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Analysis of the Complete Genome Sequence of *Zucchini yellow mosaic virus* strain A Isolated from Hollyhock

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The complete genome sequence of *Zucchini yellow mosaic virus* stain A (ZYMV-A) isolated from a hollyhock (*Althaea rosea*) was determined by using RT-PCR with a series of primer sets. The virus genome consisted of 9593 nucleotides (nt), excluding the poly(A) tract at 3' terminus of the virus genome, with 5' and 3' untranslated region of 139 and 211 nt, respectively. The deduced polyprotein of ZYMV-A consisted of 3080 amino acid (aa) residues and was 351 kDa in molecular weight. All proteolytic cleavage sites of the polyprotein of ZYMV-A were compared with those of ZYMV strains, which showed the cleavage sites were conserved among ZYMV strains. The HC-Pro contained the KITC and PTK motifs, and the DAG motif was located at CP ORF of ZYMV-A, suggesting that ZYMV-A is aphid-transmissible. Phylogenetic tree analysis based on the complete genome among ZYMV strains or CP ORFs with other potyviruses showed ZYMV strains formed a distinct group. These results clearly confirmed that ZYMV-A was another distinct strain in ZYMV population at molecular level.

Keywords : full-legnth cDNA, *Potyvirus*, sequence analysis, squash, ZYMV

The genus *Potyvirus* is the largest group of plant viruses and consists of over 200 definite and tentative species of viruses (Reichmann et al., 1992; van Regenmortel et al., 2000). Potyviruses cause severe damage to various crops in the aspect of quantity and quality. In cucurbit crops, *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* and *Papaya ringsopt virus*, belonging to potyvirus genus, have severely caused the economical damage all over the world (Hull, 2002). In particular, ZYMV, one of the most damaging viruses worldwide causes very severe mosaic symptoms on leaf and its distortion and malformation of fruit (Desbiez et al., 2002; Lecoq and Pitrat 1984; Lisa et al., 1981; Provvidenti et al., 1984). In Korea, the disease caused by ZYMV has been considered one of major limiting factors for the production of cucurbits (Kwon et al., 2005; Yoon and Choi, 1998). ZYMV as well as other species of potyviruses is transmitted by aphids in a non-persistent manner, and the virus can be seed-transmitted (Gal-on et al., 1992; Lisa and Lecoq, 1984; Yoon, 1999; Yoon and Choi, 1998). Like potyviruses, ZYMV particles are flexuous rods, 750 nm long, and contain a monopartite genome consisting of a positive-sense ssRNA with a 5' genome-linked protein and a 3' poly(A) tract (Hull, 2002; Siaw et al., 1985; van Regenmortel et al., 2000).

The entire genomes for several ZYMV strains, including three Korean strains, have been reported (Baker et al., 1992; Balint et al., 1990; Gal-On, 2000; Kwon et al., 2005; Lee et al., 1993; Lin et al., 2000; Zhao et al., 2003). A lot of sequence information for 3' terminus of the genome or partial virus-open reading frame (ORF) has been also available from various strains of ZYMV (Lecoq and Purcifull, 1992; Lee and Wong, 1998; Quemada et al., 1990). Notably, all ZYMV strains isolated from cultivated cucurbit plants have been used for determination of the complete genome sequence or partial genomic information. We previously identified and characterized the biological properties of ZYMV strain A (ZYMV-A) isolated from a hollyhock (*Althaea rosea*) showing yellowing and mosaic symptoms in 2002, Korea (Choi et al., 2002b). The phenotype of ZYMV-A was slightly different from pathological phenotypes of common strains of ZYMV on host plants. This strain also could facilitate systemic movement of a strain of movement-deficient *Cucumber mosaic virus* in zucchini squash (Choi et al., 2002a).

As next step, we sequenced further the complete genome of ZYMV-A and compared its sequence with those of other ZYMV strains and different potyviruses reported in GenBank. To the best of our knowledge, this report represents the first determination of genome sequence of ZYMV that was originated from a weed other than cucurbits.

