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Characteristics of *Ustilago maydis* Virus of SH14 Killer Strain Isolated in Korea

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SH-14, a novel killer strain of *Ustilago maydis* was isolated in Korea. It has been reported in other papers that the toxin specificity and double-stranded RNA pattern of SH-14 strain were different from other laboratory strains. In this paper, we analyzed the biochemical characteristics of *U. maydis* SH-14 virus. Three distinctive peaks were isolated from CsCl density gradient, designated as top (T), intermediate (I) and bottom (B) components. We found that the densities of each components, 1.285, 1.378 and 1.408 g/cm^3, respectively, are very similar to those of other strains. As previously reported by the analysis of dsRNA in each component, the dsRNA segments are separately encapsidated. Capsid protein of SH-14 virus consists of two proteins about 70 Kd shown by SDS-PAGE analysis. Electron microscopic examination of the virus particles revealed that UmV particles are very similar in size and morphology to all isolates as well as all lab-strains. In order to test immunological cross reactivity of UmV, western blot analysis was carried out with antiserum against A8 virus. All capsid protein had positive reaction against A8 antibody which indicated that UmV are immunologically cross-reactive with all isolates from Korea. The results presented in this paper may show that UmV isolated from SH-14 strain has very similar biochemical characteristics to those of other UmV. However, the difference in the toxin specificity and the molecular weight of toxin protein from the SH-14 strain has us to conclude that *U. maydis* SH-14 strain is a new killer type.

Key words: ***Ustilago maydis*, fungal virus, dsRNA, toxin**

Ustilago maydis virus (UmV) contains multiple double-stranded RNA (dsRNA) segments in its genome which are individually encapsidated into isometric virus particles. Virus particles were detected by Wood and Bozarth (20) in strains used earlier by Puhalla (13). The number of dsRNA segment is highly diverse depending on the strains. Most of the strains either laboratory strains or isolates from corn smut analyzed in Korea contain a typical P-type dsRNA segments as it has been previously described (9, 22). The presence of virus particles has shown to have no effects on the phenotype of the host. Some strains of *U. maydis* harboring dsRNA viruses, however, seem to produce a secretory protein which inhibits the growth of sensitive strains, called the killer phenomenon. These killer strains have been demonstrated to be resistant against their own toxin. The resistance to the killer toxin in natually occurring non-killer strains seems to be determined by nuclear genes because nonkiller strains and even sensitive strains contain virus particles or dsRNA segments. Some sensitive strains contain viral dsRNA devoid complete capsid, maybe in a form of dsRNA plasmid (3, 22). Similar system has also been reported for *Saccharomyces cerevisiae* which has been studied most extensively (19). In yeast killer system, the viral genome consists of two segments of dsRNA (L and M dsRNA) encapsidated separately. The M segment of dsRNA encodes a preprotoxin and L dsRNA encodes the major capsid protein and the RNA-polymerase (7, 14, 18). Extensive genetic analysis of yeast killer phenomenon has revealed a complex relationship of the virus and host, including host genes requiring the maintenance and expression of killer phenotype (15).

Toxin secreting strains of *Ustilago maydis* exist in about 1% of the natural population (5). Based on the toxin specificity and the distribution of dsRNA segments, three laboratory killer strains, designated as P1, P4 and

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P6, are known with no other new types of killer strains have been reported yet. Therefore, the studies of UmV have been limited to the three killer strains and some mutant strains (1, 2, 4, 8, 12, 21). The dsRNA segments are classified into heavy (H: 6.2-1.4 Kb), medium (M: 1.2-0.9 Kb) and light (L: 0.3 Kb) dsRNA. Three killer strains, contain 2~4 H dsRNAs, 2~4 M dsRNAs and 1~2 L dsRNAs. The function of these dsRNA segments have been partially elucidated either by deletion mutation or by *in vitro* translation experiments (4, 12). It has been reported that one of the M segments encodes the toxin protein and one of the H encodes the capsid protein and possibly the RNA polymerase via the read-through mechanism similar to the one in yeast killer system (7, 18). Recently, part of M and L dsRNA segment were cloned and the nucleotide sequence was reported in P4 and P6 strains (6, 10, 11, 17).

