Genomic Variation and Toxin Specificity of *Ustilago maydis* Virus Isolated in Korea

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Novel *Ustilago maydis* strains, designated as SH1 to 14 containing new types of dsRNA segments, are identified from corn smut in Korea. Among 14 isolates, 7 isolates appear to possess virus particles and the other isolates may contain dsRNA as a plasmid form. The pattern of dsRNA is highly diverse from a typical P-type containing one or more of H, M, and L dsRNAs to the one containing one or more M dsRNAs. It is likely that the strains containing H dsRNA possess virus particles which were confirmed by sucrose density gradient followed by dsRNA extraction. In the analysis of toxin specificity, 5 strains appear to have toxin activity with different range of specificity and the activity of one strain (SH14) is stronger than A4 toxin. The sensitivity of 14 isolates is also very diverse and two strains (SH10, SH11) appear to be universal sensitive strains against 5 tested toxin samples.

KEY WORDS □ ***Ustilago maydis*, dsRNA, killer system, toxin protein**

Virus particles or viral double-stranded RNAs (dsRNAs) were reported in almost every fungal species including basidiomycetous smut fungi (3, 4, 10, 12, 18). In rare cases, the presence of virus is closely related with a typical phenotypic changes in host, i.e. abnormal growth (10), hypovirulence (15) and early senescence. In most of the cases, however, the presence of virus has no effect on the growth or development of the fungi. Therefore, it is often called latent infection (3, 4). One of the most dramatic exception is the killer phenomenon of *Saccharomyces cerevisiae* and *Ustilago maydis* viruses in which killer strains containing virus particles inhibit the growth of virus-free sensitive strains (13, 22). Yeast killer system has been studied extensively and its toxin gene was cloned and complete nucleotide sequence has been reported (22). In spite of similarity of its system, however, the studies on *Ustilago maydis* killer system is at its early stage because the studies on *U. maydis* system started recently and the system is rather complicated compared to yeast killer system. The screening of strains containing virus or dsRNAs and toxin specificity of field isolates are under progress.

The interstrain growth inhibition in *U. maydis*, known as killer phenomenon, was first reported by Puhalla during genetic analysis between two different strains (19). The growth inhibition of sensitive strains by the killer strains results from the secretion of killer toxin protein. Double-stranded RNA viruses were also identified in killer strains (23) suggesting that the presence of viral particles was closely associated with the killer activity. Later the toxin gene was identified by the mutagenesis and *in vitro* translation of denatured viral genes in some of the killer strains (14, 17, 24).

Based on the distribution of dsRNAs and toxin specificity, three killer strains, designated P1, P4 and P6, and one universal sensitive strain, designated P2, were identified as laboratory strains (2, 8, 11). These killer strains are immune to its own toxin and have mutual killing activity by the secretion of toxin protein coded by viral gene. So far, most of studies on *Ustilago maydis* virus (UmV) have been carried out by using these laboratory strains and some mutant strains (1). The mechanism of killer toxin is not clearly known but some speculations have been presented in yeast system (21, 22). It is likely that toxin protein binds to receptor molecule of sensitive cell which results in disruption of membrane permeability (16).

The distribution of viral dsRNA in three killer strains is distinct. All three types of killer strains contain at least one representative of each of three size classes: heavy (H), 3.6~6.2 Kb; medium (M), 0.9~1.7 Kb; and light (L), 0.3 Kb. The amount and motility of each dsRNA are slightly different compared with those of stock culture (1, 8). The coding assignment of UmV dsRNA was partially identified either by deletion mutation of a specific segment or by *in vitro* translation of denatured

dsRNA (5, 17, 24). Segments of the H class code for capsid protein, whereas segments of the M class are thought to code for the toxin protein. These results came from genetic experiments in which loss of the M segments was correlated with the loss of ability to produce active toxin. The function of the rest of dsRNA segments was not assigned yet but some evidences have been presented that they are non-functional dsRNAs, known as satellite dsRNA (3, 4). The function of L dsRNA is still unknown but it is likely that L dsRNA is a non-functional deletion product of M dsRNA segment (8).