Materials and Methods

Virus purification. ZYMV-A, a severe strain originated

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from a hollyhock (Choi et al., 2002b), was propagated in zucchini squash (*Cucurbita pepo* cv. Black Beauty). Leaves of the zucchini squash showing typical ZYMV symptoms were ground with phosphate buffer [0.1 M sodium phosphate (pH 7.0), 5mM EDTA and 0.1% 2-mercaptoethanol]. The extract was centrifuged at 15,000×g for 10 min at 4°C. The collected extract was filtered through three layers of Miracloth (Calbiochem-Behring), and the resulting filtrate was loaded on 20% sucrose cushion in phosphate buffer. The purified ZYMV-A was finally obtained by two differential ultra-centrifugations at 150,000×g for 3 hr at 4°C.

RNA extraction and cDNA synthesis. The RNA of ZYMV-A was extracted from the virus particles using Trizol reagent (Invitrogen), according to manufacturer's instructions. The purified RNA was used as a template for cDNA synthesis (Choi et al., 1999). Basically, the cDNA corresponding to the genome of ZYMV-A was generated by combining a series of sense primers with a series of antisense primers extending from 5' untranslated region (UTR) and the 3' UTR of the ZYMV genome, as described by Yoon (1999). In addition, virus specific oligonucleotide primers were designed using published ZYMV sequences and Genbank sequence data. These primers were used for compensation of cDNA synthesis for un-amplified portion of ZYMV-A, and for double confirmation of genome sequence of the virus. The information of primers will be available on requests.

Cloning of cDNA products. PCR fragment was generated using Expand Long-Template Enzyme mixture (Roche) in a programmable thermal cycler (iCycler; Bio-Rad Laboratories). The amplified cDNA fragments were identified by agarose gel electrophoresis with EtBr staining, excised from the agarose gel, and purified using Qiaquick Gel Extraction kit (Qiagen). The purified PCR fragment was directly cloned into pGEM-T easy vector (Promega) or pCR2.1-TOPO vector (Invitrogen), according to manufacturer's instructions.

Sequencing and sequence analysis. Sequencing was carried out by using Big-dye terminator kit (Applied Biosystems) with M13-forward/reverse primers (Sambrook et al., 1989; Sanger et al., 1977). Nucleotide (nt) and amino acid (aa) sequence data were assembled and analyzed using BLAST program at NCBI website and DNASTAR software package (Madison, USA). Briefly, pairwise and multiple alignments of aa sequences were carried out using MegAlign program of DNASTAR and DNAMAN software. Phylogenetic tree analysis was carried out using the Neighbor-joining method within the Clustal-W of MegAlign program.

Results

Genome characterization of ZYMV-A. A total of 12 overlapping cDNA clones were used to determine the complete nt sequence of ZYMV-A. Each region was covered by at least two clones. The complete genome of ZYMV-A consisted of 9593 nucleotides, excluding the poly(A) tract at 3' terminus of the virus genome. A start codon (AUG) was found at nt positions 140-142, and a stop codon at positions 9379-9382 of the genome, resulting in a putative polyprotein consisting of 3080 aa. The molecular weight of the polyprotein was estimated 350,917 daltons using EditSeq program within DNASTAR. The 5' UTR was 139 nt in length. The 211-nt long 3' UTR of ZYMV-A was slightly shorter than that of other ZYMV strains. The genome organization of ZYMV-A was summarized in Fig. 1, which showed its genome was identical to that of published ZYMV strains.