In our laboratory, the distribution of dsRNA and the presence of virus particles have been studied from corn smut collected in Korea. Among 130 *U. maydis* isolated from a field, about 35 strains containing a specific dsRNA or virus particles were identified (22). Among the 35 strains, six of them were toxin producing strains. The distribution of dsRNA segments and toxin specificity were reported previously (9).

In this paper we describe the biological characteristics of UmV SH14 which appears to be most active in toxin activity and most widely active in the killing range tested in our laboratory.

Materials and Methods

Purification of virus

U. maydis SH14 was maintained on potato dextrose agar plates at 4℃. Cells for virus purification were cultured in a 5 liter fermentor (Korea Fermentor Company) at 25℃ with continuous aeration and stirring at 200 rpm on *Ustilago* complete medium (16). Cells were harvested from the culture medium by centrifugation and washed once with 0.05 M phosphate buffer (pH 7.0). Cells were broken with a bead beater (Biospecs Inc. USA) using the 0.5 mm glass bead for 20 min and the cell debries were removed by centrifugation. Virus particles were precipitated with 6% polyethylene glycol and 0.1 M NaCl on ice bath overnight. The precipitate was recovered by centrifugation and resuspended in 0.1 M phosphate buffer (pH 7.0). Then, the virus particles were collected again by ultracentrifugation at 40,000 rpm for 2 hours followed by resuspension in the 0.1 M phosphate buffer (pH 7.0). Virus particle was further purified through a linear 10~50% sucrose density gradient and the fractions containing virus were collected using a gradient fractionator (ISCO, USA). Virus particles were recovered by centrifugation and subjected to CsCl density gradient (median density=1.342 g/cm^3). The refractive index of each fractions were measured by refractometer (Fisher Scientific, USA) and density was calculated by an equation in the ISCO Manual. Fractions containing virus were pooled and dialysed twice against 0.1 M phosphate buffer.

Analysis of double-stranded RNA

The dsRNAs of UmV were extracted twice with phenol/chloroform and precipitated with 2.5 volume of cold ethanol. Then it was analyzed on a 5% polyacrylamide gel.

Analysis of capsid protein

Capsid proteins from virus samples from either sucrose gradient or CsCl gradient were analyzed on 15% polyacrylamide gel electrophoresis. Western blotting analysis were also carried out by using antibodies raised against P4 and A8 virus particles.

Electron microscopy

Virus samples from density gradient were stained with 0.3% uranyl acetate and examined with an electron microscope (Zeiss EM-109, Germany).

Western blotting of UmV capsid protein

Capsid proteins were separated on 15% SDS-PAGE and transferred to nitrocellulose paper using the Semiphore apparatus (Hoefer, USA). Antibodies raised against P4 and A8 virus were used as a primary antibody and capsid protein was detected by the alkaline phosphatase system.

Results and Discussion

Ustilago maydis SH-14 strain is one of the most active killer strains and has the most wide range of killing activity among all isolates in Korea (9, 22). In order to study biochemical characteristics of this virus, the physicochemical properties of SH-14 virion were examined. The virus peaks from 10~50% sucrose gradient were collectively pooled and analyzed on CsCl density gradient (median density=1.331 g/cm^3). Three distinctive peaks, top (T), intermediate (I) and bottom (B) were separately collected and density of each fractions was determined. The density of the T, I and B component was determined as 1.285, 1.378 and 1.408 g/cm^3, respectively (Fig. 1). Similar density profiles were also reported in P1, P4 and P6 strains (2, 21). Density profile of the SH-14 strain virus appeared to be similar to those of other

