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Short communication

Characterisation of a maize-infecting potyvirus from Spain

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Abstract. In order to characterise and classify an unknown maize-infecting potyvirus isolated from fields in northeast Spain, the entire coat protein gene and the C-terminal two-thirds of the large nuclear inclusion protein (NIb) gene were cloned and sequenced. Protein sequencing enabled the cleavage site between the two proteins to be deduced and also revealed that on storage the viral coat protein undergoes a specific degradation in which the N-terminal 39 amino acids are removed. Comparison of the nucleotide sequence of the 3′ non-coding region of the viral RNA and the predicted amino acid sequence of the coat protein with the equivalent regions of other members of the potyvirus group revealed that the Spanish virus is closely related to maize dwarf mosaic virus strain A.

Maize crops in northeast Spain are subject to severe losses due to infection with a potyvirus. These losses, estimated by deliberate inoculation of the virus on to maize plants in the field, are found to be between 28–42% of the total crop yield (Achon, unpublished data). Potyviruses which infect maize, sorghum, sugarcane and other poaceous species have been designated as strains of either sugarcane mosaic virus [SCMV; Abbot and Tippet, 1966] or maize dwarf mosaic virus [MDMV; Louie and Knoke, 1975] depending on the host from which they were originally isolated. However, on the basis of serology, some strains of MDMV have been assigned to SCMV [Shepherd, 1965; Snazelle et al., 1971; Pirone, 1972; Jarjees and Uyemoto, 1984]. Recent work, using antibodies specific to the N-terminal portion of the viral coat protein and high performance liquid chromatography (HPLC)-based peptide profiling of tryptic digests of the coat proteins [Shukla et al., 1989; McKern et al., 1991] coupled with host-range studies [Tosic et al., 1990], has suggested that viruses from the USA and Australia, which had been considered isolates of SCMV and MDMV, may actually be four distinct potyviruses.

There is little information currently available on the relationship between European isolates of potyviruses which infect poaceous species and those from the USA and Australia. Biological characterisation of the Spanish virus, which we tentatively designated MDMV-SP, suggested that although it is general similar to isolates of MDMV from the USA, it is distinct from

them in several important respects. For example, it appears to be in subdivision IV rather than I of the potyviruses based on the type of cytoplasmic cylindrical inclusion bodies formed in infected tissue [Edwardson et al., 1984; Lesemann et al., 1992]. It also differs in its host-range as it is able to infect *Bromus rubens* and causes a symtompless in *Sorghum bicolor* cv. TX2786 [Achon et al., 1993]. In order to clarify the nature of this Spanish maize-infecting potyvirus, the region of the viral RNA encoding the viral coat protein and the C-terminal portion of the large nuclear inclusion body (NIb) protein was cloned and its sequence determined.

Virus from an infected maize plant from a field in northeast Spain was propagated by mechanical inoculation on to maize plants (cv. G-4507, Funk's). Virus was purified essentially as described by von Baumgarten et al. [1981] and the virion RNA isolated as described by Wang et al. [1992]. Double-stranded cDNA specific to the 3′ terminal region of the viral RNA was synthesised by the RACE procedure of Frohman et al. [1988]. First strand cDNA was synthesised with AMV reverse transcriptase using 5′ GACTCGAGTCGACATCGA(T_{17}) 3′ as a primer, To amplify the region of the viral RNA expected to encode the carboxy-terminal part of the NIb protein and the entire coat protein the degenerate primer, 5′ GGGATCCGA (A,G)ACAGC(T,C,G,A)CT(T,C,G,A)CG(T,C,G,A)AA 3′ was synthesised. The primer consists of a *BamH1* site linked to a sequence corresponding to a region 105–120 nucleotides upstream of the coat protein gene which is partially conserved in MDMV-B, SCMV-SC and Johnson grass mosaic virus (JGMV) [Gough et al., 1987; Frenkel et al., 1991]. The second primer for the PCR reaction was 5′ GACTCGAGTCGACATCG 3′, a sequence complementary to the extreme 5′ end of the first strand cDNA. The PCR reactions were carried out in a Techne PHC-3 thermal cycler using *Taq* polymerase under conditions recommended by the manufacturer. The cycling conditions were as follows: 3 cycles consisting of 1 min at 94°, 1 min at 30° and 3 min at 72°, 30 cycles under the same conditions but with the annealing temperature increased to 57° and a final extension reaction at 72° for 15 min. When analysed by electrophoresis on agarose gels, the major product of this reaction was approximately 1.6 kb in length.