The P1 protein started with typical MASI motif and the protease cleavage site of P1 protein was identified as

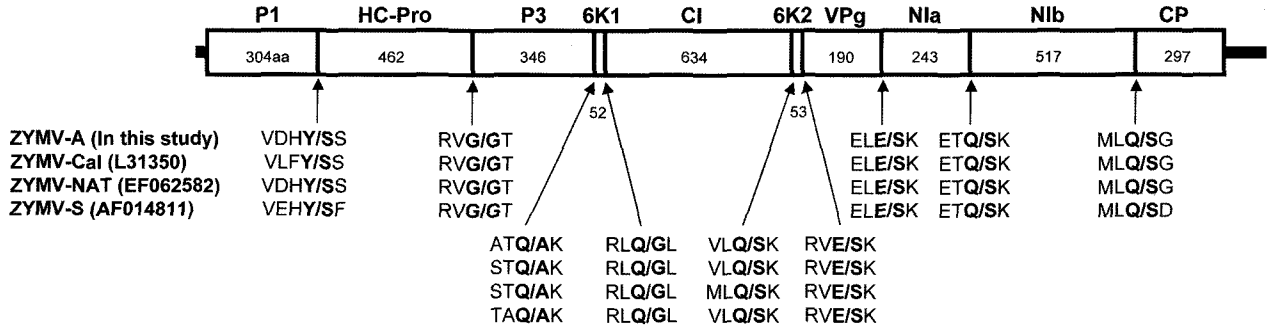

Fig. 1. Organization of the genome and predicted cleavage sites of the polyprotein of ZYMV-A. The cleavage sites of ZYMV-A polyprotein are compared with strains California (L31350), NAT (EF062582) and Singapore (AF014811). The proteolytic cleavage sites of P1 protein to CP ORFs are indicated to solid arrows mark. Each cleavage site is indicated by a slash symbol. The lengths of amino acids consisting of mature proteins are indicated.

