Korean J. Ecol. Sci., 1(2): 83~91, 2002

Short-Term Viral Evolution in Response to Passaging I. Consequences for Population Size

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ABSTRACT: The Red Queen hypothesis for the advantage of sex predicts that pathogens will evolve by increasing fitness with frequent encounters with specific host genotypes. In this study, BMV population size, measured as an indicator of fitness, was investigated during repeated passages through the same, or different host genotypes of the crop host, *Hordeum vulgare* (barley). Overall, mean BMV concentration within individual hosts was significantly higher in genetically homogeneous compared to heterogeneous host passage lines. In addition, BMV populations, passaged through a specific host variety, showed higher growth in that host variety compared to BMV passaged through varying varieties. These results supports the Red Queen hypothesis. However, the decrease in viral populations during passages contradicts the Red Queen.

Nevertheless, the results found here show that even under simplified conditions, pathogens do not evolve in simple, predictable ways. Constraints on pathogen evolution may lead to counterintuitive results.

Key words : BMV, Evolution, Host, Pathogen, Red Queen

INTRODUCTION

Considerable recent interest has centered on the short-term evolutionary potential of pathogen populations. Many factors are thought to affect pathogen evolution, including transmission mode, host population size, and host population genetic structure (Anderson and May, 1982; May and Anderson, 1983). Pathogens are under selective pressure to preferentially attack common host genotypes, which can lead to a cycling of host and pathogen genotypes driven by frequency-dependent selection (Bell, 1982; Hamilton, 1982). The Red Queen hypothesis predicts that under these circumstances, sexual reproduction can be advantageous by producing rare genotypes which escape pathogen attack (Maynard-Smith, 1978; Bell, 1982; Hamilton, 1982). In addition, if pathogens adapt to common host genotypes, then higher virulence will be found on common than rare hosts. Thus, sexual reproduction will be favored by producing novel as well as rare genotypes (Bremermann, 1980; Clay and Kover, 1996; Lively, 1996).

With several exceptions, the prediction of frequency-dependent disease incidence has been supported. Parasitic trematode (*Uvulifer* sp.) infection was proportional to clone frequency in a fish, *Poeciliopsis monacha* (Lively *et al.*, 1990) and a freshwater snail, *Potamopyrgus antipodarum* (Dybdahl and Lively, 1995). Cloned genotypes of the grass, *Anthoxanthum odoratum*, were more frequently virus-infected when common than rare in field arrays (Kelley, 1993, 1994). The percentage of the leaf covered by *Puccinia striiformis* lesions was greater in common than rare genotypes in wild (*Chondrilla juncea* ; Chaboudez and Burdon, 1995) and cultivated hosts (*Triticum aestivum* ; Finckh and Mundt, 1992). However, the severity of several rust infections (*Puccinia monoica* and *P. thlaspeos*) in *Arabis holboellii* (Roy, 1993), *Puccinia podophylli* in mayapple, *Podophyllum peltatum* (Parker, 1989), *Puccinia striiformis* in *Triticum aestivum* (Finckh and Mundt, 1992) were not correlated with genotype frequency.

In addition to frequency dependent pathogen incidence, the Red Queen hypothesis also predicts enhanced virulence in frequently infected host genotypes. Virulent strains will be favored by among host selection, if virulent pathogens acquire a selective advantage by increased transmission to new hosts (Levin and Pimental, 1981; May and Anderson, 1983; Bull, 1994; Ebert, 1994a; Ebert and Hamilton, 1996). Virulent strains may also be favored by within-host selection, if the rapid growth virulent denies resources to benign strains (Bremermann and Pickering, 1983; Levin and Bull, 1994; Nowak and May, 1994). Indirect experimental evidences in support of this hypothesis comes from reciprocal transinfection experiments with fungi, trematodes, microsporidia, and insects which show higher infection rates in hosts sampled from the same population as the pathogen (Parker, 1985; Lively, 1989; Ebert, 1994a; Mopper *et al.*, 1995, respectively). Other evidence comes from passage experiments, in which a pathogen or parasite inoculated into a series of individuals of the same geno-

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type show enhanced fitness or virulence in that host. In repeated passages with high inoculum concentration, the fitness of VSV (vesicular stomatitis virus), measured as relative growth compared to that of wild type virus, increased during passages through same host cell lines (Novella *et al.*, 1995; Elena *et al.*, 1996). However, in passages which imposed a genetic bottleneck, viral fitness continuously decreased during passages through the same host cell line (Chao, 1990). Increased invasiveness and symptom severity with repeated passages in single crop varieties have been reported with several plant viruses (reviewed by Yarwood, 1979). For example, in a study with barley stripe mosaic virus (BSMV), BSMV initially showed a low systemic infection rate in wild oats when it was transferred from barley, but the rate increased after ten passages (Chiko, 1984).

