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Isolation and Biological Characterization of *Barley mild mosaic virus* (BaMMV) Mild and Severe Strains in Korea

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Two distinct and stable isolates of *Barley mild mosaic virus* (BaMMV) designated as Naju82-S (severe) and Naju82-M (mild) were obtained. These two isolates differed in their symptomatology, virus transmission characteristics and cultivar specificity at various temperature. Thus, these isolates were referred to as strains in this study. BaMMV Naju-S strain showed severe mosaic symptoms accompanied by necrosis on the infected leaves. Naju82-S strain is more virulent demonstrated by shorter incubation period and relatively high virus concentration than Naju82-M strain. Five Korean cultivars were tested for their pathogenicity to different strains based on the rate of infection. Results showed that infection rate of cultivars to both strains did not significantly differed from each other. However, under different temperatures, the pathogenicity on the two cultivars such as cultivars Hopumbori and Sessalbori were significantly affected. Hopumbori was moderately resistant to both strains at 10-12°C and susceptible at 15-18°C. Similarly, Sessalbori was moderately resistant at 10-12°C to both strains but distinctly differentiated at 15-18°C wherein it was resistant to mild strain and highly susceptible to severe strain. Other cultivars including Baegdong, Jinyangbori and Neahanssalbori consistently showed susceptible reaction to both strains at varying temperatures tested in this study.

Keywords **:** *Bymovirus*, *Polymyxa graminis*, soilborne viruses, pathogenicity

Barley mild mosaic virus (BaMMV) is one of the important virus diseases in barley (*Hordeum vulgare* L.) causing significant yield reduction and commonly found in East Asia including China (Chen et al., 1992; Zheng et al., 1999), Japan (Kashiwasaki et al., 1990, Nomura, 1996) and South Korea (Lee et al., 1996), as well as across Europe (Huth et al., 1991). The virus belongs to *Bymovirus* genus, transmitted by the root-infecting fungus *Polymyxa graminis* Led. So far there is no known means to control the disease except for using resistant barley cultivars.

Several strains of BaMMV were reported in Japan (Nomura et al., 1996), in Germany (Huth and Adams, 1990) and in France (Hariri, 1999,). In South Korea, it has been common observation that cultivar reaction to BaMMV varied in different barley fields. This phenomenon is a possible indication that several BaMMV strains exist. Recently, two BaMMV strains in Korea were reported based on RNA1 structure analysis (NICS, RDA Project Terminal Report, 2005). This finding further indicates that BaMMV strains exist in Korea, however these strains were not isolated and its biological properties were not characterized. One of the major problems in any virus resistance breeding program is the occurrence of virulent virus strains to resistant plant. Thus, lack of knowledge on these strains often resulted in ambiguous resistance evaluation. In addition, cultivar specificity to different virus strains was also affected by temperature. Previous studies on mechanism of resistance to BaMMV showed that cultivar with known resistance genes were affected by temperature. Some genes identified to have temperature sensitive elements that inhibits virus translocation and multiplication (McGrann and Adams, 2004). Moreover, studies on climatic factors on the disease incidence of *Barly Yellow Mosaic Virus* (BaYMV) indicated that disease incidence is high at higher temperature (Park et al., 2003).

During our propagation of BaMMV infected plants in the green house by mechanical inoculation using inoculum collected from the field, various symptoms were appeared in susceptible cultivar, Baegdong seedlings. Infected plants with distinct mild and severe symptoms were selected and used as a virus source in succeeding inoculation. Apparently, all infected plants inoculated with mild isolates showed all mild symptoms and plants inoculated with severe isolates also showed all severe symptoms.

Here, we report the isolation and biological characterization of these two distinct and stable BaMMV isolates based on symptomatology, transmission characteristics, and its cultivar specificity at different temperature.

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Materials and Methods

Collection and isolation of BaMMV strains. Field-infected barley plants with BaMMV symptoms were collected in Naju province, South Korea and tested by ELISA against BaMMV, *barley yellow mosaic* (BaYMV) and soil borne *wheat mosaic virus*. BaMMV infected plants were selected and served as disease source for serial mechanical inoculation to obtain distinct and stable isolates. Virus was propagated in susceptible cultivar, Baegdong seedlings. Inoculum preparation and mechanical inoculation were done as described previously (Jonson et al., 2006a; Jonson et al., 2006b).

