Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 388-395

THE cDNA OF CUCUMBER MOSAIC VIRUS-ASSOCIATED SATELLITE RNA HAS **IN VIVO** BIOLOGICAL PROPERTIES

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Received December 11, 1987

Two isolates of cucumber mosaic virus (CMV)-associated satellite RNA, differing in their biological properties, have been reverse transcribed. One was able to induce the tomato necrotic syndrome whereas the other one attenuated fernleaf symptoms on tomato plants after co-inoculation with the helper virus. cDNAs representing partial or full-length copies have been cloned in the plasmid pAT153 and sequenced. The two RNAs showed a very limited number of variations (2 to 5 substitutions depending on the clones and a one base deletion). Full-length cDNA copies possessed the same biological properties that characterized the parent satellite RNA. Efficiency of the cDNA depended upon its form in the inoculum (circular or linear plasmid or excised cDNA) and upon the form of the helper virus (viral RNAs or virions) with which it seemed to compete for installation and/or expression.

In addition to the four usual RNA species (designated 1-4 in order of decreasing length) some CMV strains contain a fifth component of low molecular weight (~335b). This RNA has almost no sequence homology with CMV (1-3) but depends upon CMV for replication and encapsidation and is therefore considered to be satellite RNA.

The presence of satellite RNA results in a great reduction in virus yield (4-7) and in most host plants results in an attenuation of the severity of CMV-induced symptoms (5, 7-9). For the tomato at least two groups of satellite RNA can be discriminated, according to their ability to provoke either an attenuation of the CMV-induced fernleaf symptoms or the development of lethal necrosis (10-12). Satellite RNAs belonging to both groups have been sequenced (13-17) and share a high degree of homology except for a japanese isolate which differs in its central region (18). From these data, it appears that no simple relationship can be established between the nucleotide sequence of satellite RNAs and their biological function. As a prelude to a more detailed study of this relationship, we have cloned and sequenced the cDNAs of 2 satellite RNA isolates. We report in this paper that some of these cDNAs were "infectious", i.e. they produced after co-inoculation with the helper genome an RNA capable of replication and possessing the biological properties of the native satellite RNA.

MATERIAL AND METHODS

1. Virus strains. CMV I17F strain lacks satellite RNA and induces severe fernleaf symptoms on tomato. CMV I17N strain contains a "necrotic" satellite RNA while CMV R has a "nonnecrotic" one. Purifications of

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virions, viral RNAs, satellite RNA and electrophoresis on 2,4% polyacrylamide gels were as previously described (10).

2. cDNA synthesis. Polyadenylation of satellite RNA was carried out in a 100 µl reaction volume containing 50 mM Tris-HCl (pH 7,9), 10 mM MgCl2, 250 mM NaCl, 2,5 mM MnCl2, 0,25 mM ATP, 40 units of RNasin, 45 pmoles of RNA, 1,5 units of **E. coli** poly(A) polymerase for 10 min at 37°C. Synthesis of first strand cDNA was performed by the method of Gubler and Hoffman (19). The second strand synthesis was carried out according to the method of Okayama and Berg (20) with only slight modifications. The 50 µl reaction mixture contained 20 mM Tris-HCl (pH 7,5), 5 mM MgCl2, 10 mM NH4-acetate, 100 mM KCl, 40 mM of each dNTP, all of the aforesaid RNA-DNA preparation, 9 units of RNase H/ml, 240 units of **E. coli** DNA polymerase I/ml and was incubated successively at 12°C and 22°C for 1 hr each. Polyadenylation, synthesis of the first and the second strands were stopped by addition of EDTA to 20 mM and nucleic acids were extracted with phenol, twice with phenol-chloroform and precipitated in 2 M NH4-acetate with ethanol.