Previous studies did not measure changes in parasite /pathogen population size, virulence, and infectivity. Further, the majority of reciprocal transinfection and passaging experiments demonstrate pathogen adaptation at the population or species level, but not to particular host genotypes. Dawson (1967), however, showed that tomato mosaic virus cultured on a resistant tomato cultivar, increased virion number, symptom severity, and viral infection rate in that cultivar. Karban (1989) found that the specialized insect, *Apterothrips secticornis*, had higher population growth rates on the natal than other host tree clones of *Erigeron glaucus*. Mopper *et al.* (1995) showed that the leafmining insect, *Stilbosis quadricustatella*, initiated significantly more mines in natal than novel clones of *Quercus geminata*.

In addition, several studies show reduced virulence in the long associated hosts (Allison, 1982; Read, 1994). In African trypanosome epidemics, trypanosomes caused mild infection in wild ruminants, severe disease in domestic ruminants, and the most severe disease in recently imported ruminants (Allison, 1982). The rinderpest virus was relatively mild in its original hosts (cattle), but became highly virulent when introduced to wildebeest and cape buffalos. After a wildebeest and cape buffalos epidemic, virulence was reduced in those species, but increased in cattle (MacCallum and Dobson, 1995). The most famous plant disease epidemics involve novel host x pathogen combinations (chestnut blight: *Cryphonectria parasitica* in *Castanea dentata*; Dutch elm disease: *Ophiostoma ulmi* in *Ulmus americana*).

Contradictory observations and the lack of the information at host genotype level highlight the need for further study of the evolutionary consequences of host-pathogen association for pathogen evolution. Whether pathogens evolve increased virulence in common host genotypes is relevant to theories of sex (Maynard-Smith, 1978; Bell, 1982; Hamilton, 1982), disease control in agriculture (Marshall, 1977; Safeeulla, 1977; Wolfe, 1985) where higher disease incidence has been observed in genetically homogenous than heterogeneous agricultural fields (Finckh and Mundt, 1992, 1993), and mechanisms of rare species conservation (McCallum and Dobson, 1995) where epidemics of malaria in the Hawaiian avifauna, distemper in black-footed ferrets, and rinderpest in wildebeest and cape buffalos (Van Riper *et al.*, 1986; O' Brien and Evermann, 1988) may result from low genetic diversity in rare species.

We used four varieties of the crop plant (*Hordeum vulgare*) and common graminaceous virus, brome mosaic bromovirus as a model system to address the following questions: (1) Do pathogens evolve increased population size in frequently encountered host genotypes, and 2) Do pathogens evolve increased virulence, infectivity, and multiplication rates in frequently encountered host genotypes? In this study, short-term changes in a virus were measured as it was repeatedly passaged through genotypically homogeneous and heterogeneous host populations. Additional experiments were directed to identifying causes of the observed population size changes found in these experiments. The results shed light on the applicability of the Red Queen hypothesis, and on the importance of transmission dynamics to pathogen evolution.

MATERIALS AND METHODS

Host -pathogen model system

The model host used in these experiments was barley, a self-fertilizing, annual cereal (Hitchcock, 1951). Four genetically distinct varieties were used: Lewis, a two-rowed, white-aleuroned, spring, mid-season barley (Hockett *et al.*, 1985); Gaacton, a six rowed winter barley with rough awns; Venus, another six-rowed, winter barley with rough awns; and Wysor, a winter barley which varies awned to awnless (Starling *et al.*, 1987; Brown *et al.*, 1988; pers. comm. Dr. Jerry Johnson, Ag. Exp. Station, Griffin, GA).

