KOR. JOUR. MICROBIOL June, 1992 p. 177 ~ 180 Vol. 30, No. 3

Molecular Biological Characteristics of *Ustilago maydis* Virus Isolated in Korea.

Yie, Se Won and Hyoung Tae Choi

Deartment of Microbiology, College of Natural Sciences, Kang Won National University
Hyo Ja Dong, Chun Cheon Si, Kang Won Do, Korea

Among 120 *U. maydis strains* isolated in Korea 14 different strains containing specific viral dsRNA segments were analyzed for the distribution of dsRNA and the production of toxin protein. Several distinctive dsRNA patterns were identified, 9 cases of P type with typical H, M and L dsRNA and one case of non-P type, the frequency of a specific isolate was decreased with increasing number of dsRNA segments. The presence of dsRNA had no effect on the cultural or morphological phenotype of the host. Two isolates containing P type dsRNA segments appeared to produce toxin protein (killer strains) which inhibited the growth of 4 isolates (sensitive strain) with different susceptibility. Two killer strains contain unique M dsRNA segment which may code for toxin protein. However, the presence of a specific dsRNA may not related to the sensitivity of toxin protein, since the frequency of toxin-sensitive strains among dsRNA-free isolates was similar to that of dsRNA containing strains.

KEY WORDS □ ***Ustilago maydis*, Virus, dsRNA, toxin**

Viruses and viral double-stranded RNA have been described from nearly every genera of fungi including rust and smut fungi (3, 11, 2). The fungal viruses reported to date contain segmented double-stranded RNA(dsRNA) as a genome. The number of dsRNA is also widely distributed from single segment to 16 segments. Most fungal viruses, however, give rise to latent infection which cause no effect on the phenotype of the host, even if viruses replicate actively. The obvious exception is the production of killer toxin in *Saccharomyces cerevisiae* virus and *Ustilago maydis* virus (UmV).

Growth inhibition between corn smut fungi, *Ustilago maydis*, was first reported by Puhalla (14). This interstrain growth inhibition, known as killer phenomenon, was proposed to be related to a cytoplasmically inherited element. Later it has been concluded that this penomenon results from the secretion of protein substance by some strains of *U. maydis* (9). Double-stranded RNA virus or virus-like particles were identified in killer strains (16). Based on the size, three groups of the dsRNA segments are reported: heavy (H), medium (M) and light (L). These dsRNAs are separately encapsidated in 40 nm capsid protein and each killer strain contains one or more representatives of three groups of dsRNA segement (1).

There are three laboratory types of killer strains, designated P1, P4 and P6, and one universal sensitive strain, P2, which have been studied most extensively. The H dsRNA segments are encapsidated singly whereas the smaller M and L segments are encapsidated in combination of multiple segments so that the total mass of dsRNA in a virion never exceeds that of a virion containing an H segment (1).

According to the genetic analysis and in vitro translation experiments, it was suggested that toxin protein is coded by one of the M dsRNA segemnt (10) and capsid protein by one of the H dsRNA segement (4). The function of the other dsRNA segments of H and M is not known but it may codes for the same protein or they are non-functional satellite dsRNAs as proposed by Buck(3). The L segment was proposed to be involved in toxin expression without encoding its own protein (8) which is still controversial (6).

The study on the distribution of virus particles or dsRNA segments from field isolates was carried out in the United States (5). More than 80 % of isolates contained a specific dsRNAs with a great diversity which was not related any phenotypical modification of the host.

Except the original three killer strains reported earlier, no other killer strains were identified. Therefore most of the research on *U. maydis* killer phenomenon has been focused on those three strains and some mutant strains. In order to understand the diversity of dsRNA segments and

to explain the mechanism of growth inhibition by toxin protein, more killer strains with different range of killing activity and sensitive strains have to be identified.

In this paper *U. maydis* strains isolated from field were tested for the distribution of dsRNAs and the production of toxin protein. We report that the identification of 14 distinctive strains with different dsRNA band pattern and two killer strains.

MATERIALS AND METHODS

Ustilago maydis strains: Strains were isolated from corn smuts collected in Chun Cheon area. Teleospores were inoculated on *U. maydis* complete medium (15) and pure cultures were obtained by several subculture of single colony on the same medium. Among 120 isolates only strains which have a distinctive dsRNA were designated as A1 to A11 and S1 to S4. There was no cultural or morphological differences among isolates.

