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Mapping of the Seed Transmission Determinants of Barley Stripe Mosaic Virus

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The specific mechanism(s) by which some plant viruses are transmitted through seed, while others are excluded, is not known. Using infectious barley stripe mosaic virus (BSMV) RNAs transcribed in vitro from full-length cDNA clones, the viral genetic determinants of seed transmission have been mapped. Both pseudorecombinant and chimeric viruses were constructed from BSMV strains ND18 (seed transmitted) and CV17 (not seed transmitted). The markedly different seed transmissibility of these two strains facilitated the identification of RNAγ as the location of the primary determinants of seed transmission phenotype. RNAβ also played a role in seed transmission, but to a lesser extent than RNAγ. Major genetic determinants of seed transmission on RNAγ included the 5′ untranslated leader, a 369-nt repeat in the γa gene, and the γb gene. Important determinants of symptom phenotype mapped to the RNAγ leader and the γb gene as well. Some heterologous combinations of the RNAγ leader and the γb gene resulted in dramatic changes in symptomatology and seed transmission, depending on the parental source of RNAs α and β. These results suggest that a complex interaction of the RNAγ leader, the γb gene, and RNAs α and β are involved in BSMV pathogenesis. Considering the putative regulatory role of the γb gene (Donald and Jackson 1994, Plant Cell 6:1593-1606) and the *trans* effects that alterations in the γb gene have on RNAβ gene expression (Petty et al., 1990, EMBO J. 9:3453-3457), phenotypic effects attributed to elements of RNAγ could result from *cis* or *trans* interactions involving the RNAγ leader, the γb gene, and RNAs α and β. Clearly, virus replication and movement play pivotal roles in the seed transmission of BSMV.

Additional keywords: hordeivirus, *Hordeum vulgare,* nucleotide sequence.

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Nucleotide sequence data reported herein have been assigned GenBank accession numbers U13916, U13917, and U13918.

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Plant virus transmission through seed is of considerable ecological, epidemiological, and economic importance. Although the potential significance of this phenomenon was first recognized about 75 years ago (Doolittle and Gilbert 1919; Reddick 1919), the underlying mechanisms which determine the seed transmissibility of viruses have not been resolved. In fact, few attempts have been made to investigate either the host or viral genetic factors involved in seed transmission. Although Carroll et al. (1979) reported that resistance to seed transmission of barley stripe mosaic virus (BSMV) was determined by a single recessive gene in barley, multiple maternal genes have been implicated in the resistance of pea to seed transmission of pea seedborne mosaic virus (Wang and Maule 1994). Pseudorecombination studies with isolated viral RNAs have been used to link viral seed transmission phenotype to RNA 1 of raspberry ringspot and tomato black ring nepoviruses (Hanada and Harrison 1977) and also to RNA 1 of cucumber mosaic virus (Hampton and Francki 1992). An excellent, updated list of pollen and seed transmitted viruses (Mink 1993) as well as a more general review of seed transmission (Johansen et al. 1994) have recently appeared.

BSMV, the type member of the Hordeiviruses, is primarily dependent on seed transmission in barley (*Hordeum vulgare* L.) for its survival in nature. Despite this fact, significant variation exists in the efficiency of seed transmission among BSMV strains. Seed transmission of strain ND18 can reach as high as 64% in "Dickson" barley, whereas seed transmission of strain CV17 is less than 1% in the same cultivar (Timian 1974). Although some aspects of seed transmission of BSMV have been extensively studied (for review, see Carroll 1981), the viral determinants of seed transmission have not been investigated.

Identification of the genetic determinants of seed transmission may facilitate the discovery of the mechanisms by which some plant viruses are transmitted through seed, while others are excluded. More precise mapping of viral seed transmission determinants is now possible using infectious RNAs transcribed in vitro from viral cDNA clones such as those constructed for various strains of BSMV (Petty et al. 1988; 1989; Edwards et al. 1992; Weiland and Edwards 1994). I have constructed both pseudorecombinant and chimeric viruses from cDNA clones of BSMV strains ND18 and CV17 in order to identify the viral determinants of seed transmission in barley (a schematic of the BSMV genome is presented in Fig. 1). Evidence is provided that seed transmissibility of BSMV is determined primarily by three different elements of

RNAγ, including the 5′ noncoding region, an approximately 370-nt repeat in the γa gene, and the γb gene. Sequence differences which may be related to differences in the seed transmission and symptom phenotypes are also identified. Although the nucleotide sequences of the Type and ND18 strain γRNAs have been reported (Gustafson et al. 1987; GenBank M16576, M16577), the sequences presented herein were derived from infectious full-length clones with demonstrated biological activity and phenotypes representative of the "wild type" virus strains. Symptom development in relation to seed transmission is also described.

RESULTS

Location of primary determinants for seed transmission.

Seed transmissibility of pseudorecombinants derived from CV17 and ND18 clearly reflected the phenotype of the parental strain from which the RNAγ component was derived (Fig. 2). Those pseudorecombinants comprised of ND18 RNAγ together with any combination of RNAs α and β were clearly seed-transmitted at higher frequencies than their counterparts possessing CV17 RNAγ. Although more subtle than effects attributable to RNAγ, effects of RNAβ on seed transmission were apparent (Fig. 2). Factorial analysis of the data in Figure 2 revealed a statistically significant interaction of RNAs β and γ in addition to RNAβ effects ($\alpha = 0.05$, not shown).

Sequence of RNAγ.

Sequencing of the γRNAs of BSMV strains CV17 and ND18 followed assignment of the seed transmission phenotype primarily to RNAγ. For comparison, the Type strain RNAγ also was sequenced. Differences between the sequence of CV17 and those of the more efficiently seed-transmitted ND18 and Type strains were found (Figs. 3 and 4). A 369-nucleotide tandem repeat begins immediately 5′ of the CV17 γa gene and extends into it (nts 73–441 and 442–810). This repeat is nearly perfect, but does contain a single base mismatch at position 444. A similar 372-nt repeat is present in RNAγ of the Type strain and some other seed-transmissible strains, although ND18 RNAγ does not possess this repeat. A number of silent nucleotide changes were noted in both the γa and the γb genes, while relatively few amino acid substitutions were evident (Fig. 4). The greatest divergence between ND18 and CV17 was found in the 5′ noncoding region, where the sequences are essentially unrelated as a result of recombination within the CV17 genome (Edwards et al. 1992). Differences between the previously reported sequences of ND18 and Type (Gustafson et al. 1987) and the sequences of the infectious clones now reported are not compared here. This discussion is limited to the currently reported sequences, since these clones continue to be used in genetic analyses of BSMV in our laboratory and others.