The studies on the distribution of dsRNAs and toxin specificity of field isolates of *U. maydis* were not carried out extensively. It is likely that viral dsRNA is widely distributed in all *U. maydis* isolates in United States (6) and in Korea (25). The fungal viruses might be detected more efficiently by analyzing directly for dsRNA rather than looking for virus particles. It is also clear that dsRNA is extremely common in other species of smut fungi or in rust fungi (6, 7, 18). But killer strains other than three laboratory strains were not identified among natural isolates. The presence of dsRNA and the specificity of toxin protein among field isolates in Korea were reported previously (25). A typical dsRNA pattern different from laboratory strains designated A-type strains was reported. Among 14 dsRNA-containing strains, 2 strains appeared to have killer activity against 5 sensitive strains. Evidence was presented that resistance against toxin protein is not associated with the presence of a specific dsRNA. It is possible that resistant phenotype is coded by host gene not by viral gene as speculated previously. In this report, we report new *U. maydis* isolates which contain novel types of dsRNA segments and toxin specificity isolated from corn smut in Korea. Attempts were also given to purify virus particles from isolates containing specific UmV dsRNA segments.

MATERIALS AND METHODS

Ustilago maydis strains

Strains were isolated from corn smuts collected in Chuncheon area. Teliospores were inoculated on *U. maydis* complete medium (20) and pure cultures were obtained through several subcultures of a single colony on the same medium. The isolates were maintained in slant culture or in glycerol stock for further use.

Double-stranded RNA purification

Each isolates were inoculated in *U. maydis* complete broth and incubated for 3 days at 25°C with vigorous shaking. Cells were harvested and resuspended in lysis buffer (2% Triton X-100, 1% SDS, 0.1 M NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0) and dsRNA was extracted twice with mini-bead beater (Biospec Inc.) for 3 min in the presence of the same volume of phenol/chloroform. Total nucleic acid was precipitated with cold ethanol and resuspended with TE buffer. dsRNA was further purified through CF-11 column chromatography (18, 25) and analyzed either on 1.0% agarose gel or 5% polyacrylamide gel.

Virus purification

U. maydis strain was inoculated in 200 ml of *U. maydis* complete medium followed by incubation at 25°C for 2~3 days. The entire preculture was used to inoculate a 3.5 liter fermentor (Korea Fermentation Inc.) containing same medium. The culture was incubated for 2~3 days. The cells were harvested and washed with one-fourth volume of 0.05 M phosphate buffer (pH 7.0) followed by resuspending with 200 ml of same buffer. The cells were broken by grinding in a bead-beater for 15 min in ice bath. The cell debris was removed by centrifugation at 15,000 rpm for 15 min. The virus was either directly collected by ultracentrifugation in the presence of 25% sucrose cushion or precipitated by 6% polyethylene glycol in the presence of 0.15 M NaCl. Virus was resuspended with buffer and subjected to 10~50% sucrose gradient. The gradient centrifugation was carried out at 24,000 rpm for 4 hours by using Beckman SW 28 rotor (Beckman Inc.). The gradient was fractionated with gradient fractionator (ISCO Inc.) in the presence of UV monitor. The virus peak was collected and diluted with same volume of buffer followed by centrifugation at 40,000 rpm for 2 hours.

Toxin protein preparation

Culture supernatant was mixed with 1.6 volume of cold acetone and precipitant was collected by centrifugation at 10,000 rpm for 5 min. The protein pellet was resuspended with ice-cold 0.1 M phosphate buffer (pH 7.0). A4 toxin protein used as a positive control was further purified through CM-sephadex column chromatography (9).

Filter disk assay

Soft agar lawn of test organism was prepared with 2 day-old culture of isolates and a filter disk soaked with equal volume of toxin sample was placed. The plate was incubated for 36 hours and the appearance of growth inhibition zone was recorded as a sensitive strain against tested toxin.

RESULTS AND DISCUSSION

Ustilago maydis strains were isolated by serial colony isolation from corn smut collected in Chuncheon area. Isolated strains were maintained in *U. maydis* complete medium. In order to detect a specific viral dsRNA, total nucleic acid from *U. maydis* was extracted with phenol/chloroform followed by CF-11 column chromatography. The

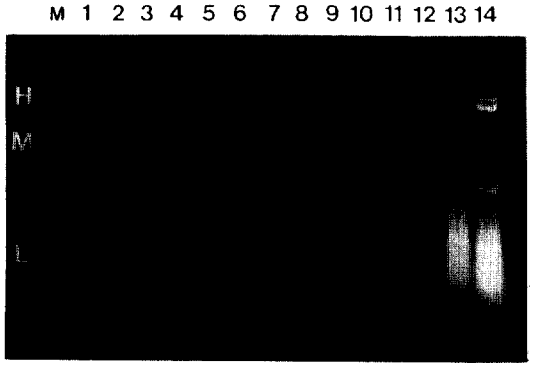

Fig. 1. *Double-stranded RNA profile of 14 isolates of U. maydis (SH1-SH14) isolated in Korea.*
Total nucleic acid was extracted with phenol/chloroform and dsRNA was purified by CF-11 column chromatography. dsRNA was analyzed in 1.0% agarose gel with DNA-ladder as a marker (M).
H, heavy dsRNA; M, medium dsRNA; L, light dsRNA.