The model virus used was brome mosaic bromovirus (BMV), a positive, single-stranded RNA virus and the type member of the bromovirus group. Plant viruses represent a plausible test of the Red Queen hypothesis. Most plant viruses have RNA genomes, with high spontaneous mutation rates (Steinhauer and Holland, 1987). Therefore, plant virus populations consist of many variants (Domingo *et al.*, 1985). Experimental studies have shown rapid changes in RNA virus fitness, measured as the relative growth rate compared to wild-type (Chao, 1990; Novella *et al* , 1995). Plant viruses are obligate parasites, and genotypic and phenotypic changes in viral populations have been shown to be driven by host factors (Kilbourne and Murphy, 1960; Chiko, 1984; Shepherd *et al*, 1987; Hajimorad *et al.*, 1991; Kilbourne, 1994). Viral vectors often shows host genotype dependent behavior (Kelley, 1993). Over 90% of plant viruses are transmitted by insect vectors (Harrison, 1987). Finally, viral pathogens are ubiquitous in wild plants (MacClement and Richards, 1956; Barrett and Gibson, 1975; Hammond, 1981; Guy, 1988) and significantly reduce fitness (Kelley, 1993). Plant viruses are likely to

be important frequency-dependent selective forces in natural and agricultural populations.

Brome mosaic bromovirus (BMV) has three genomic and one subgenomic RNA particles, with icosahedral particle structure (Ahlquist, 1994). RNA1 and 2 encode proteins involved in RNA replication and transcription. RNA3 encodes a protein needed for infecting plants. Subgenomic RNA4 is responsible for the coat protein (Ahlquist, 1994). Natural transmission of BMV has not been clearly characterized, but BMV can be transmitted by nematode or beetle in the laboratory (Lane, 1981), and by mechanical inoculation. The host range of BMV is largely restricted to the Gramineae, and BMV does not normally cause significant economic damage (Lane, 1981). Typical symptoms of BMV infection in barley include stunting and yellow streaks on leaves (Lane, 1981).

Host passage

BMV from wild-collected *Anthoxanthum odoratum* was used as a source for host passages. BMV from *Anthoxanthum odoratum* was passaged through two different lines (Fig. 1) which were distinguished as genetically homogeneous and heterogeneous host lines. In genetically homogeneous host lines, a single barley variety was used in every passage. Four different kinds of lines were used, each comprised of either Lewis, Gaacton, Wysor, or Venus. In genetically heterogeneous lines, two types of lines were used. In continuously changing genotype lines, a single barley variety was used in each passage, but the variety was changed at each passage.

In the mixed line, an equal mixture of all four varieties was used in every passage. Each passage type consisted of 3 replicate lines, thus, a total of 18 individual passages were used (6 types of passage x 3 replicates/type = 18 passages). Passage

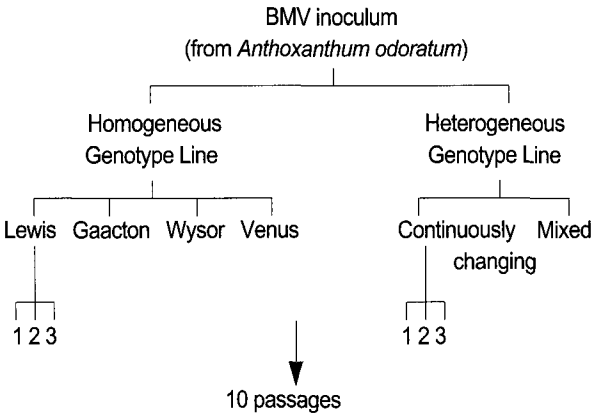

Fig. 1. Diagram of passage of BMV. Four types of homogeneous lines represent each variety used in passage. Each line has 3 replicate lines, thus, a total of 18 passages were conducted. A total of 4320 plants were used in passages (24 plants/line x 18 lines/passage x 10 passages =4320 plants).

lines were placed in trays in a completely randomized design, and varieties within mixed lines were completely randomized.

Passaging occurred every two weeks, with a total of 10 passages from June, 1994 to October, 1994. In each passage, leaves of every infected plant were harvested. Each plant was tested for infection, one week after inoculation, using ELISA (enzyme-linked immunosorbent assay). Half of the infected plant leaves were used to inoculate the plants of the next generation, and the other half were used to measure BMV concentration and then freeze-dried (-50°C, Freezemobile 5sl, Virtis Co.) for later experiments to measure virulence, infectivity, and multiplication rate. Plants in the next generation were inoculated by dusting the leaf with diatomaceous earth and then rubbing with infectious sap, obtained by grinding (1/10 dilution) fresh leaves of the infected plants from the previous generation.

