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Determination of the Length of the Poly(A) Tails of the *Arabis mosaic nepovirus* Genomic RNAs

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ABSTRACT

The genomic RNAs 1 and 2 of the NW isolate of the *Arabis mosaic nepovirus* were polyguanylated and amplified by RT-PCR, to determine the lengths of their poly(A) tails. Primers specific of ArMV-NW RNAs 1 or 2 were used in combination with a primer designed to hybridize at the junction poly(A)-poly(G) introduced at the end of the poly(A) tail during the polyguanylation procedure of the viral RNAs. The RT/PCR products were cloned and sequenced. The poly(A) tails in the different clones ranged from 10 to 81 adenosine residues for RNA 1, and from 10 to 120 adenosine residues for RNA 2, revealing an unexpected variability in the length of the ArMV genomic RNAs poly(A) tails.

Keywords: polyguanylation, yeast poly(A) polymerase

INTRODUCTION

Arabis mosaic virus (ArMV) belongs to the plant virus genus *Nepovirus* of the family *Comoviridae*. In the wine-producing areas southwest of Germany, including Neustadt an der Weinstrasse (NW), ArMV is, along with the *Grapevine fanleaf virus* (GFLV) and the *Raspberry ringspot virus* (RpRSV), two other nepoviruses, a causative agent of the grapevine fanleaf disease. Fanleaf disease is one of the most widespread and damaging virus diseases affecting grapevine. ArMV is transmitted by the nematode vector *Xiphinema diversicaudatum*, and has a wide natural host range (Wellink *et al.* 2000, and references therein).

Nepoviruses have two positive sense, single stranded genomic RNAs, RNA 1 and RNA 2, which are polyadenylated at their 3′ end, and have a covalently attached small genome-linked viral protein (VPg) at their 5′ end (for a review, see Mayo and Robinson 1996). The complete nucleotide sequences of the RNAs 1 and 2 of the grapevine isolate NW of ArMV have been reported (Wetzel *et al.* 2001, 2004). The full-length sequences of the RNAs 2 of ArMV isolates from grapevine (Loudes *et al*. 1995), butterbur, narcissus and lily (Genbank accession numbers AB279739, AB279740 and AB279741, respectively) have also been determined, as well as additional partial sequences from various isolates (Steinkellner *et al*. 1990; Bertioli *et al*. 1991; Wetzel *et al*. 2002a, 2002b).

For most eukaryotic mRNAs, which contain a 5′ cap structure (m^7G(5′)ppp(5′)N) and a 3′ poly(A) tail, efficient translation requires mRNA circularisation, which is provided by the binding of the cap and poly(A) tail to the eukaryotic translation initiation factor 4E and poly(A)-binding protein, respectively (Kapp and Lorsch 2004; Merrick 2004). The involvement of the poly(A) tail in the replication process of polyadenylated positive-strand RNA viruses has been the focus of more and more attention (Thivierge *et al*. 2005, and references therein). The deletion of the poly(A) in viral infectious clones has led in some cases to marginal infectivity only (Guilford *et al*. 1991), or loss of infectivity (Eggen *et al*. 1989; Rohll *et al*. 1995; Kusov *et al*. 1996). Thus, the presence of a poly(A) tail, but also its length have been shown to be of importance in the replicative cycle of polyadenylated positive-strand RNA viruses (Poon *et al*. 1998; Pritlove *et al*. 1998; Tsai *et al*. 1999; Kusov *et al*. 2001; Chen *et al*. 2005; Karetnikov *et al*. 2006).

In this paper, we report the use of a RT-PCR–derived method for the analysis of the length of the poly(A) tail for both genomic RNAs 1 and 2 of ArMV.

MATERIALS AND METHODS

ArMV-NW was propagated on *Chenopodium quinoa*, purified, and the viral RNAs extracted as described previously (Pinck *et al*. 1988). Purified viral genomic RNAs (100 ng) were used as templates for polyguanylation using the yeast poly(A) polymerase (PAP)(USB), as described (Kusov *et al*. 2001), with the following modifications. The RNAs were heated at 65°C for 10 min and immediately placed on ice, and then incubated 2 h at 37°C with 1 mM GTP and 1 µl (764U) PAP, in a 10 µl reaction mixture. The reaction mixture was then phenol/chloroform extracted, chloroform extracted and ethanol precipitated. The pellets were resuspended in DEPC-treated water, and used for RT-PCR using the One-Step RT-PCR system (Invitrogen). The cycling conditions were: 30 min 42°C, 5 min 94°C, 40×(94°C 20 s, 42°C 20 s, 72°C 30 s). The sequences of the different primers were (5′ to 3′): A1-7123s: ATAACCCAGTTTTAGCACTG, corresponding to nucleotides 7123-7142 on ArMV-NW RNA 1; A2-3561s: TGCTCCGAA TTTTATGCAAG, corresponding to nucleotides 3561-3580 on ArMV-NW RNA 2; oligo(dCdT): GAATTCCCCCCCCCCTTT TTT, designed to hybridize at the junction poly(A)-poly(G) introduced at the end of the poly(A) tail during the polyguanylation procedure of the viral RNAs, and containing a recognition site for the restriction enzyme *Eco*RI. The primer oligo$(dT)_{18}$V was degenerate at its 3′ end, to hybridize the last nucleotide of the 3′ non-coding regions and the beginning of the poly(A) tail. The PCR products were ligated into a pUC19 vector linearised with *Sma*I and tailed with dTTP as described by Marchuk *et al.* (1991), cloned and sequenced. Sequences were compiled and analysed using the DNASIS program package (Hitachi).