Virus-host relationships. Incubation period of virus isolates in the host were obtained by determining the time between mechanical inoculation with the virus isolate and the time when the inoculated plants showed symptoms. Percent rate of infection were obtained by calculating the proportion of infected plants based on the results of PCR or ELISA tests over the total number of inoculated plants. Visual scoring and leaf sampling were done one month after inoculation. Sampled leaves were tested by ELISA or RT-PCR following the same procedure as described previously (Jonson et al., 2006a; Jonson et al., 2006b).

Test plants and pathogenicity at various temperature. Susceptible Korean barley cultivar Baegdong was used to characterize BaMMV strains based on their biological properties. Four other Korean barley cultivars such as Hopumbori, Jinyangbori, Naehanssalbori and Sessalbori were tested for their reaction to BaMMV isolates. Pathogenicity of cultivars to the two isolates was also tested at 10-12°C and 15-18°C in the growth chamber and in greenhouse, respectively. Ten seedlings per variety were tested in all experiments and experiments were repeated twice. Virus concentrations were obtained based on ELISA at 45 DAI. The average absorbance values of all infected plants per cultivar were calculated and compared among cultivars. Data were subjected to analysis of variance (ANOVA) using GraphPad Software, San Diego California USA (www.graphpod.com). Bar graph was performed showing its corresponding error bars. Cultivar resistance was based on rate of infection: 0-30% = resistant; 31-50% = moderately resistant; and >51% = susceptible.

Results

Isolation of mild and severe isolates. Two BaMMV isolates designated as Naju82 and Naju98 collected in Naju province, South Korea were used as disease sources for mechanical inoculation to susceptible barley cultivar, Baegdong. In the first passage, all of the inoculated plants with Naju82 and Naju98 showed severe and mild symptoms, respectively. In the second passage, Naju98 showed similar mild symptoms, however, 2 kinds of symptoms were differentiated from Naju82 inoculated plants (Fig. 1). Twenty-nine percent of those inoculated plants showed severe symptoms and 71% showed mild symptoms. When sap from infected plants with mild and severe symptoms were extracted separately and was used for mechanical inoculation, the infected plants obtained showed symptoms similar to original sources. However, infection rate from succeeding passages differed from each other. Infection rate from Naju82-severe isolate was 100% while Naju82-mild isolate ranges from 84.0-97.0%. On the other hand, Naju98 isolate showed consistent mild symptoms up to the fourth passages on Baegdong plants with infection rate ranging from 45-70%, relatively lower than Naju82-mild isolate.

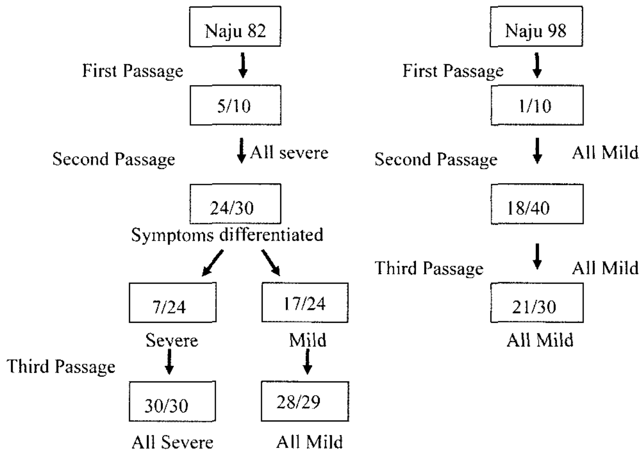

Fig. 1. Schematic diagram showing the isolation procedure in obtaining distinct and stable isolates by mechanical inoculation with 2-3 months interval after each passage on Baegdong plants, maintained at 10-12°C. Fraction number indicates number of infected plants over total number of inoculated plants. After second passage in Naju82 isolate symptoms exhibited by infected plants differentiated. From 24 infected plants, 7 showed severe symptoms and 17 showed mild symptoms and these were subsequently used as inoculum in succeeding passages.

Comparison of biological properties between isolates. The Naju82-mild and severe isolates (Fig. 2) were further characterized in this study based on their biological properties. The two isolates could be well differentiated by their symptoms such as in their plant height, tiller number and leaf infection (Fig. 2 and 3). Plants infected with severe isolate showed pronounced stunting and about 50% height reduction and tillers were reduced compared to plants infected with mild isolate (Fig. 2). Plant height and tiller number of mild isolate did not significantly vary from healthy-uninoculated plant (Fig. 2). The infected leaves of

Fig. 2. Symptoms on infected Baegdong plants with severe and mild strains inoculated at 3-4 leaf stage and tested by RT-PCR at 30 DAI. Healthy represents an uninoculated plant.