To determine the nucleotide sequence of the 3′ portion of the virus genome, the PCR products were digested with *BamH1* and *Sal1* and ligated into similarly digested M13mp18 replicative form DNA. The ligation mixture was transformed into *Escherichia coli* strain JM101 and phage harbouring inserts were identified by the *lac* complementation assay. Clones containing part of the viral poly(A) tract were identified by 'T-track' analysis [Sanger et al., 1980]. Two of these clones (M13-MDMV-5 and M13-MDMV-8) were subsequently shown to have inserts of approximately 1.6 kb while the third clone (M13-MDMV-r) had an insert of only 1.1 kb. The inserts from M13-MDMV-5 and M13-MDMV-r were oligo-labelled [Feinberg and Vogelstein, 1983] and shown to hybridise with viral RNA in northern blots (data not shown).

The sequences of all three clones were determined by the 'dideoxy' method using a variety of primers to 'walk' along the DNA. To determine the sequence on the opposite strand, the inserts from all three clones were subcloned into M13mp19 and the process repeated. A total sequence of 2143 nucleotides (excluding the poly(A) tail) could be deduced form the sequence of these clones (Fig. 1). M13-MDMV-5 and -8 had identical 5′ termini ending in the sequence of the primer. The non-primer derived portion of these clones starts at nucleotide 596 of the finally derived sequence (Fig. 1). Since this sequence is 446 nucleotides upstream of the coat protein gene rather than the anticipated 105–120, the primer must have fortuitously annealed to a site other than that expected. The two clones differed in sequence from each other at only 5 positions. The third clone, M13-MDMV-r, by contrast, had a poly(A) tract adjacent to a 3′ end corresponding to an internal sequence within M13-MDMV-5 and -8 (nucleotide 1102 in Fig. 1) and extended the sequence found in these clones in a 5′ direction by 596 nucleotides. Examination of the sequence proceding the 3′ end of M13-MDMV-r showed it to be extremely A-rich and it is reasonable to assume that first-strand cDNA synthesis was primed at this position to give rise to an apparent poly(A) tract. The different location of the 5′ end of M13-MDMV-r again presumable reflects fortuitous priming by the degenerate primer.

The contiguous sequence shown in Fig. 1 contains a single long open reading frame (ORF) sufficient to code for a polypeptide of 635 amino acids. From the known genome organisation of other potyviruses (for a review, see Reichmann et al. [1992]), it was anticipated that the coat protein gene would be contained in the carboxy-terminal portion of this sequence. To map the cleavage site for the release of the coat protein, a sample of purified virus was subjected to automated Edman degradation using an Applied Biosystems 470A gas phase protein sequencer. An unambiguous sequence of 10 amino acids was obtained which allowed the amino-terminus of the coat protein to be mapped (Fig. 1). The fact that a clear N-terminal sequence could be obtained also demonstrates that the coat protein is not blocked. Assuming that the C-terminus of the coat protein is co-terminal with that of the deduced translation product shown in Fig. 1, the calculated size of the coat protein is 32,682 Da.

When samples of denatured virus were analysed by electrophoresis on polyacrylamide gels run according to Schägger and von Jagow [1987], two forms of the coat protein could be found with estimated sizes of either 32 kDa or 29 kDa (Fig. 2). The proportion of the 29 kDa protein increased during storage of the virus at 4 °C, suggesting that this form was derived from the 32 kDa protein by proteolytic processing. To investigate this possibility further, the two forms were electrophoretically separated, transfered to a ProBlott (Applied Biosystems) membrane and subjected to gas-phase Edman degradation as before. The N-terminal sequence from the 32 kDa protein was identical to that obtained when whole virions were

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 P  I  E  K  T  E  A  N  K  T  R  T  F  T  A  A  P  L  E  T  L  L  A  G  K  V  C  V  D  D  F  N  N  Q  F  Y  S  Q  H  L
ACCCATAGAGAAAACGGAGGCAAATAAAACAAGAACATTCACAGCTGCACCACTGGAAACATTATTAGCAGGTAAAGTTTGCGTTGATGATTTCAACAATCAATTCTACTCGCAACACCT
       10                  30                  50                  70                  90                 110