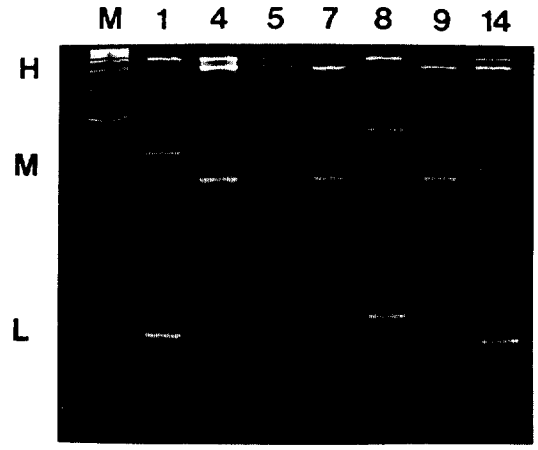

Fig. 2. *Double-stranded RNA profile of virus particles purified from strains of U. maydis.*
Among 14 strains containing specific dsRNAs, virus particles were purified from 7 strains and dsRNA was extracted with SDS-phenol. dsRNA was analyzed in 5% polyacrylamide gel with DNA-ladder as a marker (M).

dsRNA was analyzed in an agarose gel electrophoresis in the presence of marker DNA. The strains containing specific dsRNA and different from previously reported A-type strains were designated as SH1 to SH14 (Fig. 1). The isolates appeared to contain various number of H, M and L dsRNAs, and some variation was also apparent in the intermediate dsRNAs. The cultural properties of these isolates were similar to a typical *U. maydis* strain and to a virus-free strain as described previously (25). Among 14 strains containing a typical dsRNAs, 9 strains contained one or more H and M dsRNA, and the other 5 strains contained one or more M dsRNAs, none of the isolates contained only L dsRNA. This may indicate that L dsRNA is dependent on H and M dsRNA for its maintenance, which is also consistant with the report that L dsRNA is a deletion product of M dsRNA in the case of P4 (8). The number of dsRNAs and the amount of each dsRNA were different from previously reported A-type isolates (25). Since H dsRNA is known to code for capsid protein, it is likely that the strains containing H dsRNA may have typical virus particles. The purification of virus particles was carried out by using sucrose density gradient. A typical virus peak was obtained from gradient fractionation in strains 1, 4, 5, 7, 8, 9, and 14. In these strains, virus preparations appear to have a typical UV-scanning profiles. No virus peak was recorded in strains 2, 3, 6, 10, 11, 12 and 13 in which no virus profiles were shown in the UV range (data not shown). It is likely that these strains may possess dsRNA only as a satellite dsRNA which is also described in A-type series.

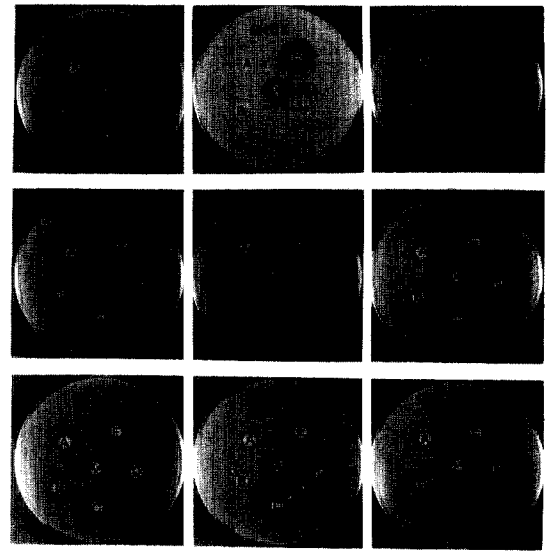

Fig. 3. *Toxin activity test of 5 killer strains against 9 sensitive strains.*
Filter disks soaked with an equal volume of toxin preparation were placed on the lawn of two day-old test strains. The plates were incubated for 36 hours with A4 toxin purified by CM-Sephdex colume chromatography as a positive control.
A, SH1 toxin; B, SH4 toxin; C, SH5 toxin; D, SH7 toxin; E, SH14 toxin; L, lawn of tested strains.