3. Cloning. The vector pAT153 was cut to completion with EcoRV. DNA was phenol extracted and sedimented through a 5-25% sucrose gradient in order to ensure a very low amount of uncut plasmid DNA in the further recombinant DNA preparation (21). Tailing of the plasmid with dCTP was carried out in a 100 µl reaction volume containing 28 mM cacodylic acid - 120 mM Tris base adjusted to pH 7,6 with KOH, 5 mg/ml of BSA, 1 mM DTT, 1 mM CoCl2, 0,1 mM dCTP, 60 µg/ml of DNA, 60 units of terminal deoxynucleotidyl transferase/ml for 10 min at 22°C. The reaction was stopped by addition of EDTA to 5 mM. A mean of 20 nucleotides was added under these conditions. Tailing of the cDNA with dGTP occurred in a similar manner in the presence of 1 mM dGTP and 540 units of enzyme/ml for 1 hr at 37°C. Vector and cDNA were hybridized without further purification under the conditions of Gubler and Hoffman (19). Competent cells of **E. coli** strain RR1 were prepared and transformed by the procedure of Hanahan (22). Recombinant clones were selected for ampicillin resistance and tetracyclin sensitivity. Insertion of cDNA into the EcoRV site of pAT153 by G:C homopolymeric tailing created a new BamHI site at this position (19). Digestion of the recombinant plasmid with BamHI generated a fragment which contained the cDNA plus 190 bp from the plasmid DNA and which was at least 600 bp long if the cDNA were a complete copy of the RNA.

4. Sequencing. The sequence of cDNA deduced from the published RNA sequences shows a common and unique ScaI site at position 151. Both cDNA-containing fragments obtained by digestion of the recombinant plasmid with BamHI and ScaI were cloned in BamHI/EcoRV-cut RF of M13 tg131 (23) and sequenced by the Sanger dideoxy chain termination method (24).

5. Biological tests. 10 young tomato plants, *Lycopersicon esculentum* cv. Monalbo, (14-16 days old) were mechanically inoculated with a mixture containing either 40 µg/ml of purified viral RNAs (RNA-1-4) or 100 µg/ml of virions of the CMV 17F strain, and DNA. The nature of DNA (uncut or linearized plasmid, excised cDNA) and its concentration are specified for each experiment. The presence of a necrotic satellite RNA was revealed by the development of necrosis by the tomato 10 to 15 days after infection. That of a nonnecrotic one was based upon the capacity of this satellite RNA to protect the plant against a further infection by a necrosis-inducing isolate (25). The 10 plants were superinfected with a preparation of I17N total RNA (40 µg/ml) 2 weeks after the first inoculation ; non protected plants died with necrosis within the next 2 weeks. Results are expressed as percents of necrosed plants (%NP) or protected plants (%PP), respectively.

RESULTS

1. Sequences of satellite cDNAs. The sequences of I17N and R satellite cDNAs are shown in Fig.1 in comparison with the known sequence of the D isolate (13) as a reference. Depending on the clones, 5 or 6 substitutions were observed between the cDNAs of the 2 "necrotic" RNAs (I17N and D) (positions 218, 224, 229, 326, 327 and 314). 3 of these positions also underwent change in the "nonnecrotic" R RNA (positions 218, 229 and 327). Moreover, this RNA exhibited additionnal differences : 3 substitutions (positions 44, 126 or 225 for clones 2 and 1 respectively, 295) and a deletion of one base (position 326). Whereas the partial copy of this RNA had the same 7 last residues at the 3'-end as the cDNAs of cDNAs of I17N, the full-length one presented a modified 3'-end ; the ACCC end of the D reference sequence was absent and 3 T substituted to the bases 329 to 331 contiguous to the ACCC sequence. In order to deter-