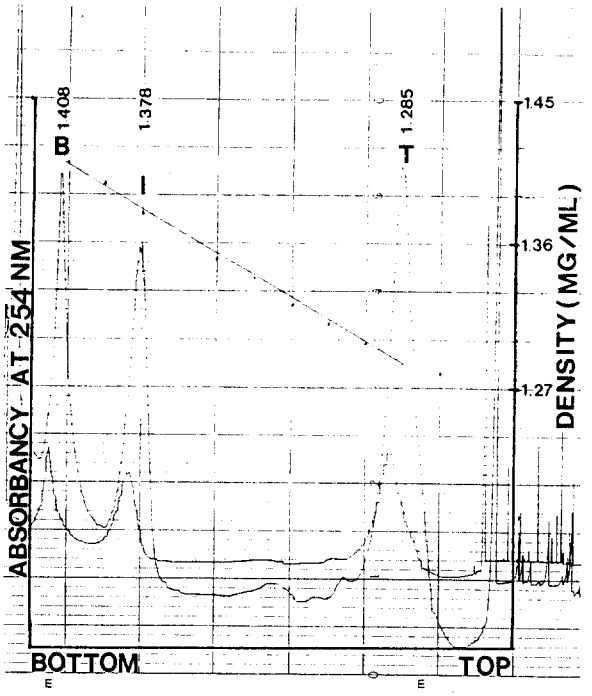

Fig. 1. Cesium chloride density gradient profile of *U. maydis* virus strain SH-14 following sucrose density gradient centrifugation. Centrifugation was carried out at 40,000 rpm in a Beckman SW-55 rotor at 20℃ for 16 hours. Virus peaks were fractionated with ISCO fractionator and the density of each peak was determined by a refractometer. T: top, I: intermediate, B: bottom component.

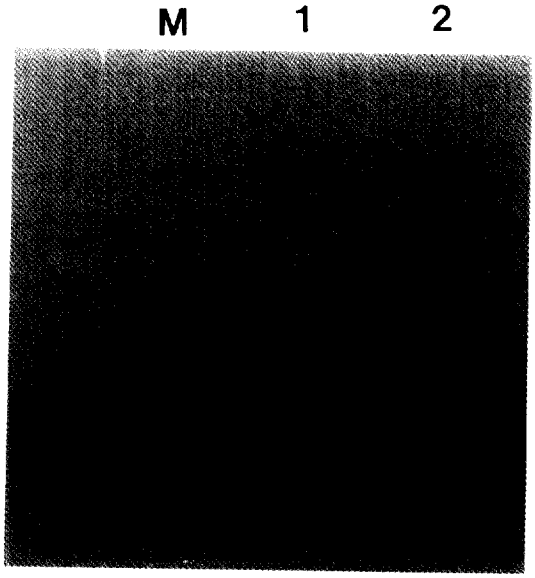

Fig. 2. Molecular weight determination of capsid protein of SH-14 virus. Virus samples from cesium chloride density gradient was analyzed on SDS-PAGE. 1: 1.0 OD at 260 nm, 2: 0.5 OD at 260 nm, M: Molecular weight marker.

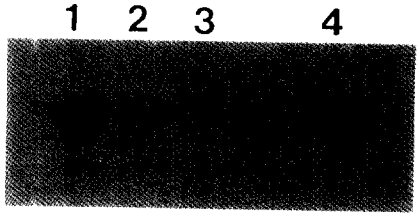

Fig. 3. Capsid protein analysis of three peaks from cesium chloride density gradient. The same amount of virus samples (0.5 OD at 260 nm) were analyzed on SDS-PAGE. 1: Top component, 2: Intermediate component, 3: Bottom component, 4: Total fraction.

laboratory strains within the error range. The density of mutant strains of UmV including T4 and 3103 also indicates that the density of UmV and the SH-14 virus may be the same (2).

Virus particles purified from CsCl gradient dialyzed against a phosphate buffer and capsid protein were analyzed on 15% SDS-PAGE. The data shows a presence of two distinctive bands with molecular weight of about 70 Kd (Fig. 2). It has been previously shown that in purified capsids, the major capsid protein of laboratory strains consists of a 75Kd single subunit and a few other proteins (2, 4). At this time we have not confirmed whether these two bands are genuine capsid protein of two subunits or degradation products of a single protein. Nevertheless, the appearance of these two bands have been consistant in all our results; moreover, analysis of other isolates also seem to contain two subunits of similar molecular weight.

