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Double-stranded RNA from rupestris stem pitting-affected grapevines

by

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Acides ribonucléiques bicaténaires obtenus de vignes atteintes de la maladie «rupestris stem pitting»

Résumé : Des acides nucléiques ont été obtenus à partir de cultures *in vitro* de 31 variétés de vigne atteintes de la maladie «rupestris stem pitting» (RSP), et de cultures de 11 variétés non-atteintes du RSP, 4 desquelles ne contenaient aucun virus, 2 desquelles étaient atteintes de fleck et 5 desquelles étaient atteintes de l'enroulement de la vigne. L'analyse électrophorétique sur gels de polyacrylamide a révélé que 21 des 31 variétés atteintes du RSP contenaient un acide nucléique, non-signalé jusqu'ici, qui était absent dans toutes les variétés non-atteintes du RSP. Au moyen d'incubations avec des nucléases, on a démontré que l'acide nucléique en question était un acide ribonucléique bicaténaire. Cet ARN était trop gros pour être un viroïde et trop petit pour être un clostérovirus. Puisque 10 des 31 variétés atteintes du RSP ne contenaient pas cet ARN, il semble qu'il existe peut-être plus d'une maladie semblable au RSP. L'arn décrit ici pourrait être associé à une telle maladie.

Key words: disease, rupestris stem pitting, virus, dsRNA, variety of vine, in vitro culture, analysis, test plant.

Introduction

The rupestris stem pitting disease (RSP) of grapevines was first identified about 10 years ago by GOHEEN and LUHN (1978) and PRUDENCIO (1985). RSP has been reported to cause a slow decline in vine vigor and total production in *Vitis vinifera* cultivars (TZENG 1984; PRUDENCIO 1985). RSP produces no obvious symptoms on the leaves and fruit of *V. vinifera* cultivars, but induces consistent strong pitting symptoms on the stem of *V. rupestris* St. George (du Lot) indicator vines (PRUDENCIO 1985). The grapevine corky bark disease (CB) also produces stem symptoms on this indicator, but it can be clearly distinguished from RSP if the indicator vine LN-33 (Couderc 1613 × Thompson Seedless) is also used. RSP induces no symptoms on LN-33, while CB produces red leaves, vine dwarfing and wood grooving.

RSP is graft-transmissible and is probably caused by a virus or virus-like agent, although the nature of the causal agent has not yet been determined. MOSSOP *et al.* (1985), working on the grapevine leafroll disease (GLR), reported a correlation between disease incidence and the presence of a high-molecular-weight double-stranded RNA (dsRNA). DODDS *et al.* (1984) have pointed out the potential value of dsRNA-based analysis for the diagnosis of virus diseases. In the investigation reported here, the dsRNA content of RSP-affected grapevine shoot tip cultures was analyzed and compared to that of RSP-free ones, in order to determine whether RSP-affected grapevine shoot tip cultures contained a dsRNA which might be associated with the disease.

Materials and methods

Grapevines and shoot tip culture

42 grapevine cultivars were used in this study (Table). 31 had been diagnosed as being affected with RSP and 11 were considered RSP-free, on the basis of symptom expression on inoculated *V. rupestris* St. George and LN-33 indicator vines. All 31 RSP-positives produced stem symptoms on St. George and none of them produced red leaves, vine dwarfing or wood grooving on LN-33. Of the 11 RSP-free controls, 4 were disease-free, 2 were fleck-affected and 5 were GLR-affected. None of these 11 cultivars produced stem symptoms on St. George.

In vitro shoot tip cultures were used as samples for RNA extraction in this investigation, as either bark or leaves from screenhouse-grown vines proved unsuitable in preliminary trials. Bark samples produced heavily smeared lanes and the results were difficult to interpret. Little or no dsRNA was obtained from leaf samples. Grapevine shoot tip cultures have proved useful in the past for virus detection and elimination (MONETTE 1988).