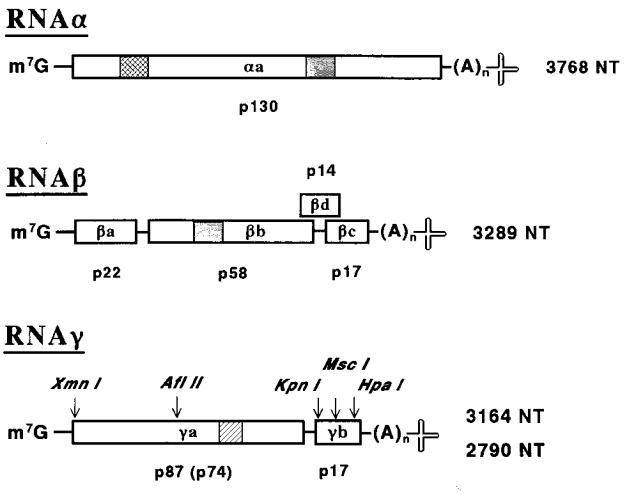

Fig. 1. A schematic of the barley stripe mosaic virus (BSMV) genome showing the relative location of restriction sites used to create RNAγ recombinants. Each RNA has a short poly-A region (A_n), a 5′ terminal cap structure (m^7G), and a 3′ terminal tRNA-like structure capable of being aminoacylated with tyrosine. Sizes of RNAs α and β and and their gene products are based on the previously reported sequences (Gustafson and Armour 1986; Gustafson et al. 1989). Open boxes represent genes. Locations of putative methyltransferase domain (cross-hatched), helicase domains (shaded), and polymerase domain (diagonal hatched) are as indicated.

Fine mapping of RNAγ seed transmission determinants.

Sequence comparisons aided the design and construction of CV17 × ND18 RNAγ recombinants by the progressive substitution of CV17 RNAγ-specific sequence into ND18 RNAγ and vice versa. Five convenient restriction sites were used for this purpose, as illustrated in Figure 1. Although the *Xmn*I site occurs within the γa ORF, exchanging 5′ termini to this point essentially resulted in an exchange of leader sequences, since CV17 and ND18 γRNAs are identical from the initiation codon downstream to the *Xmn*I site. Exchange of sequence from the *Kpn*I site to the 3′ terminus was essentially an ex-

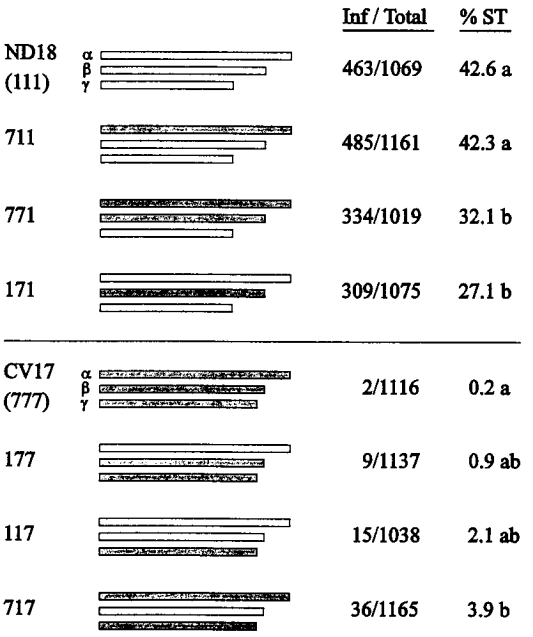

Fig. 2. Seed transmission of CV17 × ND18 pseudorecombinants in Dickson barley. Shaded and unshaded bars represent all possible combinations of RNAs α, β, and γ. Inf/total = total number of infected progeny seedlings detected/total number of viable progeny seedlings assayed (pooled from two separate experiments). %ST = Percent seed transmission detected per pseudorecombinant (average of two separate experiments; six replicates/experiment). Means in one half of the figure which are followed by the same letter were not statistically different (Bonferroni [Dunn] *t*-test, $\alpha = 0.05$).

change of γb genes, although a single amino acid difference results from γb sequence differences between ND18 and CV17 just upstream of the *Kpn*I site. Other sites employed (*Afl*III, *Msc*I, *Hpa*I) were chosen as a means of subdividing the γa and γb genes. These results are depicted in Figure 5.

Results of check inoculations of barley with 18αβ + 18γ, 18αβ +17γ, 17αβ +18γ, and 17αβ +17γ confirmed results of the earlier pseudorecombination experiments (see also Fig. 2). Deletion with *Xmn*I of the 369-nt repeat present in CV17 RNAγ resulted in an apparent increase in seed transmissibility relative to that observed when wild-type CV17 RNAγ was present (Fig. 5, 17αβ17γ vs. 17αβ +17x), although statistical significance was not established due to the low frequency of seed transmission observed. This increase was not apparent when RNAs 17γ or 17x were coinoculated with ND18αβ.

Conversely, dramatic effects on seed transmissibility occurred as a result of RNAγ leader sequence exchanges in the presence of ND18 RNAsαβ (Fig. 5, 18/17X, 18/17A, 18/17K). Seed transmissibility was greatly enhanced by the substitution of ND18 RNAγ leader sequence for that of CV17 (18/17X vs. 17X), whereas the reciprocal substitution was deleterious to seed transmission (17/18X vs. 18γ). No such leader effects were evident when the same recombinant γ RNAs were coinoculated with CV17 RNAsα and β. No further increase in frequency of seed transmission was observed as either part (18/17A) or all (18/17K) of the ND18γa gene was substituted for that of the CV17 RNAγ, regardless of the parental source of RNAs α and β.

The significant effect of RNAγ leader exchanges on frequency of seed transmission prompted a further examination of the potential role of two small ORFs present in the leader of CV17 RNAγ, but not in the leader of ND18 RNAγ (Fig. 3). Previously created (Petty et al. 1990a) RNAγ point mutants in which either, or both, of the small leader ORFs had been eliminated were evaluated for seed transmission effects. When CV17 RNAs α and β were coinoculated with these RNAγ point mutants, the frequency of seed transmission remained essentially zero (CV17αβγ: 0/856; CV17αβ + γU28: 2/851; CV17αβ + γU28A38: 0/837). This apparent lack of influence of the small leader ORFs on seed transmission was confirmed by examining the effects of point mutations designed to eliminate and/or fuse to the γa gene an analogous small leader ORF present in the Type strain RNAγ. These mutations also had no discernible effect on seed transmission (data not shown).