Each fraction from the CsCl gradient was separately collected and dialyzed against a buffer. Virus was collected again by ultracentrifugation followed by an analysis of capsid protein and dsRNA segments. Capsid protein of virions collected from each fractions were analyzed on 15% SDS-polyacrylamide gel. Since an equal amount of virus sample determined by the optical density at 260 nm was analyzed, the number of virus particles and the amount of nucleic acids would be different due to the absorbancy of dsRNA. Top component appeared to contain large amount of capsid protein consisting of two subunits compared to the other fractions (Fig. 3). Double-stranded RNA was extracted from each fractions and analyzed on 5% PAGE (Fig. 4). By comparison, top component appeared to be mostly empty particles or particles containing only small dsRNA segments, M and L dsRNAs; intermediate component appeared to contain M and L dsRNA segments as well as H1 dsRNA segment; and bottom component appeared to contain only H2 ds RNA segment. Since the number of virus in the intermediate fraction used in dsRNA analysis was much larger than that of the bottom component, the intermediate fraction may actively contain smaller amounts of dsRNA than the bottom fraction. Similar results were

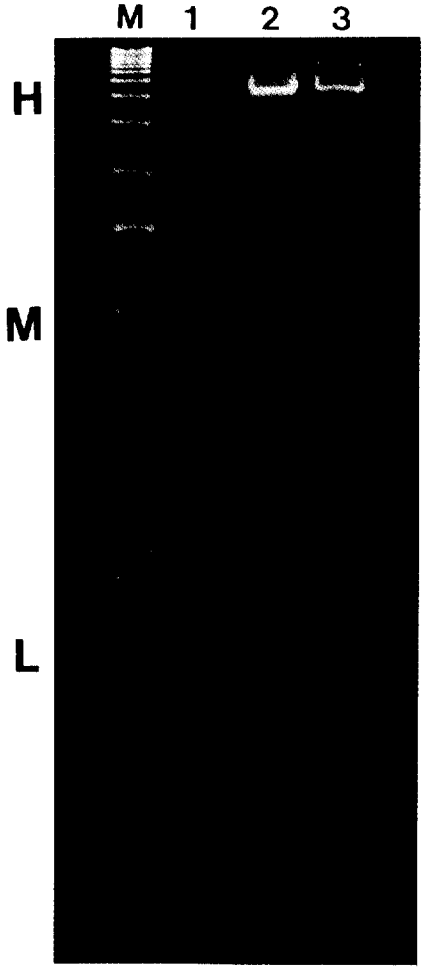

Fig. 4. Double-stranded RNA analysis of three peaks from cesium chloride density gradient. The same amount of virus samples (1.0 OD at 260 nm) were extracted with phenol/chloroform and dsRNA was analyzed on 5% polyacrylamide gel. 1: Top component, 2: Intermediate component, 3: Bottom component, M: 1Kb DNA ladder.

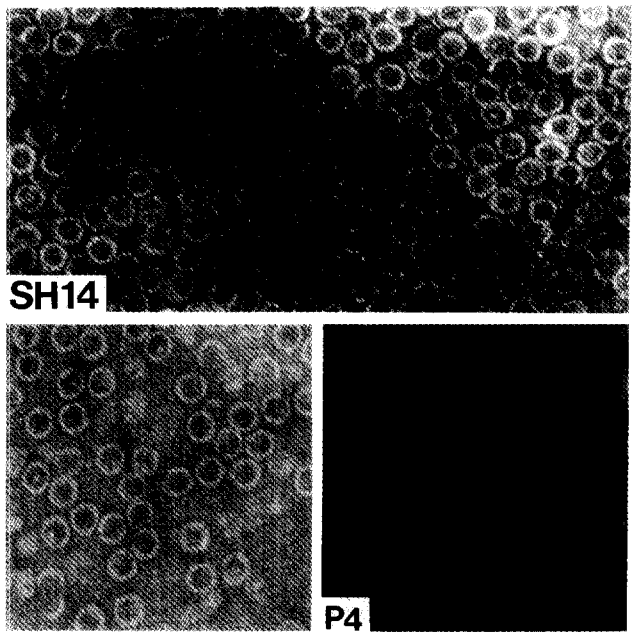

Fig. 5. Electron micrograph of three *U. maydis* virus particles. Virus particles purified from sucrose density gradient centrifugation were stained with uranyl acetate. Bar equals 100 nm.