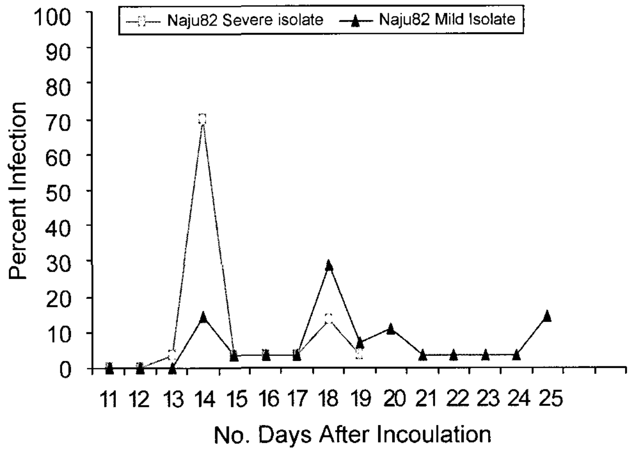

Fig. 4. Incubation periods of BaMMV severe and mild strains in susceptible cultivar, Baegdong after 1 day from inoculation. A total of 30 plants were inoculated and grown at 10-12°C.

severe isolate showed severe mosaic with necrosis (Fig. 3A) and mild infected leaves showed irregular chlorotic streaks (Fig. 3B). However, appearance of yellow flecks symptom in infected cultivar Naehanssalbori was commonly observed in mild and severe isolates (Fig. 3C). The yellow flecks later coalesced making the entire leaf wilted. In terms of virus transmission, both strains were highly pathogenic however the virus incubation period greatly varied from each other. Incubation periods of severe isolate ranges from 13-19 days and 14-25 days in mild strain (Fig. 4). About 70% of inoculated plants with severe isolate showed symptoms at 14 days after inoculation (DAI) and maximum infection attained at 19 DAI. On the other hand, mild isolate at 14 DAI showed only 10% infection and maximum infection attained at 25 DAI. This indicates that severe isolate is more virulent than mild isolate. The Naju98 isolate showed symptoms similar to Naju82 mild isolate and incubation period of the virus in the plant (data not shown).

Cultivar response at different temperature. The response of five Korean cultivars including Baegdong Hopumbori, Jinyangbori, Naehanssalbori and Sessalbori to the two isolates based on percent rate of infection did not significantly differ from each other, however, significant differences were obtained at different temperature (Table 1). Results showed that Hopumbori and Sessalbori were temperature-sensitive since it showed irregular reactions to both isolates at various temperatures. Sessalbori was moderately resistant in both isolates at 10-12°C but response to the two isolates significantly differed at 15-18°C suggesting that it could be used as differential host to BaMMV isolates at 15-18°C. Baegdong, Naehanssalbori and Jinyangbori showed consistent susceptible reactions to both strains at varying temperature indicating that these cultivars' reactions to strains were not affected by temperature. The virus concentrations between mild and severe isolates maintained at 15-18°C showed that plants infected with severe isolate generally had higher absorbance values than

Fig. 3. Typical symptoms in cultivar Baegdong infected with (A) severe strain; (B) mild strain; and (C) leaf symptoms in cultivar Naehanssalbori infected with the mild and severe strains.

Table 1. Percent infection of cultivars to mild and severe BaMMV strains by mechanical inoculation under various temperatures and tested by ELISA[a]

Cultivar	10-12°C		15-18°C	
	Mild	Severe	Mild	Severe
Baegdong	70 S[b]	100 S	60 S	60 S
Hopumbori	50 MR	40 MR	60 S	80 S
Jinyangbori	90 S	90 S	80 S	70 S
Naehanssalbori	100 S	90 S	90 S	100 S
Sessalbori	40 MR	50 MR	30 R	90 S

[a]Inoculated at 3-4 leaf stage and maintained at growth chamber (10-12°C) and temperature controlled greenhouse (15-18°C). [b]Resistance rating: 1-30% = R, resistant; 31-50% = MR, moderately resistant; and 51-100% = S, susceptible.