Sequence exchanges between the ND18 and CV17 γb genes also affected seed transmission frequencies (Fig. 5, re-

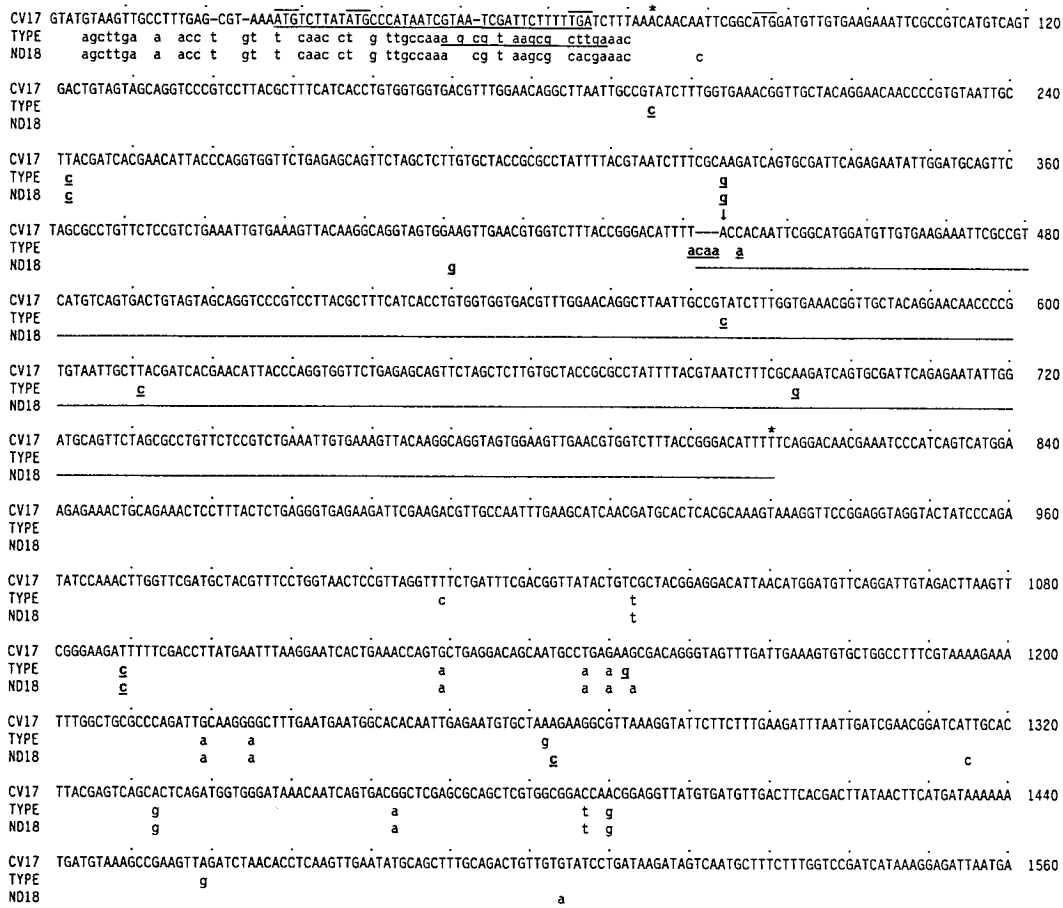

Fig. 3. Nucleotide sequences of biologically active, full-length clones of the γRNAs of barley stripe mosaic virus (BSMV) strains CV17, ND18, and Type. Only sequence deviations are noted for the Type and ND18 strains; sequence deviations which result in amino acid changes are indicated by bold underlined nucleotides. A gap is indicated by (–). Initiation and termination codons of the γa and γb genes, and of the small ORFs present in the 5′ leader, are overlined. The sequence encompassing the small leader ORFs present in the CV17 and Type strains is underlined. The first and last nucleotides of a 369-nt tandem repeat (372 nt in Type strain) are indicated by an asterisk (*), while the first nucleotide of the second repeat unit is indicated by a downward arrow.

combinants 18/17M, H, and 17/18M, H; Fig. 6, 17/18MX, HX). Substitution of ND18 γb sequence for that of CV17 resulted in an increased frequency of seed transmission, while the reciprocal substitutions resulted in a decreased frequency of seed transmission. These trends were independent of the parental source of RNAs α and β, although the most striking effects were evident when γb recombinants (18/17K vs. 18/17M, H; 17/18K vs. 17/18M,H) were coinoculated with CV17 RNAs α and β.

Initial substitutions of CV17γa sequence, in whole or in part, for ND18γa sequence resulted in reduced frequency of seed transmission (Fig. 5, 17/18A or K vs. 17/18X). However, this sequence substitution resulted in the addition of the 369-nt tandem repeat to the RNAγ construct and not simply the substitution of CV17γa sequence. To distinguish whether the reduction in seed transmission was due to the introduction of the repeat or to the CV17γa sequence itself, the repeat was deleted from the 17/18A, K, M, H RNAγ recombinants using *Xmn*I. Elimination of the repeat to give the AX, KX, MX, or HX RNAγ recombinants resulted in higher frequency of seed transmission whether these recombinants were coinoculated with CV17 RNAs α and β or ND18 RNAs α and β (Fig. 6). Most significantly, no reduction in seed transmission was observed when CV17γa sequence was either partly or wholly substituted for ND18γa sequence (17/18X vs. 17/18AX or KX).

Symptom determinants/development.

The parental strains used in these studies differ significantly in the type of symptoms induced in infected barley as well as in their seed transmissibility. CV17 induces a bleached chlorotic to necrotic streak in leaves of infected barley plants, whereas ND18 induces a more uniform chlorotic stripe mosaic symptom. ND18 does not induce the extensive striped chlorosis and necrosis associated with CV17 infection (Fig. 7E,F). Also, symptoms of ND18-infected plants tend to fade as plants mature, whereas the streaks induced by CV17 tend to remain quite evident as plants mature.