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Short Communication

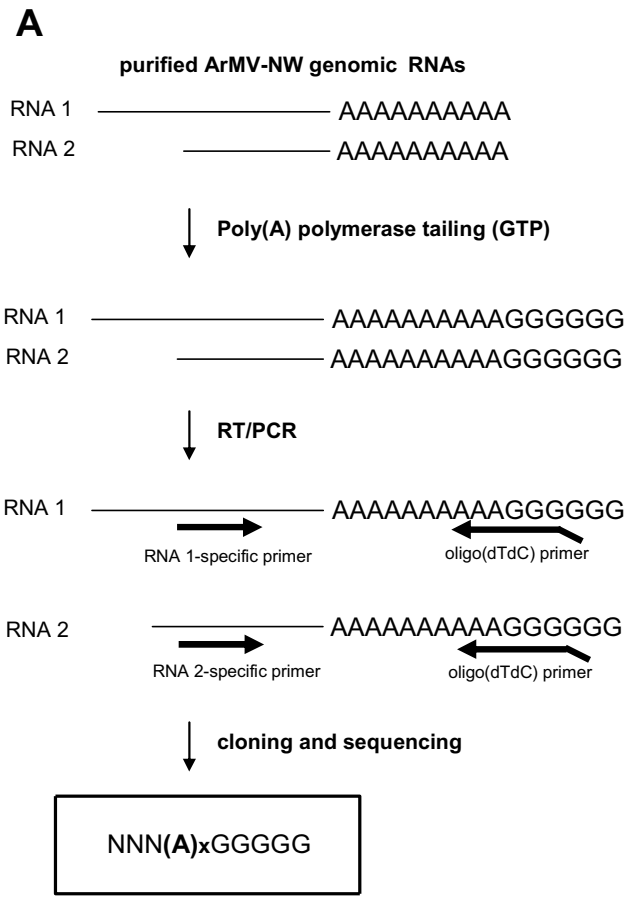
RESULTS AND DISCUSSION

The G-tailing RT-PCR method used in this study is based on the ability of the yeast poly(A) polymerase to catalyze the addition of uridine or guanosine residues at the 3′ end of poly-adenylated RNAs, although with reduced efficiency when compared to the addition of adenosine residues (Martin and Keller 1998). The ArMV-NW genomic RNAs, which have a poly(A) tail at their 3′ ends, were enzymatically tailed with guanosine residues prior to RT-PCR. This procedure, which to our knowledge has not been used for plant viruses, offered the advantage over previously described methods (Poon *et al*. 1998; Pritlove *et al*. 1998; Chen *et al*. 2005) to amplify the entire poly(A) tails, thus allowing a precise determination of the number of adenosine residues (Kusov *et al*. 2001). The primer combinations used for RT-PCR from purified polyguanylated viral RNAs were A1-7123s/oligo(dT)$_{18}$V and A1-7123s/oligo(dCdT), for ArMV-NW RNA 1, and A2-3561s/oligo(dT)$_{18}$V and A2-3561s/oligo(dCdT), for ArMV-NW RNA 2. RT-PCR products of the expected length, 225 bp and 277 bp, were obtained with the primers combinations A1-7123s/oligo(dT)$_{18}$V and A2-3561s/oligo(dT)$_{18}$V for RNAs 1 and 2 respectively (**Fig. 1**), which included a poly(A) tail of 18 adenosine residues introduced by the primer oligo(dT)$_{18}$V. The primers combinations A1-7123s/oligo(dCdT) and A2-3561s/oligo(dCdT) produced a more diffuse range of RT-PCR products (**Fig. 1**, lanes 1b and 2b), mainly of bigger sizes than those obtained with the previous primers combinations, for both RNAs 1 and 2. Identical results were obtained in two independant repeats for each of the ArMV-NW genomic RNAs, suggesting the presence of poly(As) of various sizes, some of them being very short, and some being larger than 100 adenosine residues (**Fig 1**).

