogenic ND18 strain pathogenic to oat, it is reasonable to speculate that the CCA (ND18) to ACA (CV42) codon change, represented in mutant 18αT-724, was instrumental in the acquisition of oat pathogenicity by BSMV. The virulence of this mutant to oat was poor, but was raised to near wild-type levels by locally clustered changes in the αa gene, suggesting that these changes may have resulted from natural selection for higher virulence in the pathogen. Thus, although BSMV strain CV42 possesses nucleotide substitutions relative to ND18 in other regions of RNA α, as well as in RNAs β and γ, the major determinants of oat pathogenicity appear to reside within a 61-amino-acid block in the αa protein.

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