 N  G  P  W  T  V  G  I  T  K  F  Y  G  G  W  N  K  L  L  E  K  L  P  D  G  W  I  Y  C  D  A  D  G  S  Q  F  D  S  S  L
GAATGGTCCATGGACGGTTGGAATTACCAAGTTCTACGGTGGATGGAACAAGCTACTCGAGAAGCTGCCAGATGGCTGGATTTATTGTGATGCGGATGGTTCACAATTCGATAGTTCACT
      130                 150                 170                 190                 210                 230

 T  P  Y  L  I  N  A  V  L  N  I  R  L  Q  F  M  E  P  W  H  I  G  E  Q  M  L  K  N  L  Y  T  E  I  V  F  T  P  I  A  T
CACTCCGTATTTGATAAATGCAGTTCTCAACATTCGATTACAGTTCATGGAGCCATGGCATATTGGTGAACAAATGCTCAAGAATTTGTATACGGAAATCGTTTTCACACCAATTGCAAC
      250                 270                 290                 310                 330                 350

 P  D  G  S  V  I  K  K  F  K  G  N  N  S  G  Q  P  S  T  V  V  D  N  T  L  M  V  I  I  A  F  N  Y  A  L  L  S  C  G  I
ACCCGATGGATCTGTTATCAAGAAGTTCAAGGGCAACAATAGCGGACAACCGTCAACAGTTGTTGATAACACATTGATGGTAATCATAGCATTCAACTATGCACTATTATCATGTGGCAT
      370                 390                 410                 430                 450                 470

 D  L  K  E  A  D  E  V  C  R  M  Y  A  N  G  D  D  L  L  I  A  V  N  P  A  H  V  D  I  L  D  E  F  G  K  H  F  A  T  L
CGACTTGAAGGAGGCTGATGAAGTATGCCGAATGTATGCAAATGGGGATGATTTGCTAATCGCAGTAAACCCAGCACATGTTGACATTCTAGATGAGTTTGGTAAACACTTCGCAACGTT
      490                 510                 530                 550                 570                 590      <---

 G  L  N  F  E  F  E  S  R  T  K  D  K  S  E  L  W  F  M  S  T  R  G  I  E  H  E  G  I  Y  I  P  K  L  E  M  E  R  I  V
AGGACTAAACTTCGAATTCGAATCACGAACAAAAGACAAATCAGAACTATGGTTTATGTCAACGCGAGGCATCGAACACGAGGGAATATACATTCCAAAATTGGAAATGGAACGAATCGT
      610                 630                 650                 670                 690                 710

 A  I  L  E  W  D  R  S  L  L  P  Q  Y  R  L  E  A  I  C  A  A  M  V  E  A  W  G  Y  K  D  L  L  H  E  I  R  K  F  Y  A
TGCAATCCTCGAGTGGGATCGATCGCTACTCCCTCAATACCGTCTTGAGGCAATTTGTGCAGCAATGGTTGAGGCGTGGGGATATAAGGATTTGCTCCATGAGATACGTAAATTTTATGC
      730                 750                 770                 790                 810                 830

 W  L  L  E  M  Q  P  F  A  G  L  A  K  E  G  L  A  P  Y  I  A  E  S  A  L  K  N  L  Y  T  G  A  K  V  S  E  D  E  L  N
ATGGTTGCTCGAAATGCAACCTTTCGCTGGCTTGGCAAAAGAGGGATTAGCACCATACATAGCTGAATCAGCCCTCAAAAATTTATACACTGGTGCTAAAGTTTCGGAAGATGAGTTGAA
      850                 870                 890                 910                 930                 950

                                                                 ↓  *  *  *  *  *  *  *  *  *  *
 V  Y  A  R  Q  F  F  D  D  L  P  N  Y  L  A  D  E  V  I  D  V  K  H  Q  A  G  E  N  V  D  A  G  Q  K  T  D  A  Q  K  E
TGTTTACGCGCGACAATTCTTTGACGATCTTCCAAACTATTTGGCTGATGAAGTCATAGATGTCAAACATCAAGCTGGTGAAAATGTTGATGCGGGACAGAAGACTGATGCACAAAAGGA
      970                 990                1010                1030                1050                1070