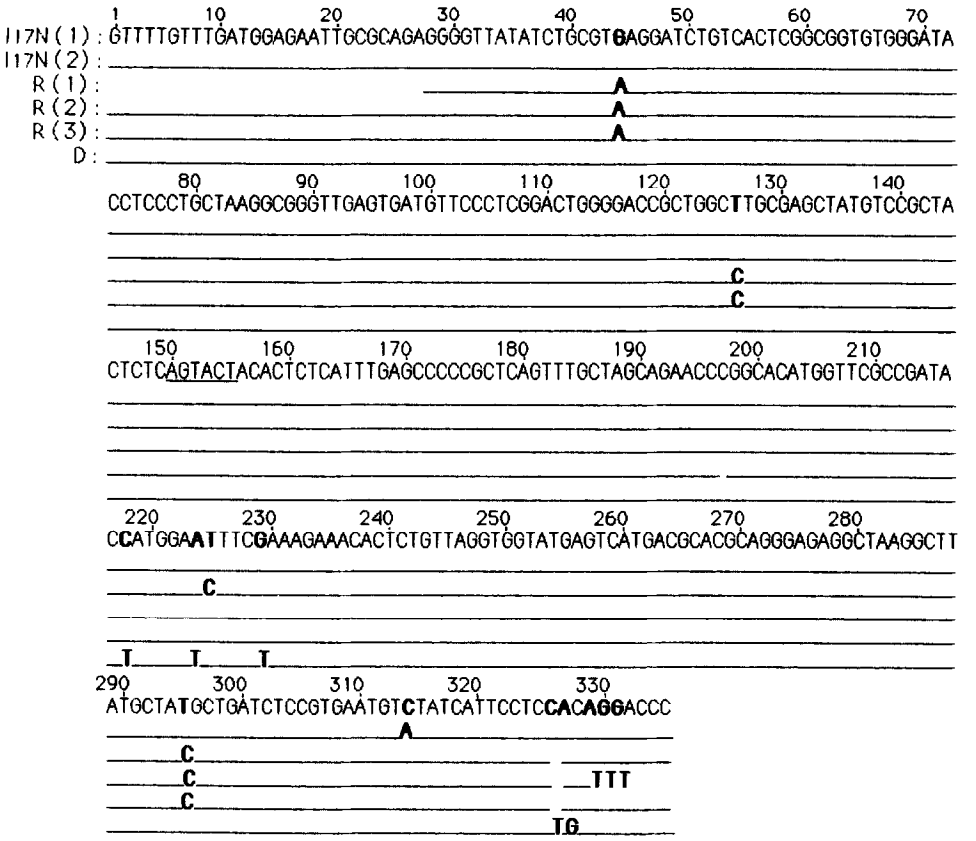


Fig. 1 . Comparison of the sequence of I17N, R and D satellite cDNAs.
4 full-length cDNA copies of I17N satellite RNA have been sequenced ; among them 3 were represented by I17N (1) sequence and one by I17N (2) sequence. R (1) and (2) corresponded to 2 different copies of R satellite RNA. R (1) was a partial copy stretching from residue 28 to the 3'-end. R (3) was a recombinant clone constructed from clones (1) and (2) digested by ScaI (the position of this site within the sequence is underlined) and carried the 3'-end of the cDNA of clone (1) and the 5'-end of the cDNA of clone (2). D sequence, deduced from the known sequence of the RNA (13) is used as reference. A horizontal line means no change while an interrupted line indicates a deletion. Base substitutions are indicated by the corresponding letters.

mine if these modifications represented a variation of the R satellite RNA or a wrong initiation of reverse transcription, a recombinant between the 2 cDNA copies has been constructed. The new clone (3) contained a cDNA having the 5'-end of the former full-length copy and the 3'-end of the partial one. Finally, this cDNA differed from I17N (1) cDNA by 3 substitutions (positions 44, 126 and 295) and the one base deletion (position 326).

2. Biological properties of satellite cDNAs. Satellite RNA has a very high biological activity. It is replicated under optimal conditions when present at a concentration of 4,5 pM in the inoculum and still provokes the development of necrosis (or attenuated fernleaf symptoms) by some of the infected plants when present at lower doses (7). For the validity of our biological tests, it was necessary to be sure that the modified symptoms on infected tomatoes did not result from a contamination of the preparations of viral RNAs or virions by a satellite RNA. In each experiment dealing with a cDNA of a "necrotic" RNA, 10 plants were inoculated with one of these preparations alone and 10 others with the same inoculum con-

Table 1 : Biological properties of the cDNA of I17N satellite RNA

plasmid DNA µg/ml	helper virus as : RNAs 40 µg/ml			virions 100 µg/ml		
	Nb.P[a]	%IP[a]	%NP[a]	Nb.P[a]	%IP[a]	%NP[a]
100	-	-	-	70	100	57,1
50	20	0	-	80	100	61,2
20	20	0	-	80	100	77,5
10	30	86,7	15,4	80	100	67,5
5	52	100	17,3	60	100	48,3

a : Nb.P : number of inoculated plants ; %IP : percent of infected plants ; %NP : percent of necrosis among the infected plants.

taining 5 to 20 µg/ml of a purified preparation of pAT153. None of the controls developed the symptoms characteristic of the presence of a satellite RNA of either type. For the study of cDNA copies of the "nonnecrotic" RNA, a third set of control plants was first infected with I17F strain and then superinfected with the necrosis-inducing strain. All the plants died from necrosis, confirming that no "nonnecrotic" satellite RNA contaminated the viral preparations and indicating that the superinfection was achieved.