Pseudorecombination studies revealed that the primary determinant(s) of the streak vs. mosaic phenotype was located on RNAγ, as were the determinants for seed transmission. Progeny of plants infected with pseudorecombinants created from all possible combinations of RNAs α, β, and γ developed symptoms typical of the parent from which the RNAγ component was derived (Fig. 8).

During attempts to map more precisely the seed transmission determinants using RNAγ recombinants, symptom de-

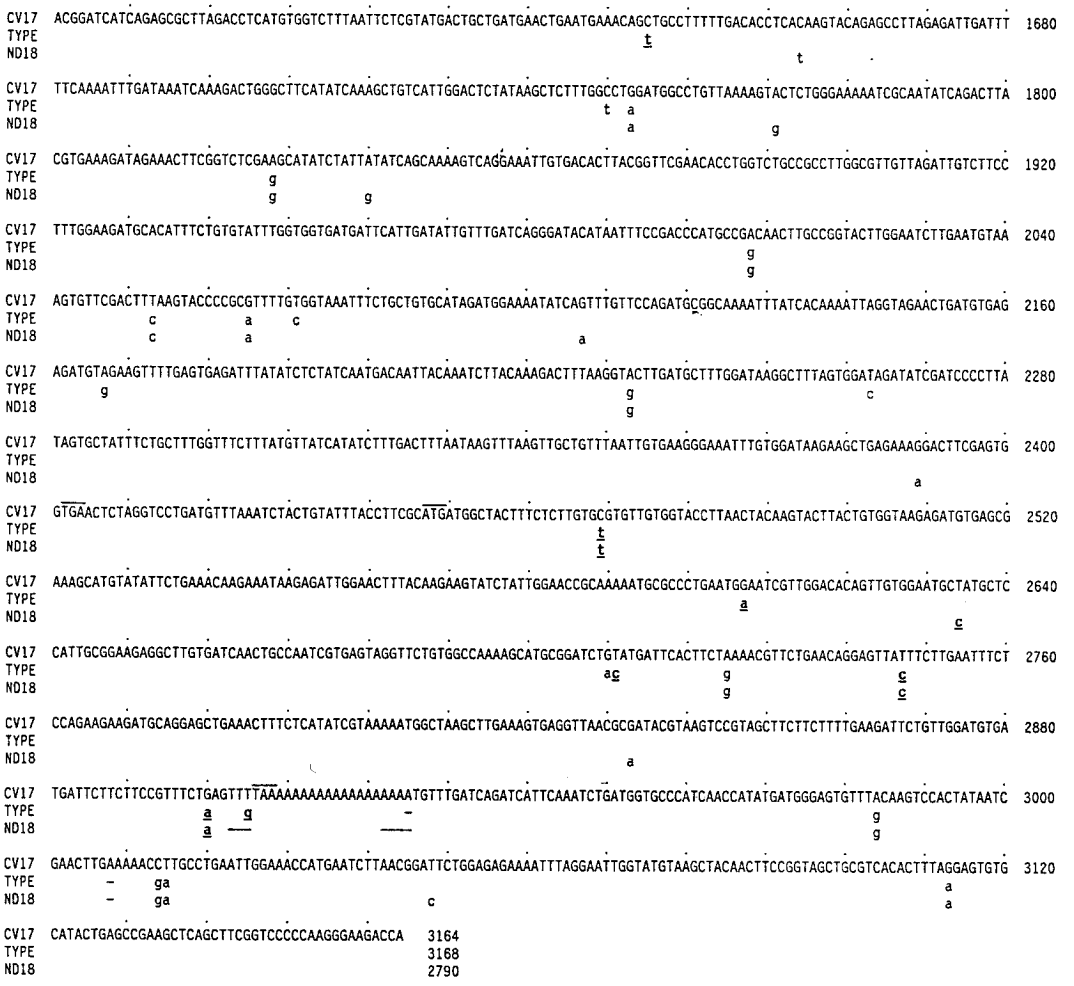

Fig. 3. *(continued from preceding page)*

terminants segregated as well. Although the presence or absence of the 369-nt repeat in the γa gene affected seed transmissibility, no discernible effect was found on symptom type. Furthermore, no prominent changes in symptom phenotype could be attributed to the γa gene itself.

The RNAγ leader and the γb gene were found to influence symptomatology, however. Both of these regions played prominent roles in determining whether symptoms of infected plants included a bleached chlorosis and necrosis or simply milder chlorosis and mosaic. Although influence of the RNAγ leader or the γb gene on symptom phenotype was not entirely independent of RNAs α and β, RNAs α and β did not have an appreciable influence on symptom type when the RNAγ leader and the γb gene were derived from the same parent. Some degree of CV17-like streaking resulted in plants infected with any combination of RNAs α and β if the source of RNAγ was CV17 (Fig. 7F, 8). Similarly, pseudorecombinants with an ND18 RNAγ induced a mosaic reminiscent of wild type ND18 (Fig. 7E, 8).

Parental source of RNAs α and β also did not profoundly affect symptoms of plants inoculated with RNAγ recombinants 17/18X, A, K, M, or H (not shown). Although recombinants with CV17 RNAs α and β tended to induce slightly more necrosis than their counterparts with ND18 RNAs α and β, the symptom type did not differ as a result of the parental source of RNAs α and β. The extent of streaking and necrosis did increase markedly as greater portions of the CV17γb gene were incorporated into the recombinants (i.e., 17/18X, A, or K vs. 17/18M, H, or 17γ).

Parental source of RNAs α and β was a significant factor in determining the symptom severity of recombinants 18/17X, A, and K (Fig. 7A,B). A severe, patchy, bleached chlorosis developed when any of these γ recombinants was coinoculated with CV17 RNAs α and β (Fig. 7B). When coinoculated with ND18 RNAs α and β (Fig. 7A), very mild symptoms were induced on plants, although occasional short bleached chlorotic streaks were present to a limited extent. Although no such prominent RNAαβ influence was observed with recombinants 18/17M or H, these γ recombinants still induced slightly more severe symptoms, including some necrosis, when coinoculated with CV17 RNAs α and β than with ND18 RNAs α and β (Fig. 7C,D). As ND18 sequence was substituted for CV17 sequence within the γb gene (18/17X, A, or K vs. 18/17M or H), symptoms in infected plants bore greater resemblance to those induced by ND18 (i.e., chlorosis, mosaic). Thus, the heterologous combination of an ND18 RNAγ leader sequence and a CV17 γb gene resulted in the most striking effects on symptom type. Together, the above results clearly suggested an interaction between the RNAγ leader, the γb gene, and either or both RNAs α and β.