A second host passage experiment was conducted between March and May, 1997 with inoculum consisting of either the same amount of BMV each generation (0.06mg/ml, total 0.6mg in 10ml sap) or of equally-diluted (1/10) infectious sap (original protocol). BMV from wild-collected *Anthoxanthum odoratum* was passaged through Venus variety four times. Each passage line had two replicates, with two inoculum concentrations. At the time of passage, all leaves of infected plants were harvested from the previous generation. Half were used to measure BMV concentration within individual plants with quantitative ELISA (McLaughlin *et al*, 1984), and the other half used for passage to the plants of next generation. A total of 384 plants were inoculated in the experiment (24 plants/passage line x 4 passage lines x 4 passages = 384 plants).

A third host passage experiment was conducted between December, 1996 to March, 1997 using equally diluted inoculum (1/10 dilution) and the same protocol outlined above. BMV from wild-collected *Anthoxanthum odoratum* was passaged through Venus variety four times. Each passage line had two replicates. A total of 192 plants were inoculated in the experiment (24 plants/passage line x 2 passage lines x 4 passages = 192 plants).

A fourth host passage experiment was conducted between March and April, 1996. BMV from *Anthoxanthum odoratum* was passaged through Lewis and Wysor varieties with inoculum of equally diluted BMV (1/10 dilution), four times. Each passage line had two replicates. All of the passage protocols were same as described at first passage experiment. A total of 384 plants were inoculated in the experiment (24 plants/passage line x 2 replicate lines/passage x 2 varieties x 4 passages = 192 plants).

Population size measurement

BMV population size was measured as BMV concentration (mg BMV/g fresh leaf weight) within an individual infected plant, at the time of passaging. In every passage, the same amount of fresh leaf (0.03g) was taken from each infected plant, and then

BMV concentration was measured by quantitative ELISA. The BMV was purified using Lane' s method and used as a standard for calculating concentration (McLaughlin *et al* , 1984; Lane, 1986).

Experimental conditions and data analysis

Each plant was grown in a tube (2.5 cm in diameter x 12.5 cm in height) filled with commercial soil (Farfard mix 3-B). A total of 200 tubes were placed in each tray. All plants in the experiment (healthy and inoculated) were cultured in a controlled environment (growth chamber: model number CMP 3244, Conviron Co., 25℃ during day, 20℃ during night, 16 hrs light period), and watered daily.

Data were statistically analyzed using SAS version 6.11 (SAS Institute 1995). Data for BMV concentration were ln-transformed to normalize the distribution. Since BMV concentration in each generation was affected by the concentration of the previous generation, repeated measures of analysis of variance were performed using a general linear model for testing statistical significance of the difference in BMV concentrations between homogeneous and heterogeneous host passages (Spector *et al.* 1985).

RESULTS

Change in BMV population size

In both homogeneous and heterogeneous passages, BMV infection percentage varied between 18% and 40% in the first generation, and then it increased in the second generation (60-100%). This high infection percentage was maintained in almost all passage lines over all generations (Table 1a). No significant difference in the percent infected was found between homogeneous and heterogeneous (mixed and changing) passage lines (Table 1b). BMV population size within individual plants, measured as BMV concentration per gram fresh leaf weight, increased between generation 1 and 2 in both homogeneous and heterogeneous passage lines (Fig. 2). This trend can be visualized as the percentage of change in BMV concentration between generations (Table 2). After the second generation, BMV concentration within individual plants decreased in both homogeneous and heterogeneous passage lines (Fig. 2). The slope of linear regression of BMV concentration against passage generation after the second generation was negative in every passage line (Fig. 2 and Table 2). However, BMV concentration significantly decreased in 2 out of 12 lines of homogeneous host passage and in 2 out of 6 heterogeneous host passage lines (Table 2).

Overall, mean BMV concentration in genetically homogeneous passage lines was 11% higher than that of genetically heterogeneous passage lines, and this difference was statistically significant by repeated measures analysis of variance (Table 3a and

Table 1. Mean infection rate during genetically homogeneous and heterogeneous host passages. The difference in infection percentage was tested for statistical significance by repeated measures analysis of variance

a.