The co-inoculation of the helper virus and a full-length cDNA copy of I17N satellite RNA could induce necrosis among the infected plants. The results of several tests are summarized in table 1. Data obtained with inocula containing either of the 2 full-length copies did not differ significantly and have been cumulated. One of the unusual features of DNA preparations was their capacity when at concentrations higher than 10 µg/ml to inhibit infection with viral RNAs. On the other hand, when cDNA was at lower concentrations, only a few plants developed the syndrome characteristic of the replication and the expression of necrotic RNA. This problem could be overcome by using purified virions instead of viral RNAs. Under these conditions both infection of all the plants was ensured whatever the quantity of DNA and the biological activity of the DNA was enhanced. Nevertheless, cDNA appeared 8,000 folds less active than satellite RNA itself if one considers that a similar percentage of necrotic plants occurred by inoculation of a solution containing satellite RNA at 0,45 pM (7) or 10 µg/ml of recombinant plasmid (approximately at 3,8 nM ds cDNA). The reduction of the proportions of necrosed plants when DNA was present at concentrations higher than 20 µg/ml constituted an unexpected result that remains to be explained. In any case, it did not seem possible to induce necrosis of all the plants in the conditions reported here.

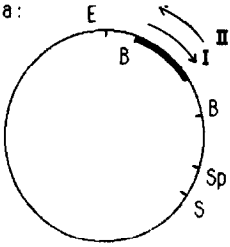
The orientation of the cDNA insert in the pAT153 vector did not influence its expression (table 2). Similar results have been obtained with cDNAs of STNV (Satellite Tobacco Necrosis Virus) (26) and of viroid (27, 28). Thus, no particular sequence of the plasmid vector seemed to be involved in the infection process of these cDNAs. When cDNA was inoculated as linearized recombinant plasmid, the cutting site was of importance. Results presented in table 2 show that the linearization had to occur near the 3'-end of the (+) strand of the cDNA to keep the cDNA active. As satellite RNA probably replicates using the RNA-RNA pathway, its cDNA must serve **in vivo** as template for an host DNA-dependent RNA polymerase. Moreover, our results suggest that the RNA transcript has to be a (+) stranded one. The cDNA-containing fragment generated by total digestion with BamHI proved to be less active that the uncut plasmid (table 2). Although efficiency of the former could be increased, by reducing the concentration of virions (50 µg/ml) or by co-inoculating it with viral RNAs, no more than 20% of the plants developed necrosis (data not shown).

Table 2 : Effect of orientation of the cDNA insert and of linearization of the recombinant plasmid on the biological properties of the cDNA of I17N satellite RNA.

cDNA as[a] :	insert orientation[a]			
	I		II	
	Nb.P[b]	%NP[b]	%NP[b]	%NP[b]
uncut plasmid 20 µg/ml	120	68,3	30	53,3
EcoRI-cut plasmid 20 µg/ml	100	7,0	30	56,7
SphI-cut plasmid 20 µg/ml	60	66,7	-[c]	-
SalI-cut plasmid 20 µg/ml	100	55,0	30	13,3
BamHI fragment 2 µg/ml	60	10,0	-	-

virus was provided as purified virions (100 µg/ml).

a :



E : EcoRI, B : BamHI, Sp : SphI, S : SalI
The arrow indicates the orientation of the DNA copy the transcription of which would yield a (+) RNA strand

b : Nb.P : number of inoculated plants, %NP : percent of necrosed plants
c : - : not tested

The tests of the biological activity of the R satellite cDNA clone 2 were all negative. No RNA possessing either of the two functions of satellite RNA could be recovered from the inoculated tomatoes. All of these plants developed severe fernleaf symptoms. This suggests that the modified 3'-end of this cDNA copy ocurred presumably by reverse transcriptase miscopying. On the other hand, inoculation of tomato plants with an inoculum containing cDNA clone 3 allowed the complete protection of some of them against a further infection by the necrosis-inducing strain. This possibility is characteristic of the presence of a "nonnecrotic" satellite RNA in the protected plant (25). As shown in table 3, this cDNA of R satellite RNA