The RT-PCR products generated with the primers combinations A1-7123s/oligo(dCdT) or A2-3561s/oligo(dCdT) were cloned and twenty clones sequenced. The sequences obtained were between 98% and 100% identical to the corresponding sequences on ArMV-NW RNA 1 or RNA 2, respectively. It is unknown if this variability reflected natural variability in the viral population or were due to mutations introduced during the RT-PCR procedure. The poly(A) tail lengths obtained for ArMV-NW RNA 1 ranged from 10 to 81 adenosine residues, and from 10 to 120 adenosine residues for ArMV-NW RNA 2 (**Table 1**), which reflects the heterogeneity in sizes observed for the RT-PCR products (**Fig. 1**). Most of the clones however contained poly(A) tails between 20 and 30 adenosine residues. It is unclear if this reflects the distribution of lengths of the poly(A) tails of the viral genomic RNAs *in planta*, or the fact that RT-PCR products containing short homopolymeric sequences are easier to clone than those with longer ones. The range of sizes observed for ArMV-NW is much wider than those observed for the viral RNA of *Hepatitis virus A* (HVA), which ranged between 41 to 60 adenosine residues (Kusov *et al*. 2001), but much narrower than those observed for *Influenza virus* (Poon *et al*. 1998; Pritlove *et al*. 1998) or *Bamboo mosaic virus* (Chen *et al*. 2005), with poly(A)s of lengths of 300 adenosine residues. It remains to be determined if ArMV-NW RNA molecules with very short poly(A) tails still have retained some infectivity, or show reduced or loss of infectivity, and which would be the minimal size of a poly(A) to guarantee an efficient viral replication process. It is also unknown if the ArMV-NW RNA 2 has longer poly(A)s than the ArMV-NW RNA 1, or if this result was mainly due to the cloning procedure.

Table 1 Length of the poly(A) tails of *Arabis mosaic virus* genomic RNAs. The number of adenosine residues in the poly(A) tails of the different clones generated by RT-PCR from polyguanylated viral genomic RNAs is indicated.

RNA 1:									
10	15	19	27	29	30	30	31	67	81
RNA 2:									
10	19	20	25	29	30	30	80	119	120



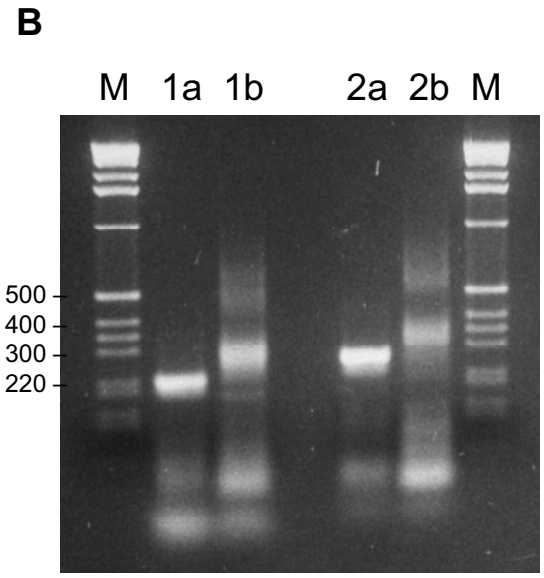


Fig. 1 RT/PCR from the polyguanylated genomic RNAs of the isolate NW of *Arabis mosaic virus*. (**A**) Experimental procedure. (A)x: number of adenosine residues of the poly(A) tail; G: guanosine residues introduced during the tailing procedure; N: viral nucleotides upstream of the poly(A) tail. (**B**) Agarose gel electrophoresis of the RT-PCR products obtained from polyguanylated ArMV-NW genomic RNAs, with primer combinations A1-7123s/oligo(dT)$_{18}$V and A1-7123s/oligo(dCdT) for ArMV-NW RNA 1 (lanes 1a and 1b respectively), and with primer combinations A2-3561s/oligo(dT)$_{18}$V and A2-3561s/oligo(dCdT) for ArMV-NW RNA 2 (lanes 2a and 2b respectively). M: 1kb DNA Ladder.

The construction of full-length infectious clones of ArMV would allow the insertion of poly(A) tails of various lengths in the viral genomic RNAs, and also different lengths between RNAs 1 and 2, along with additional muta-

tions in the 3′ non-coding region (Karetnikov *et al*. 2006). Their availability would greatly contribute to a better understanding of the viral replication process, and the function(s) of the poly(A) tails.

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