	Infection percentage (%)					
	Homogeneous			Heterogeneous		
Generation	Lewis	Gaacton	Wysor	Venus	Changing	Mixed
1	18.1	38.9	22.2	38.9	33.3	29.2
2	61.1	95.8	94.4	93.1	95.8	90.3
3	90.3	84.7	94.4	84.7	98.6	91.7
4	68.1	98.6	97.2	98.6	90.3	93.1
5	75.0	98.6	95.6	87.5	80.6	87.3
6	77.8	94.4	94.4	94.4	97.2	88.7
7	65.3	91.7	94.4	93.1	86.1	77.8
8	75.0	81.9	91.7	87.5	93.1	68.1
9	51.4	59.7	95.8	84.7	54.2	68.1
10	97.2	87.5	90.3	97.2	93.1	72.2

b.

Between homogeneous and heterogeneous			Among homogeneous, mixed, and changing		
Source	d.f.	F	Source	d.f.	F
Passage type	1	0.73	Passage type	2	3.02
Error	4		Error	6	

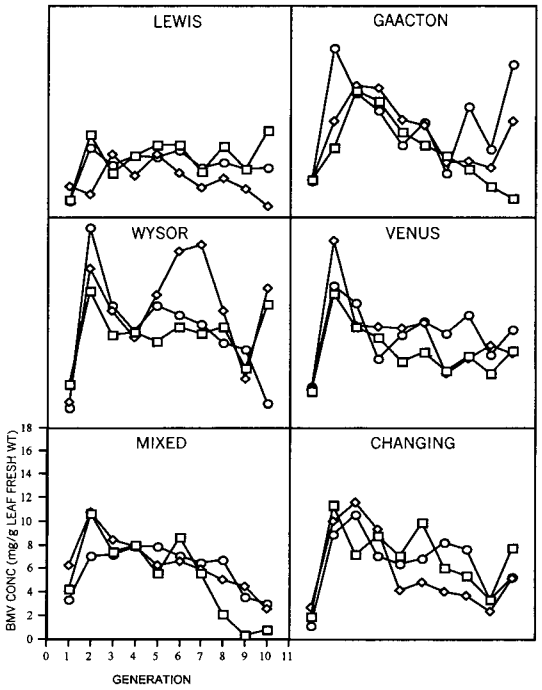

Fig. 2. Change in BMV concentration within infected plants during homogeneous and heterogeneous host passages. Square, circle and diamond lines represent each of three replicate lines. Each point in the line is the mean BMV concentration within infected plants.

Table 2. Percentage of change in BMV concentration between the first and second generations, and the slope of linear regression of change in BMV concentration after second generation. Percentage of change in BMV concentration was calculated by the equation: {(mean BMV concentration in second generation - mean BMV concentration in the first generation) / mean BMV concentration in the first generation} *100. Ln-transformed data were used for each regression. The difference in percentage of change in BMV concentration between homogeneous and heterogeneous passages was tested for statistical significance by t-test and analysis of variance[1].

Passage type	Line	Line replicate	Percentage of change between 1st and 2nd generations (%)	Slope of decrease after 2nd generation
Homogeneous	Lewis	1	396.13	-0.1462
		2	-25.87	-0.0458
		3	352.22	-0.0355
	Gaacton	1	85.58	-0.3288*
		2	152.73	-0.1180
		3	363.15	-0.0654
	Wysor	1	223.56	-0.0270
		2	516.39	-0.0807
		3	907.37	-0.1739*
	Venus	1	275.69	-0.0851
		2	407.73	-0.0932
		3	253.04	-0.0660
Heterogeneous	Mixed	1	463.20	-0.6322*
		2	259.25	-0.2177*
		3	643.66	-0.0940
	Changing	1	154.60	-0.0708
		2	73.35	-0.1468
		3	110.70	-0.1393

Symbol: 1, the difference between homogeneous and heterogeneous passages was tested by t-test (T=-0.356, d.f.=16, p=0.73). The difference among homogeneous, mixed, and changing passages was tested by analysis of variance (F=1.97, d.f.=2, 15, p=0.17). *, significant at 0.0028 significance level by Bonferroni multiple comparison.

Table 3. Average BMV concentration within individual plants in homogeneous and heterogeneous passage lines. Each value represents mean ± standard error. The difference in mean BMV concentration between homogeneous and heterogeneous passage lines was tested for statistical significance by repeated measures analysis of variance using ln-transformed data

a.