These results also demonstrated that symptom type/severity can be somewhat loosely correlated with seed transmission of BSMV. Those isolates which induced more necrosis tended to be transmitted at lower frequency than those with milder symptoms (i.e., more chlorotic than necrotic). However, recombinants which induced the mildest symptoms were not necessarily seed transmitted at the highest rates (e.g., 18αβ +18/17X, A, or K vs. ND18), nor were some recombinants

A

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CV17γa  MDVVKKFAVMSVTVVAGPVLTLSSPVVVTFGTGLIAVSLVKRLLQEQPRVIAYDHEHYPGGSESSSSSCATAPILRNLSQDQCDSENIGCSSSACSPSEI  100
TYPEγa                                      A               H                          R                      100
ND18γa                                                      H                          R                      100

CV17γa  VKVTRQVVEVERGLYRDIL-PQFGMDVVKKFAVMSVTVVAGPVLTLSSPVVVTFGTGLIAVSLVKRLLQEQPRVIAYDHEHYPGGSESSSSSCATAPILRN  200
TYPEγa                    YKQ                                       A               H                         201
ND18γa          G         -----------------------------------------------------------------------------------  118

CV17γa  LSQDQCDSENIGCSSSACSPSEIVKVTRQVVEVERGLYRDIFQDNEIPSVMEEKLQKLLYSEGEKIRRRCQFEASTMHSRKVKVPEVGTIPDIQTWFDAT  300
TYPEγa    R                                                                                                   301
ND18γa  -----------------------------------------                                                             177

CV17γa  FPGNSVRFSDFDGYTVATEDINMDVQDCRLKFGKIFRPYEFKESLKPVLRTAMPEKRQGSLIESVLAFRKRNLAAPRLQGALNEWHTIENVLKKALKVFF  400
TYPEγa                                    T                    R                                              401
ND18γa                                    T                                                         T         277

CV17γa  FEDLIDRTDHCTYESALRWWDKQSVTARAQLVADQRRLCDVDFTTYNFMIKNDVKPKLDLTPQVEYAALQTVVYPDKIVNAFFGPIIKEINERIIRALRP  500
TYPEγa                                                                                                        501
ND18γa                                                                                                        377

CV17γa  HVVFNSRMTADELNETAAFLTPHKYRALEIDFSKFDKSKTGLHIKAVIGLYKLFGLDGLLKVLWEKSQYQTYVKDRNFGLEAYLLYQQKSGNCDTYGSNT  600
TYPEγa                  V                                                                                     601
ND18γa                                                                                                        477

CV17γa  WSAALALLDCLPLEDAHFCVFGGDDSLILFDQGYIISDPCRQLAGTWNLECKVFDFKYPAFCGKFLLCIDGKYQFVPDAAKFITKLGRTDVRDVEVLSEI  700
TYPEγa                                                                                                        701
ND18γa                                                                                                        577

CV17γa  YISINDNYKSYKDFKVLDALDKALVDRYRSPYSAISALVSLCYHIFDFNKFKLLFNCEGKFVDKKLRKDFEW  772
TYPEγa                                                                            773
ND18γa                                                                            649
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B

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CV17γb  MMATFSCACCGTLTTSTYCGKRCERKHVYSETRNKRLELYKKYLLEPQKCALNGIVGHSCGMLCSIAEEACDQLPIVSRFCGQKHADLYDSLLKRSEQEL  100
TYPEγb         V                                             E                                  H             100
ND18γb         V                                                      P                                       100

CV17γb  FLEFLQKKMQELKLSHIVKMAKLESEVNAIRKSVASSFEDSVGCDDSSSVSEF  153
TYPEγb  L                                                  KL  153
ND18γb  L                                                  K-  152
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Fig. 4. Amino acid sequences of the γa and γb genes of BSMV strain CV17 in comparison with those of the Type and ND18 strains. Only amino acids which differ are noted for the Type and ND18 strains. A dash (–) indicates a gap.

which induced quite severe symptoms seed-transmitted at the lowest rates (e.g., CV17αβ +18/17X, A, or K vs. CV17).

DISCUSSION

The preponderance of evidence concerning seed transmission of BSMV suggests that seed transmission does not occur unless the virus is able to invade the reproductive tissues prior to fertilization (Carroll 1981; Carroll 1972; Carroll and Mayhew 1976a, 1976b), although it appears that some seed transmission of BSMV may occur as a result of direct embryo invasion (Crowley 1959). In either case, isolates that are not seed transmitted apparently are deficient in the ability to invade tissues which provide a crucial gateway to the embryo. This is consistent with recent evidence that virus invasion of nonvascular testa tissues was limited in a pea cultivar resistant to seed transmission of pea seedborne mosaic virus (Wang and Maule 1994).

Our data clearly indicate that BSMV genes with known roles in virus replication and movement determine seed transmission phenotype. The influence of the RNAγ 5′ noncoding region is of particular interest because the CV17 RNAγ is apparently a chimeric molecule wherein the 5′ leader is derived from RNAα through recombination (Edwards et al. 1992). In addition, the presence of two small overlapping ORFs within this leader region was shown previously to preclude systemic movement in *Nicotiana benthamiana*, perhaps by reducing the efficiency of translation of the γa gene (Petty et al. 1990a). The fact that RNAγ point mutations designed to eliminate these small leader ORFs had no significant influence on the frequency of seed transmission indicates that the observed 5′ leader effects on seed transmission may be intrinsic to the leader sequence rather than to the presence of the small ORFs. Alternatively, although the influence of the 5′ leader ORFs was not discernible in either the CV17 or Type genetic background, the presence of these small ORFs may have greater effect in a different genetic background (e.g., ND18 RNAs α and β, not tested).

As a result of the sequence divergence in this region, the predicted secondary structure of the CV17 RNAγ leader is quite distinct from that of the ND18 RNAγ leader (not shown; RNAFOLD, Zuker and Stiegler 1981; Jacobsen et al. 1984). RNA secondary structure, particularly near the 5′ and 3′ termini, may play an important role in viral replication processes. For example, stem-loop structures within the 5′-noncoding regions of brome mosaic virus and beet necrotic yellow vein virus appear to be critical for plus-strand RNA synthesis (Pogue and Hall 1992; Gilmer et al. 1993). Thus, seed transmission effects linked to the RNAγ leader could be due to disruptions in replication or movement as a result of differences in secondary structure.