Double-stranded RNA purification: Each isolates were inoculated in *U. maydis* complete broth and incubated for 3 days at 30 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., USA) 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 either through CF-11 column chromatography (13) or simply by RNase treatment (0.1 μg/m*l*) at 25 C for 10 min. in the presence of 2xSSC(1XSSC: 0.15 M NaCl, 0.05 M sodium citrate, pH 7.2). DsRNA was analyzed on 1.3% agarose gel.

Toxin protein preperation: Culture supernatant was mixed with 1.6 volume of cold acetone and precipitant was collected with centrifugation. The protein pellet was resuspended with ice cold 0.1 M phosphate buffer (pH 7.0). Toxin protein was further purified through CM-sephadex column chromatography (7).

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

RESULT AND DISCUSSION

Among 120 *Ustilago maydis* strains isolated from corn smut, about 50% of isolates were found to contain more than one specific dsRNA. Based on the dsRNA band pattern and the area of sample collected 14 typical strains were designated as A1

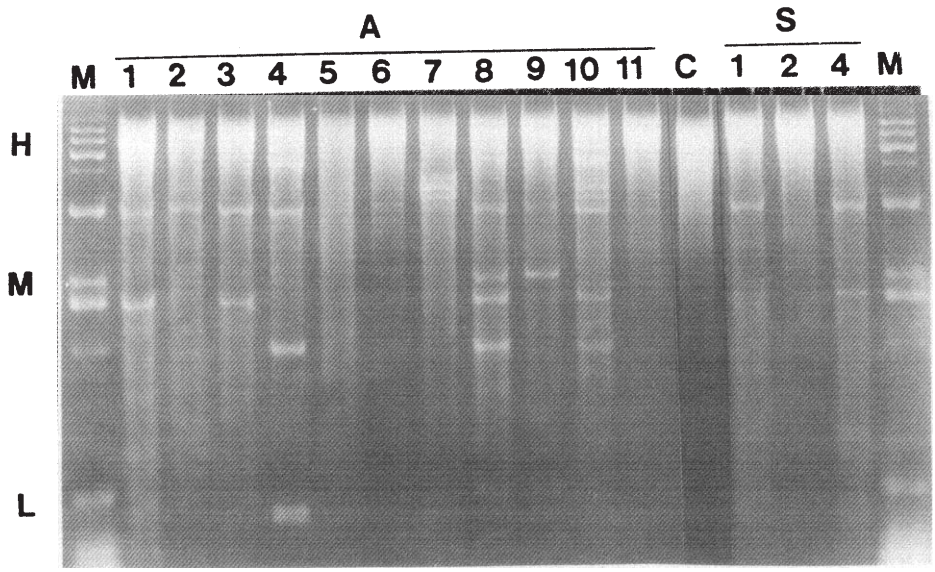

Fig. 1. *Double-stranded RNA pattern of 14 U. maydis strains isolated from corn smuts. Total nucleic acid was directly extracted and treated with RNase (0.1 vg/ml) at 25°C for 10 min. DsRNA was analyzed in 1.3% agarose gel. Different strains were designated as A1 to A11, dsRNA-free strain (C) and S1, S2 and S4. Moleclar weight marker was A8 dsRNA purified through CF-11 chromatography. H: Heavy dsRNA, M: Medium dsRNA and L: Light dsRNA.*

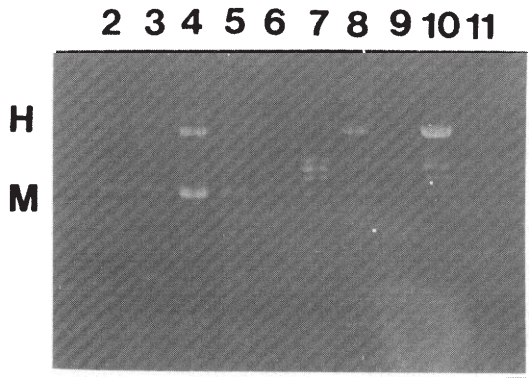

Fig. 2. *Double-stranded RNA from some of 14 isolates (A2-A11) was further purified through CF-11 chromatography. DsRNA was analyzed in 1.3% agarose gel and electrophoresis was carried out extensively to increase the resolution of H and M dsRNA segments.*