Passage type	Average BMV concentration within individual plants (mg BMV / g fresh leaf weight)
Homogeneous (n=2257)	7.396 ± 0.124
Heterogeneous (n=1107)	6.642 ± 0.149
Mixed (n=539)	6.293 ± 0.209
Changing (n=568)	6.973 ± 0.211

b.

Between homogeneous and heterogeneous			Among homogeneous, mixed and changing		
Source	d.f.	F	Source	d.f.	F
Passage type	1	9.66*	Passage type	2	2.04
Error	4		Error	6	

Symbol: *, p<0.05

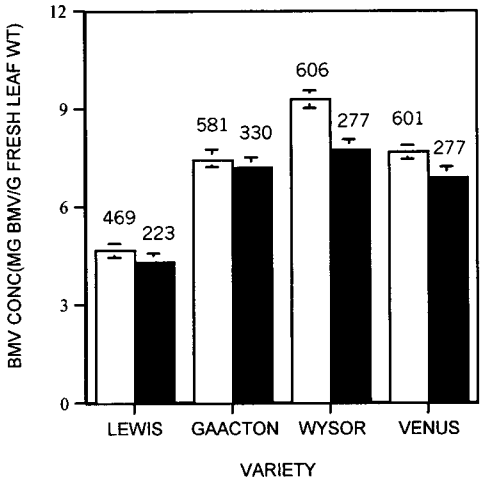

Fig. 3. Average BMV concentration within individual plants between homogeneous and heterogeneous passage lines shown in each variety. Open and closed bars represent the mean BMV concentration in homogeneous and heterogeneous passage lines, respectively. Each bar represents the mean and standard error. The number above the bar represents the number of infected plants. The difference in BMV concentration between homogeneous and heterogeneous passage lines was tested for statistical significance by repeated measures analysis of variance. Significance was decided at 0.0125 significance level by Bonferroni multiple comparison.

b). On average, BMV concentration between the first and second generations increased more in homogeneous compared to heterogeneous lines (325.6% increase in homogeneous and 284.1% in heterogeneous), and significant decreases after the second generation occurred in a higher proportion of heterogeneous than homogeneous passage lines (Table 2, 17% of homogeneous passage lines and 33% of heterogeneous passages lines). However, the difference in BMV concentration among homogeneous, mixed, and changing passage lines was not statistically significant (Table 3b). BMV was at a higher concentration in homogeneous than both mixed and changing passages, and higher in changing than mixed passages. In each variety, mean BMV concentration was higher overall in homogeneous than heterogeneous passage lines, but this difference was not statistically significant in every variety (Fig. 3).

Repeatability of the changes in BMV concentration within individual host

When BMV was passaged using the same protocol as used in the original passage experiment (1/10 dilution of infected leave material used as inoculum), BMV concentration within individual plant followed the same pattern in 3 out of 4 experiments (Fig. 4). BMV concentration within individual plants increased in the second generation and then decreased (Fig. 4). However, in one case, BMV concentration monotonically increased until the fourth generation (Fig. 4d).

In the passages with same amount of BMV used as inocula each generation, the pattern of BMV concentration within individual plant was similar to that observed in host passages with equally diluted inoculum. BMV concentration increased at the second generation and then decreased (Fig. 5a and b).

Viral population growth between generations was estimated by dividing the BMV concentration within individual plant by the estimated BMV inoculum concentration. This ratio increased at the second generation, and then decreased in the passages with inoculum of equal virus concentration and of equal dilution (Fig. 5c and d). However, the rate of decrease after the second generation was greater in the passages with same dilution than with same amount of BMV (Table 4). In the original passages, BMV growth rate was estimated as the BMV concentration within individual plants divided by the mean BMV concentration in previous generation, because BMV concentration in inoculum was not measured. This calculation is reasonable if the BMV concentration in the inoculum was proportional to that in individual plants in the previous generation (which it should have been, since half of these plants were used as the inoculum source). Using this measure of growth rate, BMV growth increased at the second generation, and then dramatically decreased in the third generation (Fig. 6). After the third generation, no obvious changes were observed.