Although the potential translational effects of the small leader ORFs on expression of γa had no discernible effect on seed transmission, the presence of the 369-nt tandem repeat in the γa gene was deleterious to seed transmission. The absence of this repeat may increase the activity of the γa-encoded replicase, resulting in improved replication and movement. Deletion of the γa repeat in previous studies resulted in phenotypic effects consistent with this hypothesis (Petty et al. 1994; Petty et al. 1990). Time of appearance of local lesions on *Chenopodium amaranticolor* was greatly accelerated following deletion of the γa repeat from the Type strain (Petty et al. 1994), while systemic pathogenicity of the Type strain to barley, which had been lost upon deletion of the γb gene, was regained upon deletion of the γa repeat (Petty et al. 1990).

The observed variation in seed transmission and symptom phenotype as a result of sequence substitution within the γb gene is consistent with previous observations that deletion of the γb gene negatively impacts systemic movement, possibly through *trans* effects on RNAβ gene expression. Significant reduction in the accumulation of RNAβ gene products has been observed after mutation or deletion of the γb gene (Petty et al. 1990; Donald and Jackson 1994). Although little is actually known about the precise role of the γb gene in replica-

	ND18αβ		CV17αβ	
	Inf / Tot	% ST	Inf / Tot	% ST
CV17γ	6/295	2.1 a	0/574	0.0 a
17X	12/565	2.1 a	37/580	6.4 a
18/17X	179/578	30.9 bc	47/544	7.5 a
18/17A	161/573	28.0 bc	44/530	8.5 a
18/17K	138/583	23.7 b	23/547	3.2 a
18/17M	193/554	34.5 cd	169/555	30.0 b
18/17H	247/561	43.9 de	213/568	38.3 b
ND18γ	252/531	47.2 e	103/283	36.5 b
ND18γ	252/531	47.2 a	103/283	36.5 a
17/18X	149/525	28.0 b	172/506	33.2 a
17/18A	60/564	10.6 c	95/567	16.8 b
17/18K	31/554	5.6 cd	72/545	13.1 b
17/18M	24/581	4.1 cd	16/558	2.9 c
17/18H	6/576	1.1 d	5/576	0.9 c
CV17γ	6/295	2.1 d	0/574	0.0 c

Fig. 5. Seed transmission in Dickson barley of RNAγ recombinants inoculated in combination with either ND18 RNAsα and β or CV17 RNAs α and β as indicated. Inf/Tot = total number of infected progeny seedlings detected/total number of viable progeny seedlings assayed (pooled from two separate experiments). %ST = percent seed transmission detected (average of two separate experiments; six replicates/experiment). Means within a quadrant and followed by the same letter were not significantly different (Bonferroni [Dunn] *t*-test, α = 0.05).

	ND18αβ		CV17αβ	
	Inf / Tot	% ST	Inf / Tot	% ST
ND18γ	289/567	50.9 a	184/577	31.8 a
17/18X	188/519	36.6 b	165/520	31.8 a
17/18AX	213/514	41.5 ab	196/503	38.8 a
17/18KX	169/522	32.3 b	184/505	36.3 a
17/18MX	110/577	19.1 c	208/585	35.5 a
17/18HX	69/569	12.1 cd	79/571	13.8 b
17X	24/580	4.2 d	55/584	9.4 b

Fig. 6. Seed transmission in Dickson barley of CV17 × ND18 recombinants after deletion of the 369-nt repeat from the γa gene. Each RNAγ recombinant identified at left was inoculated in combination with either ND18 RNAs α and β or CV17 RNAs α and β, as indicated. Inf/tot = total number of infected progeny seedlings detected/total number of viable progeny seedlings assayed (pooled from two separate experiments). %ST = Percent seed transmission (average of two separate experiments; six replicates/experiment). Means within a column and followed by the same letter were not significantly different (Bonferroni [Dunn] *t*-test, α = 0.05).

tion and genome expression, its small cysteine-rich product is believed to play a key role in pathogenesis and the regulation of viral gene expression (Donald and Jackson 1994; Koonin 1991).

Comparison of the γb sequences of strains CV17 and ND18 revealed four amino acid substitutions (Fig. 4). Two of these, Ala^{8}/Val^{8} and Leu^{63}/Pro^{63}, occur within the C1 and C2 cysteine-rich clusters, respectively (Fig. 9, and Donald and Jackson 1994). The lack of silent nucleotide changes within this region and downstream to the *Msc*I site suggests that the Leu^{63}/Pro^{63} substitution, either alone or in combination with the Ala^{8}/Val^{8} substitution, significantly affected seed transmission frequency. It is interesting to note that both the CV17 and Type strains possess leucine at position 63 in the C2 cluster. When introduced into a Type strain mutant, this Leu^{63} to Pro^{63} change produced pleiotropic effects on viral RNA genomic ratios, virus accumulation, and symptom development in barley (Petty et al. 1994).