                                                                 +  +  +  +  +  +  +  +  +  +
 A  E  K  K  A  A  E  E  K  K  A  K  E  A  E  A  K  Q  K  E  T  K  D  K  A  T  E  K  A  D  T  G  G  S  T  G  K  D  K  D
AGCAGAAAAGAAAGCAGCCGAAGAAAAGAAAGCAAAAGAAGCTGAGGCCAAGCAAAAAGAAACTAAGGATAAAGCAACTGAAAAGGCTGATACTGGTGGATCTACAGGAAAAGACAAAGA
      1090      --->      1110                1130                1150                1170                1190
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 V  D  A  G  T  S  G  S  M  S  V  P  K  L  K  A  M  S  K  K  M  R  L  P  Q  A  K  G  K  N  I  L  H  L  D  F  L  L  K  Y
TGTAGATGCTGGAACTTCAGGTTCAATGTCAGTACCGAAGCTTAAAGCTATGTCTAAGAAGATGCGTTTACCTCAGGCAAAAGGAAAGAACATTCTCCATCTCGACTTCCTTTTGAAATA
      1210                 1230                 1250                 1270                 1290                 1310

 K  P  Q  Q  Q  D  L  S  N  T  R  A  T  R  A  E  F  D  R  W  Y  E  A  V  Q  K  E  Y  E  L  D  D  T  Q  M  T  V  V  M  S
TAAACCACAACAACAAGATTTATCAAACACCCGAGCAACCAGGGCTGAATTTGATAGGTGGTATGAAGCAGTACAGAAAGAATACGAACTTGATGACACACAAATGACAGTTGTCATGAG
      1330                 1350                 1370                 1390                 1410                 1430

 G  L  M  V  W  C  I  E  N  G  C  S  P  N  I  N  G  V  W  T  M  M  D  G  D  E  Q  R  T  F  P  L  K  P  V  I  E  N  A  S
TGGACTGATGGTTTGGTGTATTGAAAACGGTTGCTCACCGAACATCAATGGTGTCTGGACCATGATGGACGGAGATGAACAGAGAACATTTCCTTTAAAACCAGTTATTGAGAATGCATC
      1450                 1470                 1490                 1510                 1530                 1550

 P  T  F  R  Q  I  M  H  H  F  S  D  A  A  E  A  Y  I  E  Y  R  N  S  T  E  K  Y  M  P  R  Y  G  L  Q  R  N  L  T  D  F
TCCAACTTTCAGACAAATTATGCATCATTTTAGTGATGCAGCTGAAGCGTATATCGAGTATAGAAACTCAACAGAAAAATATATGCCGAGGTATGGACTTCAGCGCAACTTAACCGACTT
      1570                 1590                 1610                 1630                 1650                 1670

 S  L  A  R  Y  A  F  D  F  Y  E  I  S  S  R  T  P  V  R  A  K  E  A  H  M  Q  M  K  A  A  A  V  R  G  S  N  T  R  L  F
TAGCCTTGCACGTTATGCATTTGATTTCTATGAGATATCATCTCGAACTCCAGTGCGTGCAAAGGAAGCCCACATGCAGATGAAGGCAGCAGCAGTCCGTGGTTCAAACACACGGTTGTT
      1690                 1710                 1730                 1750                 1770                 1790

 G  L  D  G  N  V  G  E  A  H  E  N  T  E  R  H  T  A  G  D  V  S  R  N  M  H  S  L  L  G  V  Q  Q  G  H  *
TGGTCTTGATGGGAATGTCGGAGAAGCCCACGAAAATACAGAACGCCATACAGCTGGCGATGTTAGTCGCAATATGCACTCCCTCCTGGGAGTTCAGCAGGGGCACTGAGACGGGGTTGA
      1810                 1830                 1850                 1870                 1890                 1910

ACTTCTACGCAGTAATTTAGTAATATATACGTAAGCTATTGTGGTGAGGTTTTACCTCGTTAGTTTTATATATATATTATGCTATGTTCCTACTATGTCTGCAAGTGAGTGAGGTTACAC
      1930                 1950                 1970                 1990                 2010                 2030

CTCGACACTTATGGTAGGCACTATTACTAGCTTCGAATCACGATACGGACGGTCCATTGAGTGGTTCTACCACGAGGATGCAGCGAGTTTCGTGGTGAGAGAC--poly(A)
      2050                 2070                 2090                 2110                 2130
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Fig. 1. Nucleotide sequence of the 3′ terminal region of the Spanish maize-infecting potyvirus. The deduced amino acid sequence of the translation product of the single long ORF is shown above using the standard one-letter code. The sequence of the N-terminus of the coat protein as deduced by Edman degradation of virus particles is indicated by **** allowing the cleavage site between the NIb and coat protein to be deduced. The N-terminal 10 residues of the truncated 29kDA coat protein are indicated (++++). The 5′ ends of clones M13-MDMV-5 and -8 and the 3′ end of clone M13-MDMV-r are indicated below the nucleotide sequence by ← and →, respectively.