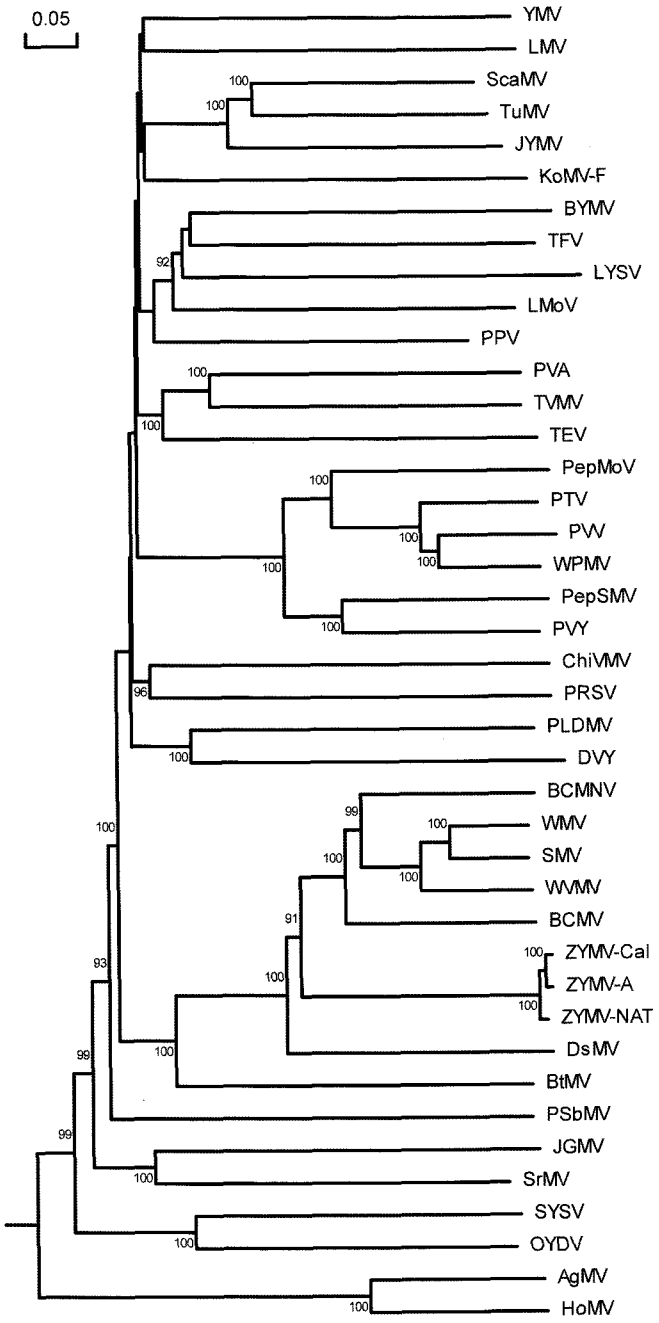

Fig. 2. Phylogenic analysis of the complete polyprotein amino acid sequences of 41 potyviruses using DNAMAN program. The polyproteins were translated *in silico* from the nucleotide sequences. *Agropyron mosaic virus* (AgMV, NC_005903); *Bean common mosaic necrosis virus* (BCMNV, NC_004047); *Bean common mosaic virus* (BCMV, NC_003397); *Bean yellow mosaic virus* (BYMV, NC_003492); *Beet mosaic virus* (BtMV, NC_005304); *Chilli veinal mottle virus* (ChiVMV, NC_005778); *Daphne virus* Y (DVY, NC_008028); *Dasheen mosaic virus* (DsMV, NC_003537); *Hordeum mosaic virus* (HoMV, NC_005904); *Japanese yam mosaic virus* (JYMV, NC_000947); *Johnsongrass mosaic virus* (JGMV, NC_003606); *Konjac mosaic virus* (KoMV-F, NC_007913); *Leek yellow stripe virus* (LYSV, NC_004011); *Lettuce mosaic virus* (LMV, NC_003605); *Lily mottle virus* (LmoV, NC_005288); *Onion yellow dwarf virus* (OYDV, NC_005029); *Papaya leaf-distortion mosaic virus* (PLDMV, NC_005028); *Papaya ringspot virus* (PRSV, NC_001785); *Pea seed-borne mosaic virus* (PSbMV, NC_001671); *Pepper mottle virus* (PepMoV, NC_001517); *Pepper severe mosaic virus* (PepSMV, NC_008393); *Peru tomato mosaic virus* (PTV, NC_004573); *Plum pox virus* (PPV, NC_001445); *Potato virus* A (PVA, NC_004039); *Potato virus* V (PVV, NC_004010); *Potato virus* Y (PVY, NC_001616); *Scallion mosaic virus* (ScaMV, NC_003399); *Shallot yellow stripe virus* (SYSV, NC_007433); *Sorghum mosaic virus* (SrMV, NC_004035); *Soybean mosaic virus* (SMV, NC_002634); *Thunberg fritillary virus* (TFV, NC_007180); *Tobacco etch virus* (TEV, NC_001555); *Tobacco vein mottling virus* (TVMV, NC_001768); *Turnip mosaic virus* (TuMV, NC_002509); *Watermelon mosaic virus* (WMV, NC_006262); *Wild potato mosaic virus* (WPMV, NC_004426); *Wisteria vein mosaic virus* (WVMV, NC_007216); *Yam mosaic virus* (YMV, NC_004752); *Zucchini yellow mosaic virus* (ZYMV-NAT, EF062582 and ZYMV-Cal, L31350).

VDHY/SS, resulting in mature protein consisting of 304 aa. The putative HC-Pro/P3 cleavage site (G/G) was found in RVG/GT motif that was conserved in all potyviruses. The HC-Pro was 462 aa long, and both KITC and PTK motifs in HC-Pro that played a role in aphid transmission (Atreya and Pirone, 1993) were found in ZYMV-A. The alignment with previously sequenced ZYMV strains resulted in identification of proteolytic cleavage sites for generation of mature functional proteins: P3/6K1 cleavage site was Q/A,; 6K1/CI cleavage site was Q/G, which was conserved in all potyviruses; the CI/6K2 cleavage site was Q/S, which was typical of most potyviruses; VPg/NIa cleavage site was E/S; NIa/NIb and NIb/CP cleavage sites were Q/S, which was conserved in potyviruses (Fig. 1).