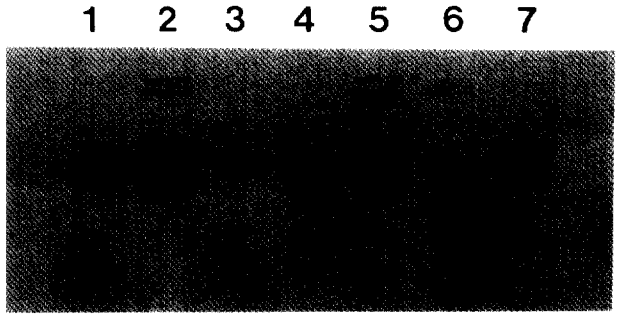

Fig. 6. Western blot analysis of capsid protein from *U. maydis* SH-14 virus and other isolates. Virus particles from sucrose density gradient were separated on SDS-PAGE and capsid proteins were detected with rabbit antibody raised against A8 virus particle. 1: SH-11 strain 2: SH-12 strain 3: SH-13 strain 4: SH-14 strain 5. P4 strain 6. A7 strain 7. A10 strain.

also reported in the laboratory which analyzed P1 and P6 strains (2, 4, 21). These results support the hypothesis that the density of UmV is determined by the size of dsRNA encapsidated in the virion and also suggests that UmV dsRNAs are separately encapsidated as previously described (2, 21).

Electron microscopic observation of each purified sample revealed that all three fractions contain morphologically identical icosahedral virion of approximately 45 nm in diameter. Other isolates were also examined by electron microscope. All the isolates appear to have morphologically identical virion (Fig. 5). The viruses purified from P1, P4 and P6 strains were morphologically indistinguishable, and antibodies against one virus particle cross-reacted with the others (4).

It has been shown that three killer strains have the same immunological cross reactivity. In order to test serological reactivity among UmV isolates, ELISA test was carried out by using antibodies raised against A8 virus particle. The analysis has shown cross reactivity among all isolates tested with different range of specificity (Data not shown). To confirm immunological cross reactivity among isolates, especially, for SH-14 strain, western blot analysis of capsid proteins by using A8 antibody was carried out. All viral capsid protein had positive reaction against A8 antibody which suggest that UmV are immunologically cross reactive with all isolates from Korea (Fig. 6).

The results presented in this paper may indicates that

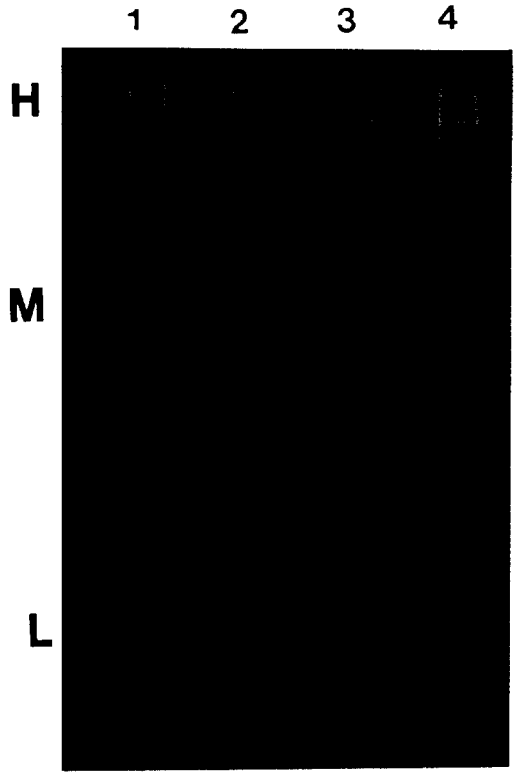

Fig. 7. Double-stranded RNA band pattern of three laboratory strains (P1, P4 and P6) of *U. maydis* virus and SH-14 strain isolated in Korea. DsRNA extracted from virus particle was analyzed on 5% polyacrylamide gel electrophoresis. 1: P1, 2: P4, 3: P6, 4: SH-14 strain.

UmV isolated from SH-14 strain has very similar density distribution of virus particles to that of laboratory strains and its capsid protein has immunological cross reactivity with other isolates from Korea. The comparison of dsRNAs from SH-14 virus and those of the three laboratory strains revealed that SH-14 virus has typical P-type dsRNA pattern (2H, 2M and 2L dsRNAs) although the mobility of each dsRNA was slightly different (Fig. 7). The analysis of SH-14 toxin protein, however, has revealed a completely different specificity (9), molecular weight and amino acid composition (in preparation). Therefore we conclude that UmV from SH-14 strain is a new killer type.

Acknowledgement

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