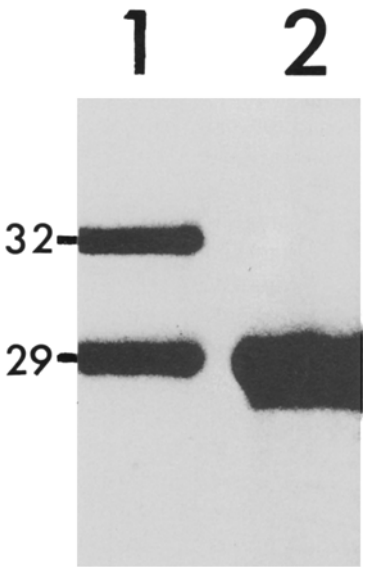

Fig. 2. Coomassie-stained SDS/polyacrylamide gel of the coat protein of the Spanish maize-infecting potyvirus showing the effect of storage. The virus was stored in 50mM sodium phosphate buffer, pH 8.0 at 4° for either 1 week (Lane 1) or 2 weeks (Lane 2) prior to analysis on a 16.5% polyacrylamide gel [Schägger and von Jagow, 1987]. The positions of the 32 and 29kDa forms of the coat proteins are indicated.

analysed (Fig. 1). The N-terminus of the 29 kDa product could also be unambiguously identified (Fig. 1) and starts at residue 40 of the coat protein. This result implies that the 29 kDa protein arises from the 32 kDa protein by the loss of precisely 39 amino acids from the N-terminus. The effect of this loss reduces the calculated size of the viral coat protein to 28,468 Da, in good agreement with size reduction as observed on polyacrylamide gels. The reduction in size of potyviral coat proteins has been reported previously [Hiebert et al., 1984; Lain et al., 1988; Shukla et al., 1988] and the lability of the N-terminal region is presumably due to the fact that it is exposed on the surface of the potyvirus particle [Allison et al., 1985; Shukla et al., 1988].

To clarify the taxonomic position of MDMV-SP, the nucleotide sequence of the 3′ non-coding region was compared with those from a number of monocot-infecting potyviruses. By far the greatest identity (94.9%) was

found with the 3′ non-coding region of mazie dwarf mosaic virus strain A (MDMV-A; J. Jilka and J. M. Clark, unpublished data) from the USA. By contrast the identity with the 3′ non-coding region of sugarcane mosaic virus (SCMV-SC) and MDMV strain B [Frenkel el al., 1991] was approximately 50%. Similarly, when the amino acid sequences of the coat proteins were compared, that of MDMV-SP showed an identity of 96.2% with MDMV-A as opposed to approximately 70% when compared with SCMV-SC and MdMV-B. The homology between MDMV-SP and MDMV-A is in the range of variability found between viruses which have been designated as strains of JGMV [Shukla et al., 1992] and is similar to the variability found between strains of other potyviruses [Shukla and Ward, 1988; Frenkel et al., 1989]. The high degree of identity between the sequences of the N-terminal 70 amino acids of the coat proteins of the Spanish virus and MDMV-A (63 out of 70 residues identical) is also similar to the level of identity found in this region between strains of JGMV. This is particularly striking since this region is known to be extremely variable between strains of SCMV [Frenkel et al., 1991; Xiao et al., 1993]. From these homology studies we conclude that MDMV-SP is, indeed, a strain of MDMV and is closely related to MDMV-A.

To our knowledge this is the first example of a European isolate of a maize-infecting potyvirus being characterised at the molecular level. The work also illustrates the usefulness of PCR-based cloning methods in the field of virus taxonomy and epidemiology. The biological significance, if any, of the proteolyic cleavage of the coat protein which occurs on storage of virus is unclear though its occurrence may explain the variation in estimates of the size of the coat protein of MDMV-A [von Baumgarten and Ford, 1981; Jensen et al., 1986].

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