Table 1. *Summary of toxin activity test of 5 killer strains (SH 1, 4, 5, 7, 14) and A4 of Ustilago maydis against other isolates containing a specific dsRNA.*

Killer strains \ Isolates	SH1	SH2	SH3	SH4	SH5	SH6	SH7	SH8	SH9	SH10	SH11	SH12	SH13	SH14	A4
SH1	*	S	R	R	R	R	S	S	S	S	S	S	R	R	S
SH4	R	R	R	*	R	R	R	R	R	S	S	R	R	R	R
SH5	R	S	R	R	*	R	S	S	S	S	S	S	S	R	S
SH7	R	R	R	R	R	R	*	R	R	S	S	R	R	R	R
SH14	R	R	R	R	R	S	S	S	S	S	S	S	S	*	R
A4	R	R	R	R	R	S	S	S	S	S	S	S	S	S	*

Unencapsidated dsRNAs were also reported from rust fungi including *Puccinia* and *Melampsora* species (3, 7, 18). The dsRNAs from virus peak were extracted with SDS-phenol and analysed in 5% polyacrylamide gel electrophoresis (Fig. 2). The pattern of dsRNA segments was similar to that of the total nucleic acid analysis which indicates that all the dsRNAs are encapsidated in a capsid protein. In the case of SH8, however, some of M dsRNA was not appeared in virus fraction suggesting that these dsRNAs were not encapsidated. Partial encapsidation of dsRNA segments was also reported in other fungal species (7, 18) and A-type isolates (25). The results may indicate that encapsidation is not obligatory for the maintenance of dsRNA segments in fungi. The relationship between encapsidated dsRNA and satellite dsRNA is not completely known. To investigate dsRNA segments and their encapsidation, immunological approach and electron microscopic observation are under process in this laboratory.

In order to analyze toxin specificity among isolates, filter disk test was carried out (Fig. 3). Crude toxin preparations from 14 culture supernatants were tested against all 14 test lawns. Toxin samples from A4 strain which is a mutant strain of laboratory type P4 was used as a positive control. A distinctive growth inhibition zone from toxin preparation of 5 strains (SH1, 4, 5, 7, and 14) was identified with different range of killer activity. The other 9 strains were sensitive strains with different range of sensitivity. The results were summarized in Table 1. Four strains (SH1, 3, 4 and 5) were resistant against all tested toxins including A4 and two strains (SH 10 and 11) were universally sensitive against 5 toxin samples. The other four strains (SH 7, 8, 9 and 12) showed same range of sensitivity. The pattern of dsRNA genome appears to be not associated with killer activity or sensitivity ranges which are also reported in A-type strains (25). The evidences also indicate that all killer strains possess virus particles encapsidated with all three classes of dsRNA segments. Present study has not shown any evidence of coding assignment of toxin protein. The examination of cross reactivity of toxin protein between SH-series and A-series are under process in this laboratory. Since SH14 produces relatively large amount of toxin protein, biochemical characterization of this protein is making some progress in this laboratory.

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초 록: 한국에서 분리된 *Ustilago maydis* 바이러스의 유전자의 변이와 독소의 특이성

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한국에서 채집한 옥수수 깜부기에서 분리된 *Ustilago maydis* 균주들 중 이미 보고된 A-type 균주와 다른 특이한 dsRNA를 갖는 새로운 14균주를 분리하여 SH1에서 SH14으로 명명하고 이 균주의 유전자 다양성과 독소단백질의 특이성을 연구하였다. 분리균들 중 일부만 한개 이상의 M dsRNA만을 가지고 있고 나머지는 모두 전형적인 P-type (H, M and L dsRNA)의 유전자를 가지고 있는 것으로 확인되었다. 이들 중 7균주는 바이러스 입자를 갖는 것으로 확인되었으나 나머지는 dsRNA-plasmid 상태로 존재하는 것으로 여겨진다. 독소 단백질의 특이성을 조사한 실험에서는 4개의 균주(SH 1, 4, 5, 7, 14)가 각기 다른 특이성을 갖는 독소의 활성을 보였으며 특히 SH14 균주는 A4보다 강한 활성을 보였다. 각 균주들의 감수성은 2균주는 실험한 모든 독소에 대하여 성장저해 현상을 보였으며 나머지는 각기 다른 양상의 감수성을 보였다.