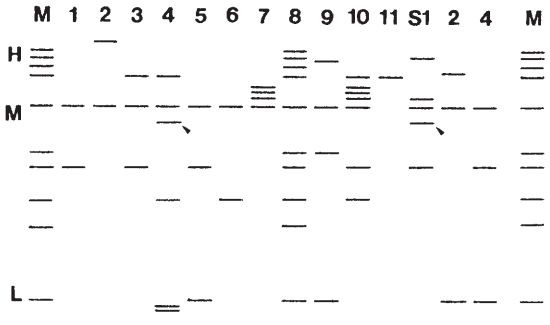

Fig. 3. *Schematic representation of 14 strains of U. maydis isolated in Korea. A typical P type with H, M, and L dsRNA and non-P tyep(A7) was presented as a results of different types of gel analysis. Two toxin producing strains(A4 and S1) contain a unique M dsRNA(arrow) which may code for toxin protein.*

to A11 and S1, S2 and S4(Fig. 1). Among dsRNA containing strains about 30% of isolates contained one M dsRNA which is not used in this study, or one M dsRNA plus one H dsRNA(A2, S2 type). Overall the more diverse the pattern of dsRNA was the less number of isolates was recorded. Among typical P type isolates, A4, A8, A9, A10 and one non-P type(A7) was isolated only once. The presence of dsRNA has no effect on the cultural or morphological characteristics of *U. maydis* strain. The resolution of band pattern was completely dependent on the preparation and gel analysis. Some of H dsRNA are only detected on 2.5% polyacryamide gels and some of L and M dsRNAs are detected only in 1.5% agarose gel. Since dsRNA is independently encapsidated the

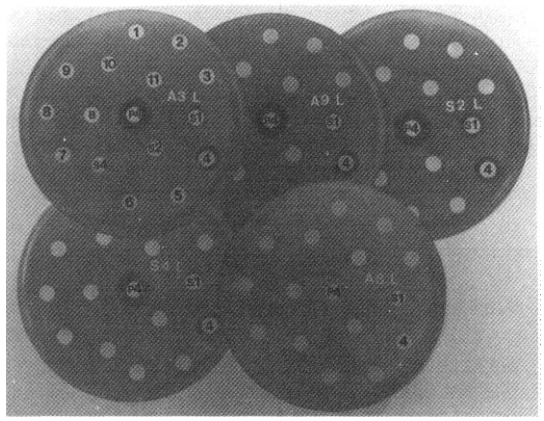

Fig. 4. *Toxin sensitivity test: 4 sensitive strains(A3, A9, S2 and S4) and one of resistant strain (A8). The lawn was prepared with two day old test organism. Filter disks soaked with an equal volume of toxin preparation were placed and the plates were incubated for 2 days. P4: A4 toxin purified through CM-sephadex. B: Filter disk soaked with phosphate buffer.*

amount of each dsRNA shown on gel analysis was variable. In order to determine the exact patterns of dsRNA, different types of gel analysis were carried out with different amount of sample loaded(data not shown). Since dsRNA sample from direct extraction appeared to contain large amount of chromosomal DNA which interfered the resolution of H dsRNA(Fig. 1), the dsRNA was further purified through CF-11 column chromatography (13). This method also resulted in the loss of some of small segments, only H and M dsRNA segments are shown in Fig. 2. As a result of different types of gel analysis, the distribution of dsRNAs are schematically represented on Fig. 3.

All the isolates except one (A11) contained 1 M dsRNA in common which indicate that this segment may have essential role to the maintenance of the rest of dsRNAs. Nine out of 14 isolates contained at least one H dsRNA segment with different size. Since it has been reported that H dsRNA codes for capsid protein (4) the presence of H dsRNA may indicate the presence of virus particle. The attempt to purify virus particles from isolates was partially successful, only from A4, A7 and A10 (data not shown). The distribution of various dsRNA segments in A8 strain may be particulary useful as a possible candidate for a molecualr marker of dsRNA.

Crude toxin preparations from culture supernatant were tested against all 14 isolates. Only two isolates (A4 and S1) have growth inhibitory effect on A3, A9, S2 and S4 strains with different size of inhibition zone(Fig. 4). Among sensitive

strains S4 was least sensitive against A4 and S1 toxin. DsRNA-free strains were also tested against A4 and S1 toxin and the frequency of occurence of sensitive strains was similar to that of dsRNA-containing strains indicating that toxin resistant factor (also called immunity factor) may be coded by chromosomal DNA not by viral genome as proposed by Peery (12).

In order to explain the diversity of dsRNA and toxin activity in detail, these characteristics had to be compared with those of laboratory strains. Unfortunately, however, the laboratory strains are not available and efforts are given to recover those strains (personal communication).