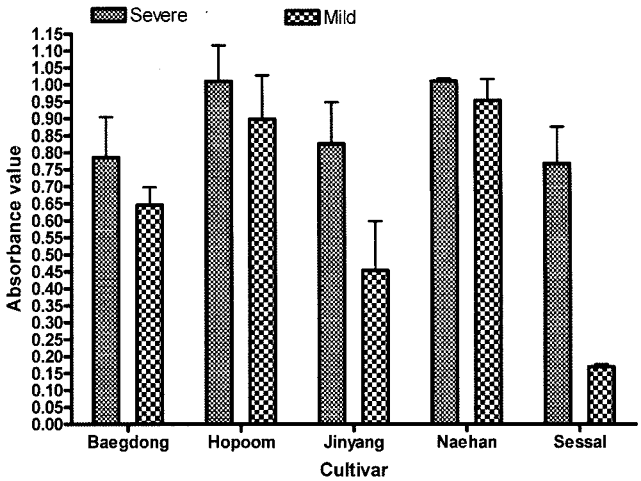

Fig. 5. Absorbance (405 nm) values of the five Korean barley cultivars infected with mild and severe strains of BaMMV at 45 DAI grown at 15-18°C.

those of plants infected with mild isolate (Fig. 5).

Discussion

In this study, we have demonstrated the occurrence of BaMMV mixture of strains collected from the field. Many studies have shown that a mixture of virus strains usually occurs in nature and causing the diverse symptoms in the field (Rocha-Pena et al., 1995). Symptom variations in mixture of strains may depend on the proportion of each strain present in the infected plant (Matthews, 1981). Like in this study, the mild isolate was not immediately notice from the initial passage probably the proportion of severe isolate was higher than those of mild isolate. In addition the number of plants inoculated were small (only 10 seedlings) and all 5 infected plants have severe mosaic symptoms. However, in the second passage, we have inoculated 30 seedlings and this time infected plants with mild symptoms were being manifested (Table 1). Strains differentiation is also influenced by the interaction of environment and host response. The symptoms may be mild under certain conditions and quite different or much more severe in another environmental conditions especially the temperature (Park et al., 2003; Romancer and Nedellec, 1997; University of Illinois Extension, 1991). Results of our study further proved that pathogenicity of both strains on some cultivars was variably affected by temperature. For instance in this study, Hopumbori showed moderately resistant to both strains at 10-12°C but susceptible at 15-18°C. This result is similar to findings of Park et al. (2003) that disease incidence increased at higher temperature. And among cultivars tested, Sessalbori showed resistance differentiation to the two strains at different temperature suggesting that Sessalbori could be used as differential host to differentiate mild and severe strains at 15-18°C (Table 1). The varying resistance reactions of Hopumbori and Sessalbori to strains at varying temperature further indicates that these varieties possessed resistant genes to BaMMV. Hopumbori was reported to possessed *rym5* gene to BaYMV but not mentioned on BaMMV (Jonson et al., 2006b) and no report on Sessalbori. However, further study is suggested to fully understand the mode of action of host response to soilborne barley mosaic virus complex.

The two biologically distinct strains were isolated after three passages by sap mechanical inoculation. Both mild and severe BaMMV strains are highly pathogenic. However, severe strain is more virulent characterized by rapid transmission and severe infection with relatively high virus concentration than mild strain. The apparent longer incubation period of the mild isolates than the severe isolate is not well understood. However, this phenomenon is inherent and normal for mild infections of plant viruses in that their symptoms could not be easily and readily noticed (Gibbs and Harrison, 1976). For instance, those mild isolates were already in the plant but were not probably of high enough virus concentration to elicit much more pronounced response (i.e. symptoms) that was readily noticeable. The virus concentration in severe isolates was higher than mild isolate (Fig. 4). This result proved further the work of Skaria et al. (1985) that is, symptom severity of *barley yellow dwarf virus* in cereals was correlated to virus concentrations in a plant.

In Naju barley field, two mild isolates were present; Naju98 and the mild isolate obtained from Naju82 mixtures (Fig. 1). Although, both were similar in symptomatology and transmission, we still can not conclude that Naju98-mild and the Naju82-mild isolates were similar isolates, unless their nucleotide sequence were made available and compared. In this study, however, the significant differences in the biology such as symptomatology, virus transmission, virus concentration, and cultivar pathogenicity reaction between mild and severe isolates obtained from Naju82 mixtures indicated that these isolates can be regarded as strains in this study. Although, further studies that would deal on the structural criteria using more sensitive and discriminating procedures and serology should be undertaken. Consequently, the recognition of the occurrence of strain mixtures in nature and its isolation and characterization are important in providing information in epidemiology, formulating effective control strategies and developing screening methodology for resistance.

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