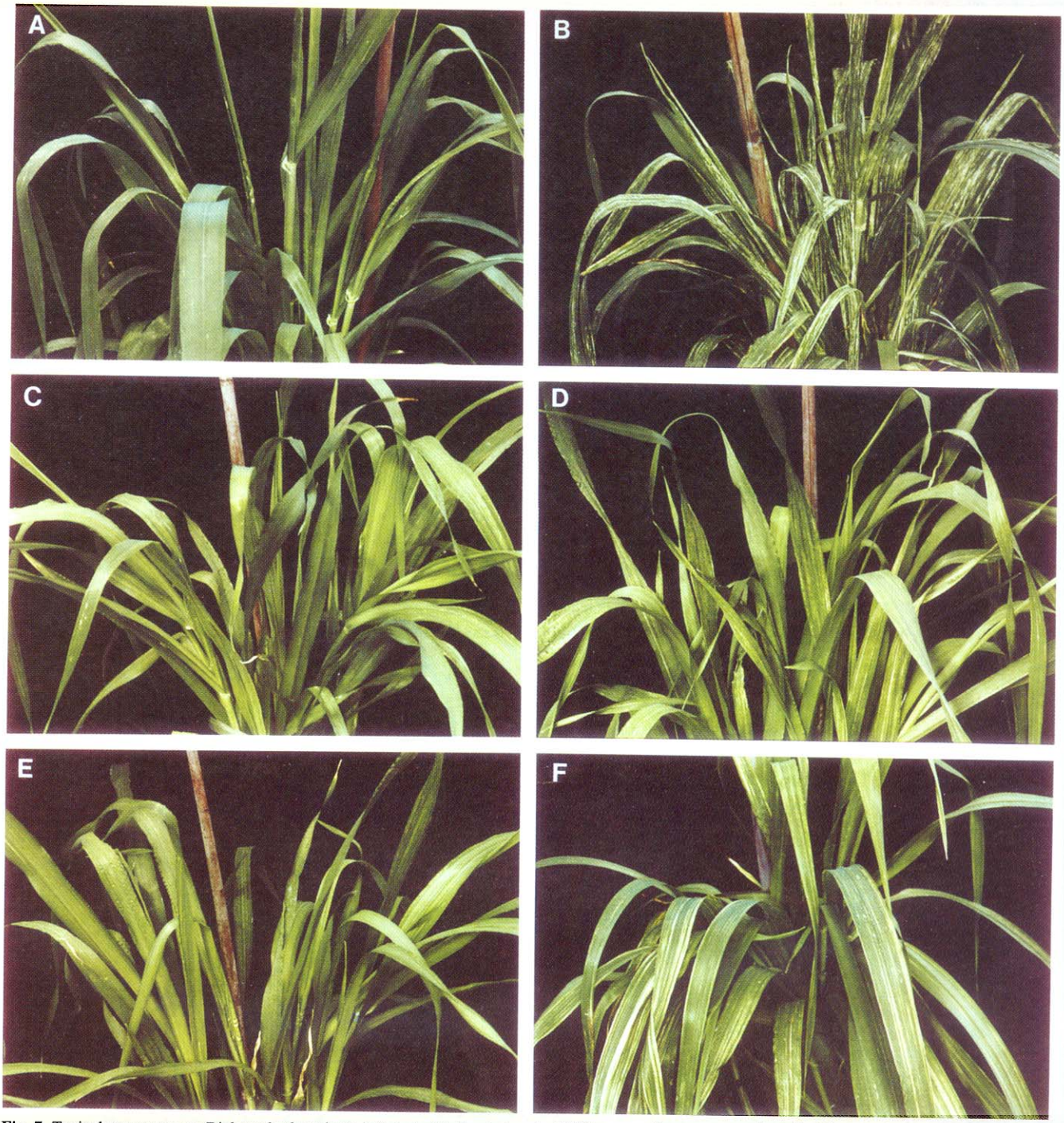

Fig. 7. Typical symptoms on Dickson barley plants infected with the designated RNAγ recombinants, approximately 35 to 40 days after inoculation. **A,** ND18αβ + 18/17X,A, or K; **B,** CV17αβ + 18/17X,A, or K; **C,** ND18αβ + 18/17M or H; **D,** CV17αβ + 18/17M or H; **e)** ND18 check; **F,** CV17 check.

While the A^8/V^8 and L^{63}/P^{63} codon changes clearly affected the seed transmission phenotype, seed transmission levels of recombinants did not approach those of the wild-type without incorporation of additional downstream sequence. Thus, the other amino acid substitutions in γb also may influence this phenotype. Because amino acid differences at the γb carboxy terminus were not separated from the 3′ noncoding region in these experiments, an additional contribution by the 3′ noncoding region cannot be excluded (although no statistically significant differences were observed between 'H' recombinants and the parental γRNAs).

Symptom development was more clearly affected by the L^{63}/P^{63} codon change. Since there are no silent nt differences between ND18 and CV17 in the γb gene upstream of the *Msc*I site, the significant shift in symptom type observed between X, A, or K recombinants (Fig. 7A,B) and M or H recombinants (Fig. 7C,D) apparently was due to the L/P codon change. Although the mechanism underlying the phenotypic effects of the L/P substitution is not clear from these results or those of Petty et al. (1994), the L/P substitution could reasonably be expected to change the conformation of the γb protein and thus affect its activity. Comparative analysis of the CV17 and ND18 γb protein sequences using several protein secondary structure prediction methods (Garnier et al. 1978; Chou and Fasman 1979; Gascuel and Golmard 1988) suggests such a conformational change is likely (not shown). Donald and Jackson (1994) found pronounced symptom effects as a result of mutations within the γb cysteine-rich clusters and the basic motif linking the two. The symptom effects observed in their study do not appear to be the same as observed here; nevertheless, both studies demonstrate the importance of the γb gene in disease development.

Although the primary determinants of the seed transmission and symptom phenotypes studied here mapped to elements of RNAγ, these phenotypes were not completely independent of interactions between these elements of RNAγ and RNAs α and β (or their gene products). Certain heterologous combinations of the RNAγ 5′ leader and the γb gene resulted in dramatic changes in symptomatology and seed transmission, but the specific phenotypic effects were also clearly dependent on an interaction with RNAs α and β. It is not surprising that readily discernible phenotypic effects arose as a result of these interactions, given the known *trans* effects of γb on RNAβ gene expression and that RNAβ encodes both the coat protein and the "triple gene block" proteins implicated in virus movement. It is also reasonable to propose that substantial sequence changes in the RNAγ leader might alter a potential interaction with the αa protein, an essential component of the viral replicase, in the replication of RNAγ. The fact that symptoms unlike those induced by either parental strain were induced by the ND18γ leader/CV17γb recombinants, but not by the pseudorecombinants, further suggests a possible interaction between the RNAγ 5′ leader and the γb gene. Such an interaction could be direct, or indirect as a result of an interaction between the γb gene and RNAs α or β.

It appears that recombination between the leader regions of CV17 RNAs α and γ may have forced compensatory changes in certain other elements of the CV17 genome. The phenotypic effects of disrupting these adaptations provides biological evidence for the molecular interactions among different genomic elements. The complexity of these interactions hampers the distinction of viral features which directly influence the seed transmission phenotype from those which influence the phenotype through indirect effects on other viral processes. Analysis of the ability of recombinant or mutant viruses to replicate and spread in vegetative versus reproductive barley tissues should facilitate the identification of viral features that may be specifically involved in the seed transmission phenotype.

Fig. 8. Typical symptoms on Dickson barley seedlings infected with ND18 × CV17 pseudorecombinants at 7 days after inoculation. Plant at the left is healthy; the next two are representative of seedlings infected with pseudorecombinants possessing an ND18 RNAγ; the two seedlings on the right are representative of those infected with pseudorecombinants possessing a CV17 RNAγ.