ACKNOWLEDGMENT

This reserch was supported by the grant from the Ministry of Education (Genetic Engineering Research, 1991)

REFERENCES

1. **Bozarth, R.F., Koltin, Y., Weissman, M.B., Parker, R.L., Dalton, R.F. and Steinlauf, R.,** 1981. The molecular weight and packaging of dsRNA in the mycovirus from *Ustilago maydis* killer strain. *Virol.* **113**, 492-502.
2. **Brown, G.G. and Finnegan, P.M.,** 1989. RNA plasmids. *Inter. Rev. Cytol.* **117**, 1-56.
3. **Buck, K.W.,** 1986. Fungal virology. CRC Press Inc. Florida.
4. **Dalton, R.E., Podila, G.K., Flurkey, W.H. and Bozrth, R.F.** 1985. *In vitro* translation of the major capsid polypeptide from *Ustilago maydis. Virus Res.*, **3**, 153-163.
5. **Day, P.R.,** 1981. Fungal virus populations in corn smut from Connecticut. *Mycologia* **73**; 379-391.
6. **Field, L.J., Bruenn, J.A., Chang, T.H., Pinchai, O. and Koltin, Y,** 1983. Two *Ustilago maydis* viral dsRNAs of different size code for the same product. *Nucleic Acids Res.*, **11**, 2765-2778.
7. **Ganesa, C., Flurkey, W.H., Randhawa, Z.I. and Bozarth, R.F.,**1991. *Ustilago maydis* virus P4 killer toxin:Characterization, partial amino terminus sequence, and evidence for glycosylation.
8. **Kandel, J. and Koltin, Y.,** 1978. Killer phenomenon in *Ustilago maydis*: Comparison of the killer proteins. *Exp. Mycol.*, **2**, 270-278.
9. **Koltin, Y. and Day, P.R.** 1976. Inheritance of killer phenotype and double-stranded RNA in *Ustilago maydis. Pro. Nat. Acad. Sci. USA*, **73**, 594-598.
10. **Koltin, Y., Mayer, I. and Steinlauf, R.,** 1978. Killer phenomenon in *Ustilago maydis*: Mapping viral functions. *Mol. Gen. Genet.*, **178**, 173-178.
11. **Koltin, Y.,** 1988. The killer systems of *Ustilago maydis*: secreted polypeptides encoded by viruses. *In Viruses* of fungi and simple eukaryotes. pp. 209-242. Koltin, Y and Leibowitz, M.J. Eds. Marcel Dekker Inc. New York.
12. **Peery, T., Koltin, Y. and Tamarkin, A.,** 1982. Mapping the immunity of the *Ustilago maydis* P1 virus. Plasmids **7**, 52-58.
13. **Pryor, A., Boelen, M.G., Dickinson, M.J. and Lawrence, G.J.,** 1989. Widespread incidence of double-stranded RNAs of unknown function in rust fungi. *Can. J. Bot.*, **68**, 669-676
14. **Puhalla, J.E.,** 1968. Compatibility reactions on solid medium and interstrain inhibition in *Ustilago maydis. Genetics*, **60**, 461-474.
15. **Stevens, R.B.,** 1974. Mycological guidebook. Univ. of Washington Press, Seattle, USA.
16. **Wood, H.A. and Bozarth, R.F.,** 1973. Heterokaryon transfer of virus-like particles associated with a cytoplasmically inherited determinant in *Ustilago maydis. Phytophath.* **63**, 1019-1021.

(Received March 23, 1992)
(Accepted March 29, 1992)

초 록: 한국에서 분리한 *Ustilago maydis* Virus의 분자생물학적인 특성

이세원 · 최형태 (강원대학교 자연대학 미생물학과)

춘천근교에서 분리한 옥수수 깜부기균의 원인균인 *Ustilago maydisa*에서 double-stranded RNA 의 분포 양상과 toxin protein 의 분비 여부를 조사하였다. 약 50 % 의 분리균에서 dsRNA 의 존재가 확인되었으며 분포 양상에따른 14 종의 특이한 viral dsRNA 가 확인되었다. 대부분의 분리균이 M dsRNA 를 공통적으로 가지고 있었으며 9 종은 각기 특이한 H dsRNA 를 가지고 있어 한 종외에는 전형적인 P-type dsRNA 분포 양상을 보였다. 분리균들 중 2 종이 toxin protein 을 분비하여 4 종의 성장을 억제 하였으며 이러한 현상은 dsRNA 를 갖지 않는 분리균에서도 비슷한 양상으로 나타났다. 따라서 자연계에서 분리되는 *U. maydis*에는 다양한 분포의 dsRNA 가 존재하며 toxin protein 에 대한 저항성은 균주에 따라 다른 것으로 여겨진다.