All functional protein motifs of RNA-dependent RNA polymerase (Ia to VIII) were conserved in NIb protein of ZYMV-A and motifs of typical helicase (I, Ia and II to VI) were found in the CI protein (data not shown). The N-terminal region of the CP ORF contained the conserved DAG motif required for aphid transmission of the potyviruses (Atreya et al., 1991; Shukla et al., 1988).

Sequence analysis between ZYMV-A and potyviruses. Sequence analysis between ZYMV-A and other ZYMV strains showed over 89% sequence identity and that ZYMV-A was closely related to the California strain of ZYMV. The aa sequence identity of polyprotein between ZYMV-A and other potyviruses was ranged from 64 to 47% (data not shown).

To determine the genetic relationships of ZYMV-A to other potyviruses, phylogenetic tree analysis was analyzed on the basis of CP ORF sequences. Comparisons of CP ORFs showed that ZYMV was closely related to *Bean common mosaic virus, Watermelon mosaic virus, Soybean mosaic virus, Wisteria vein mosaic virus*, and *Bean common mosaic necrosis virus*. In addition, ZYMV was formed a distinct cluster in the phylogenetic tree (Fig. 2), indicating that ZYMV-A was a typical strain, regardless of the isolation host.

Discussion

The genome of ZYMV-A consists of 9593 nts and its sequence was deposited to Genbank (AB098081 and AJ429071). The sizes of all ORFs of the ZYMV-A genome were conserved like other strains of ZYMV. The 6K1 protein, NIa/VPg, and the 3' UTR region appear to be the most conserved regions among all ZYMV strains. Proteolytic cleavage sites on ZYMV-A were also identified high conservations, similar to ZYMV strains. In addition, motifs associated with aphid transmission, such as DAG motif in CP ORF or FITC motif in HC-Pro, were highly conserved in ZYMV-A, suggesting that the virus is aphid-transmissible. It remains to determine if the virus is authentically transmitted by aphids.

Regardless of some variations on ZYMV genomes, phylogenetic tree analysis and multiple alignment data based on CP ORFs showed no distinct close relationships between ZYMV strains and other potyviruses, suggesting that the ZYMV population appears to have a stable ecological status (Desbiez et al., 2002; Glasa et al., 2007; Lecoq and Purcifull, 1992). Although ZYMV isolates could easily spread to geographically adjacent areas, the variability of ZYMV isolates might not be relevant to ZYMV isolates found in different countries (Pfosser and Baumann, 2002).

So far, the complete genomes for three strains of ZYMV (designated ZYMV-PA, PE and PS) have been determined and analyzed (Kwon et al., 2005). Based on sequence alignment results, high sequence identity between ZYMV-A and the three ZYMV strains were observed. However, it is interesting that symptoms and infectivity of ZYMV-A were different from those of strains PA, PE and PS on *Nicotiana benthamiana* and *Gomphrena globosa* (Choi et al., 2002b; Kwon et al., 2005). ZYMV-A failed local infection on *N. benthamiana*, while strains PA, PE and PS infected locally. On the other hand, ZYMV-A could infect systemically *G. globosa*, but the other three strains failed in the systemic infection of the host. Therefore, some subtle interactions between ZYMV-coding proteins or between ZYMV protein and host protein(s) are likely to play a role in infection and symptom development of ZYMV (Desbiez et al., 2003; Gal-On, 2000), though we did not find any significant amino acids or region in virus-encoded ORFs as symptom or pathogenicity determinants by sequence analysis among ZYMV Korean strains. In spite of low variability among ZYMV strains based on analyses of phylogenetic tree and multiple alignments, it is assumed that plants around cucurbit fields play a role in the transmission of ZYMV or the existence of unrecognized reservoirs for ZYMV in the field. It remains to find the relationships between host range and alternate epidemiological patterns of ZYMV in the field.

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