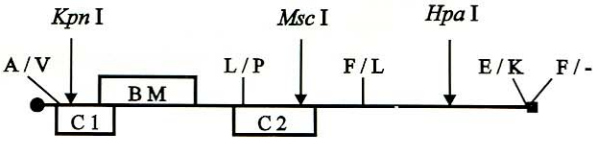

Fig. 9. Schematic representation of the γb gene product showing relative locations of the cysteine-rich regions (C1 and C2), the basic motif (BM), the restriction sites used in this study (*Kpn*I, *Msc*I, *Hpa*I), and the amino acid differences between CV17 and ND18 (CV17-encoded amino acids are to the left of the slash, ND18-encoded amino acids are to the right of the slash).

MATERIALS AND METHODS

Virus and cDNA clone sources and maintenance.

Virus isolates were removed from storage in liquid nitrogen, propagated in "Black Hulless" barley (*Hordeum vulgare* L.) in a greenhouse with a 16-h photoperiod at 27°C, and purified as previously reported (Edwards and Timian 1986). BSMV strain CV17 was originally derived from an isolate infecting wheat in Wyoming. Strain ND18 was originally isolated from Type strain by R. G. Timian; Type strain was originally isolated by passage through tobacco by H. H. McKinney.

The procedures of Petty et al. (1988) were used to construct full-length clones of CV17 (Edwards et al. 1992). The full-length nature of the cDNA clones, the integrity of the cDNA/plasmid junctions, and the authenticity of the clones were verified by sequencing and infectivity assays. Clones representing the ND18 and Type strains were kindly provided by A. O. Jackson (Petty et al. 1988, 1989).

Sequencing of γRNAs.

Nucleotide sequence of CV17 RNAγ was determined by sequencing both strands of an infectious full-length cDNA clone. Sequence of the plus strand (viral sense) was obtained using a nested deletion strategy as follows. A 3.1-kb *ClaI*/*MluI* fragment from clone pCV17γ2 was blunt-ended and transferred into the *Eco*RV site of pBluescript II SK- (Stratagene). Nested deletions were generated using Exo III and S1 nucleases. Deletion clones were selected by size and sequenced using deazaGTP and standard dideoxynucleotide sequencing methods (Sanger et al. 1977). Universal forward and reverse primers were used to obtain sequence corresponding to 5′ and 3′ termini as well as to sequence deletion clones. Sequence of the minus strand was obtained by primer walking with custom primers designed on the basis of the known sequence. Determination of the minus-strand sequence in the 5′ proximal repeated region was accomplished by using primers binding near the end of the repeat (nts 826 to 840) and adjacent to the junction of the two repeat units (nts 444 to 458). Confirmation of sequence corresponding to the 5′ terminus was achieved using the latter primer and also by using the γ826 primer to sequence a clone in which the repeat had been deleted with *Xmn*I. The initial single-strand sequence of the minus strand of the ND18 and Type clones was obtained utilizing the same primer set used for CV17 RNAγ. Differences with previously published sequences of these two strains were further verified by sequencing of the opposite strand.

Construction of recombinants.

Capped BSMV α, β, and γ RNAs were produced by in vitro transcription of the respective full-length clones for each virus strain exactly as previously described (Petty et al. 1989). Pseudorecombinants were created by simply mixing equal amounts of the appropriate combinations of capped transcripts. All possible combinations were created and evaluated.

True recombinants were constructed by the progressive substitution of CV17 RNAγ-specific sequence into ND18 RNAγ and vice versa. Full-length γ clones of strains ND18 and CV17 were double-digested with one of the five restriction enzymes depicted in Figure 1 and with *Mlu*I (which cuts exactly at the viral 3′ terminus) according to the conditions specified by the enzyme supplier. The restriction digests were then electrophoresed in 1% agarose gels containing 1× TAE and 0.5 μg/ml of ethidium bromide. The appropriate fragments were pooled and the DNA extracted from the gel using GeneClean (Bio 101). The DNA fragments were then ligated together and the ligated DNA was used to transform *E. coli* DH5αF′ according to standard protocols (Sambrook et al. 1989). Individual colonies were selected, grown overnight, and plasmid DNA was extracted using the alkaline lysis method. The recombinant nature of plasmid inserts and the integrity of the ligation sites were verified by RFLP mapping with *Rsa*I and by DNA sequencing (Sanger 1977).

An *Xmn*I site appearing within the repeated region of CV17 RNAγ (nts 73 to 441 and 442 to 810) was used to excise the repeat from the CV17 RNAγ-derived sequence in some recombinants. After *Xmn*I digestion, these plasmids were gel purified, religated, propagated, and purified as described above.

Plasmids which contained the appropriate recombinant inserts were linearized with *Mlu*I, transcribed, and resultant γ transcripts were then mixed with the appropriate α and β transcripts and inoculated to plants as previously described (Petty et al. 1989).

RNAs were inoculated directly to 5- to 6-day-old Dickson barley plants, except in one of the pseudorecombination experiments in which RNAs were inoculated to Black Hulless barley and isolates were subsequently transferred to Dickson barley after a 1-week incubation period. No differences were observed in either seed transmission or symptom phenotype when virus isolates were inoculated directly to Dickson barley or inoculated to Dickson barley after a single passage in Black Hulless barley.

Plant maintenance.

Dickson barley seed was planted in artificial media in 6-inch pots. Plants were maintained at 2 per pot with 6 plants total per virus isolate per experiment. Plants were held in a greenhouse at approximately 24 to 27°C for 1 to 2 weeks while initial symptoms developed, and then temperature was reduced to about 16 to 21°C to promote normal tillering. Temperature range was then allowed to rise to 21 to 27°C until plants reached maturity. Day-length was extended to 16 h with supplemental lighting from sodium vapor lamps. Benlate and Temik were applied as necessary to control powdery mildew and insect pests. Plants were fertilized weekly with a water soluble fertilizer (20-20-20).

Seed harvest and assay for seed transmission.

After drying, heads were harvested and seed was threshed and cleaned by hand to prevent any possible cross-contamination. To assess seed transmission rates, approximately 50 to 100 seeds per mother plant (300 to 600 per virus isolate per experiment) were planted and observed for symptoms typical of infection for up to 10 to 14 days after planting. ELISA was then used to verify the absence of virus in apparently healthy seedlings. Standard double antibody sandwich ELISA protocols were used (Clark and Adams 1977).

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