Table 3 : Biological properties of the cDNA of R RNA : protection against a superinfection with CMV

plasmid DNA µg/ml	Nb.P[a]	%PP[b]
100	50	28
50	50	38
20	80	55
10	50	42
5	50	34

Virus was inoculated as purified virions (100 µg/ml). During each experiment, as a control of cDNA expression, 10 plants were infected with an inoculum containing 20 µg/ml of I17N cDNA : a mean of 53,3% of the plants developed necrosis.
a : Nb.P : number of inoculated plants.
b : %PP : percent of protected plants after superinfection with I17N strain.

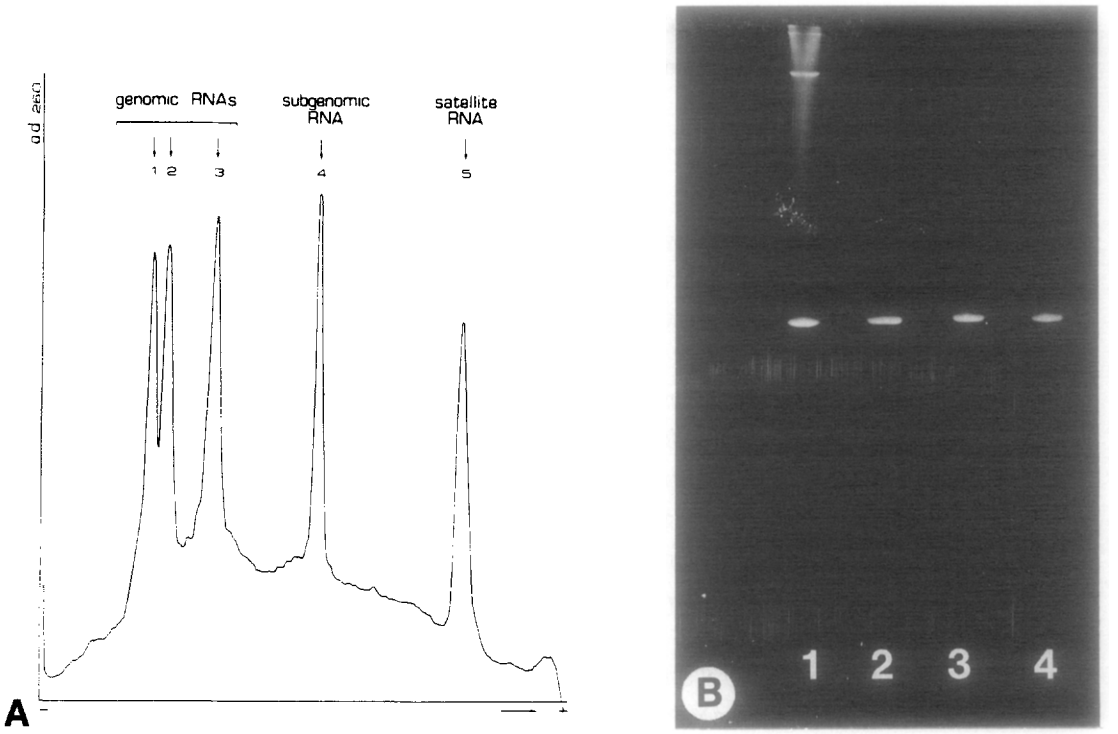


Fig. 2 . Analysis of produced RNA after infection with an I17N cDNA-containing inoculum
A. U.V. electrophoregram of the RNA extracted from the recovered nucleoproteins and analyzed in a 2,4% polyacrylamide gel.
B. Analysis of satellite RNA by oligo(dT)cellulose chromatography. The different RNA fractions were electrophoresed through a 5% polyacrylamide gel in 8 M urea. lane 1 : total viral RNAs ; lane 2 : sucrose gradient purified fifth component ; lane 3 : pass-through fraction of the oligo(dT)cellulose chromatography ; lane 4 : native satellite RNA.

presented a biological activity similar to that of the full-length cDNA copies of I17N satellite RNA. In particular, a maximum of efficiency was observed when the plasmid was provided at 20 µg/ml in the inoculum.