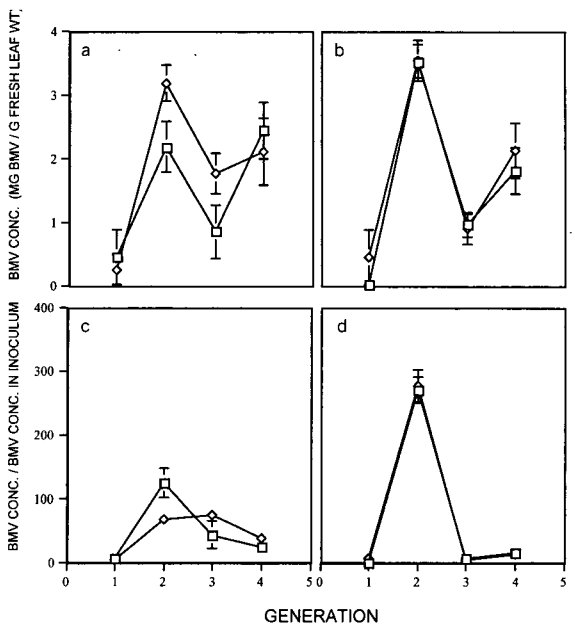

Fig. 5. Changes in BMV concentration within individual plant during host passages (a and b) and BMV concentration within individual plant divided by BMV concentration in inoculum (c and d) when BMV was repeatedly passaged through Venus variety with same amount (a and c) and dilution (b and d). Square and diamond lines represent each replicate line.

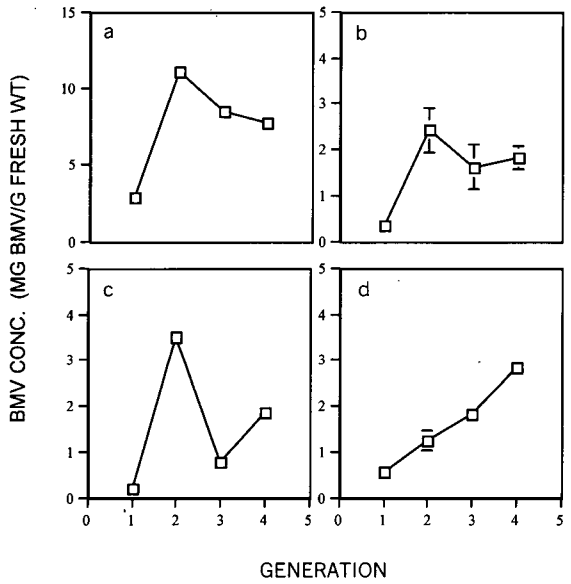

Fig. 4. Changes in BMV concentration during passages. Changes in BMV concentration are shown in each of four genetically homogeneous host passages conducted separately. The graph a is the result from the original host passage experiment shown in only genetically homogeneous passage up to the 4th generation. In b, passage was done by Dai Kio.

Table. 4. The results of linear regression of changes in BMV concentration within individual plant per capita BMV inoculated during repeated passages through Venus variety with same amount and dilution of BMV. Linear regression was conducted for the change in BMV concentration after 2nd generation. LN-transformed data from 2nd, 3rd, and 4th generations were used in linear regression

Inoculum treatment	Line replicate	Slope	R2	T for H0: slope=0	p
Same amount	1	-0.752	0.051	-1.735	0.088
	2	-1.251	0.159	-3.499	0.0008
Same dilution	1	-1.919	0.244	-4.542	0.0001
	2	-2.058	0.220	-4.351	0.0001

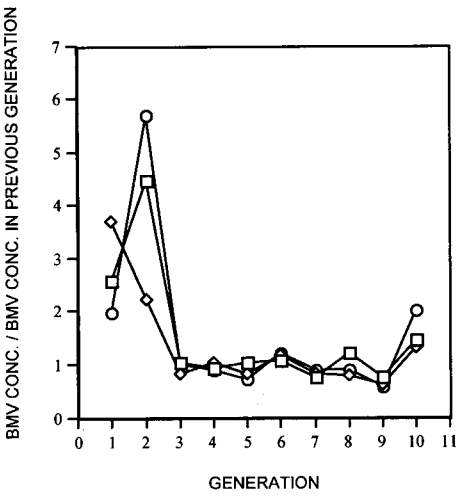

Fig. 6. Change in BMV concentration within individual plant divided by mean BMV concentration within individual plant in previous generation. Square, diamond, and circle lines represent genetically homogeneous, mixed, and changing passages, respectively.

Table. 5. The rate of initial increase in BMV concentration between the fourth and seventh day after inoculation, and BMV concentration at the 14th day after inoculation. The rate of initial increase in BMV concentration within a host was measured as the amount of increase in concentration between fourth and seventh day from inoculation per day (conc./day), which was calculated as (concentration at the seventh day - concentration at the fourth day) / 3days. The number in the parenthesis is sample size

Generation	rate of initial increase in BMV concentration (conc./day)	BMV concentration at the 14th day (mg/g fresh leaf weght)
1	0.208 ± 0.0389 (28)	1.011 ± 0.0805 (161)
2	0.207 ± 0.0523 (28)	0.935 ± 0.0910 (152)
3	0.232 ± 0.151 (4)	1.719 ± 0.273 (22)
8	0.226 ± 0.161 (3)	0.961 ± 0.253 (23)
9	0.109 ± 0.0374 (24)	0.877 ± 0.109 (142)

DISCUSSION

A significantly higher mean BMV concentration within individual plants observed in the passages of homogeneous compared to heterogeneous host genotypes is consistent with the prediction of the Red Queen hypothesis that pathogens which continuously encounter the same host genotypes will achieve higher population sizes. This might be the consequence of selection for higher BMV growth in the host genotype, with which it is in frequent contact, which is not possible in heterogeneous passages because of the changing host environment. In each variety used in host passages, the BMV population, passaged through only a specific host variety, showed a trend to increase its growth within individual plant compared to BMV passaged through varying genotypes. The results presented here affirm previous findings of increased viral particle density in a particular plant variety after the virus was cultured in it (Dawson, 1967).

However, the decrease in BMV concentration after the second generation contradicts the Red Queen hypothesis, because increased viral populations over time were predicted. Actually, BMV concentration increased at the second generation. The increase in BMV concentration between first and second generations may have resulted from selection of a strain(s) compatible with barley, since the original host plant was a different species (*Anthoxanthum odoratum*). Similar results have been shown previously with barley stripe mosaic virus (BSMV), transferred from barley to wild oats, in which the rate of systemic infection was initially low in wild oats, but increased after 10 passages with 20-25 days cycle (Chiko, 1984). However, the decrease in viral concentration after the second generation in the passage through even same host genotypes is not consistent with that predicted from strain selection during host passages. Repeated selection for the beneficial mutants, with higher replication ability within a host is known to result in the increase in viral fitness during genetically homogeneous host passages (Chiko, 1984; Novella *et al.*, 1995; Elena *et al.*, 1996). But why an increase in viral fitness should lead to decreased population sizes is not immediately clear.

The decrease in BMV concentration after the second generation observed in original host passages was repeated when viruses were passaged under the same conditions in three cases out of four separate passage experiments. In the one experiment with a different result, a continuous increase in BMV concentration within individual plants was observed to four generations. This result may be related to the unusual symptoms which were present in plants of the third and fourth generation of this experiment. Leaves were vaguely chlorotic with necrotic spots, although the cause of these foliar symptoms was not identified. Interestingly, the BMV growth rate estimated as the BMV concentration within individual plant divided by BMV concentration in inoculum decreased at the third generation and no further changes occurred.

The BMV population decline was not likely to have been caused by the accumulation of deleterious mutations from genetic bottleneck passages (Chao, 1990), because the viral population size used in the passages was not small (1/10 diluted ground sap was used in each passage).

One interesting comparison in the experiment was that between mixed and changing passages. Although virus encounters different host genotypes in both passages, BMV showed a trend of higher concentration within individual plant in changing than mixed passage lines. This result might suggest an advantage of mixed culture rather than using different homogeneous

plantings in sequence in order to control diseases in agriculture.

In conclusion, viral population sizes were larger in homogeneous than heterogeneous passages, overall, which supports the Red Queen hypothesis. However, the predicted increase in viral population size with subsequent passages did not occur. If increases in viral fitness lead to increased population sizes, then the decrease in population size with passages is problematical. The results here indicate that pathogen evolution is complex, and influenced by many factors, not all of which have been elucidated. The Red Queen may represent an oversimplistic view of pathogen-host evolution.

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(Received May 6, 2002, Accepted July 2, 2002)