Nucleoproteins purified from tomato plants that had developed necrosis after infection with an I17N cDNA-containing inoculum contained a fifth component migrating like satellite RNA during electrophoresis through a 2,4% polyacrylamide gel and representing 18% of the encapsidated RNAs (figure 2A). This RNA was further purified by sucrose gradient centrifugation and assayed for its ability to bind to oligo(dT)cellulose ; as it was not retained on the oligo(dT)cellulose presumably it did not carry the poly(A) tail (18 residues long) present at the 3'-end of the (+) strand of the cDNA used in this case. Consequently the poly(G) tail located 3' to the poly(A) would also presumably be absent. Furthermore, when electrophoresed through a 5% polyacrylamide gel under denaturing conditions which would allow a difference of 20 to 45 bases (length of each tail) to be clearly observed, the RNA had the same electrophoretic mobility as native satellite RNA (figure 2B). Moreover, it induced the same typical necrotic syndrome on tomatoes as native satellite RNA (data not shown). Analysis of viral progeny had also been carried out from tomatoes inoculated with a R cDNA-containing inoculum. In a first experiment 10 plants were analyzed for the presence of satellite RNA within the nucleoproteins while 10 others were superinfected with the necrotic inoculum. A fifth component migrating like satellite RNA under denaturing conditions was observed in 8 preparations among 10 in the former case whereas 7 plants among 10 were pro-

tected against the challenge infection in the latter one. In an independent experiment it was observed that all the protected plants exhibited the presence of satellite-like RNA within extracted virions.

DISCUSSION

13 isolates of CMV satellite RNA have been sequenced to date (13-18, this paper). Except for the japanese Y strain, homologies among them extend from 84 to 99%. Detailed comparison of the observed changes does not make possible the correlation of structural and biological properties of these molecules (17). Moreover, it appears that the french isolates share greater homology to each other than to any of the satellite RNAs isolated in other countries, independently of their respective biological characteristics. The minimal differences between a "necrotic" RNA (117N) and a "nonnecrotic" one (R) were 2 substitutions (residues 44 and 295) and a one base deletion (residue 326). The additional substitutions observed between 2 cDNAs clones of the same RNA could reflect a weak variability within the RNA population but a miscopying by the reverse transcriptase cannot be ruled out as they appeared in only one of the clones. In the case of 117N satellite RNA, the unique variation between 2 full-length cDNAs located at position 314 did not modify the expression of the cDNA.

Plant infection with cloned viral cDNAs has been successful in only a few cases, i.e. viroids (29) and satellites (26, 30). COLLMER and KAPER (31) have reported the biological activity of RNA transcripts of CMV satellite RNA, but the corresponding cDNAs failed to induce the expected symptoms when coinoculated with the helper genome. The discrepancy between their results and ours can be explained by a recent observation of VAN EMMELO and al. (26). These authors have indeed shown that the presence of homopolymeric dG/dC tails longer than 5 bp at the ends of a cDNA of the Satellite Tobacco Necrosis Virus are essential for its infectivity. Whereas homopolymeric tailing has also been used by us (typical dG/dC tails length of about 20 bp), another strategy was used by COLLMER and KAPER for cloning the cDNAs in the plasmid vector.

The RNA progeny recovered from plants infected with a cDNA-containing inoculum seemed similar to native satellite RNA in that it had the same length - as judged by its electrophoretic mobility - and the same biological function. Since we have not sequenced the ends of this RNA nor looked for a cap at its 5'-end, we cannot definitively confirm its identity with satellite RNA. Nevertheless, **in vivo** processing of the RNA transcript is thought to occur in all the models so far studied and the production of an RNA progeny similar to native RNA has been clearly demonstrated as well for viruses of higher plants (31-33) as for viruses of animal cells (34).

Study of viral RNA genomes is now based on the use of recombinant DNA techniques. Work in our laboratories is taking advantage of the very limited number of changes between the sequences of our RNA isolates and of the feasibility of the direct bioassay of satellite cDNA reported in this paper to study the structure-function relationship of the CMV satellite RNA in the necrotic syndrome.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the "Centre National de la Recherche Scientifique" ATP 1387, from the "Fondation pour la Recherche Médicale", from the "Ministère de l'Education Nationale" and from the "Institut National de la Recherche Agronomique".

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