For the initiation of grapevine shoot tip cultures, 3-cm shoot tips were cut from plants grown in a shadehouse. Expanded leaves were removed and shoot tips were surface sterilized by vigorous stirring for 20 min in a 0.6 % solution of commercial sodium hypochlorite containing 0.1 % Tween-20. Shoot tips were rinsed 3 times in sterile distilled water, aseptically trimmed to 2—4 mm and placed on an initiation medium. For the initial establishment of shoot tips in culture, MURASHIGE and SKOOG (1962) medium (MS) was diluted to 3/4 strength and supplemented with (per l) 60 mg adenine sulfate, 128 mg $NaH_2PO_4 \cdot H_2O$, 0.023 mg indole-3-butyric acid (IBA), and 2 mg 6-benzylaminopurine (BAP). This medium was adjusted to pH 5.7, 0.7 % Difco Bacto agar was added and 15 ml was dispensed into 25 × 150 mm culture tubes capped with polypropylene closures (Bellco Kaputs). 6 weeks after initiation, the cultures were transferred to proliferation medium. For the proliferation of grapevine cultures MS medium was used at full strength and supplemented with (per l) 80 mg adenine sulfate, 170 mg $NaH_2PO_4 \cdot H_2O$, 0.03 mg IBA, and 2 mg BAP. This medium was adjusted to pH 5.0, 15 ml or 30 ml was dispensed into 125 or 500 ml Erlenmeyer flasks, respectively, and these were capped with aluminum foil. Both agar-gel and liquid media were autoclaved at 121 °C for 15 min at 1.4 at (142 kPa). Cultures were maintained at 23 ± 2 °C with a 16-h photoperiod under 48 (agar medium) or 30 (liquid medium) $\mu E\ m^{-2}s^{-1}$ (400—700 nm) provided by cool-white fluorescent lights. The liquid cultures were placed on a device that tilted the flasks in opposite directions, 30 ° from the vertical, every 30 s. Proliferating cultures were transferred into fresh medium every 2 weeks, first in 125 ml then in 500 ml flasks. When the mass of a shoot tip culture reached about 7 g (F.W.) the culture was blotted dry and stored at −80 °C.

Nucleic acid extraction

Extraction of dsRNA was based on a method described by MORRIS *et al.* (1983). Cultures were removed from the freezer and homogenized for about 1 min in a blender containing (per 7 g of culture) 14 ml 2 × TSE (2 × TSE = 0.2 M Tris-HCl, 0.4 M NaCl, 4 mM EDTA pH 7.0), 20 ml 90 % phenol, 16 mg bentonite and 2 ml 10 % sodium dodecyl sulfate. The homogenate was filtered through 4 layers of cheesecloth and centrifuged for 20 min at 10,000 *g*. The upper (aqueous) phase was carefully removed and anhydrous ethanol added to it to a concentration of 15 %. Whatman CF-11 phosphocellulose was then added (0.25 g/20 ml) and the mixture was stirred on ice for 60 min, after which it was centrifuged for 10 min at 6,000 *g*. The supernatant was discarded and the pellet was stirred for 2 min in 10 ml TSE/15 % ethanol. The phosphocellulose was pel-

PAGE analysis of RNA extracts from RSP-affected and RSP-free grapevines

Analyse électrophorétique sur gels de polyacrylamide des acides ribonucléiques obtenus de vignes atteintes ou libres de la maladie «rupestris stem pitting»

Cultivar	Disease diagnosed on woody indicators	Stem pitting-associated RNA	Cultivar	Disease diagnosed on woody indicators	Stem pitting-associated RNA
Ontario	RSP	M	Bacchus	RSP	–
Landot 244	RSP	M	Maréchal Foch	RSP	–
Muscat d'Alsace	RSP	M	Faber	RSP	–
Auxerrois clone 56	RSP	M	Schoenburger	RSP	–
Weisser Burgunder	RSP	M	Festival	RSP	–
Faberrebe	RSP	M	Oraniensteiner	RSP	–
Verdelet	RSP, corky bark	M	Reichensteiner	RSP, fleck	–
Florental	RSP	M	1616 E	RSP	–
Gamay Rouge de la Loire	RSP, fleck	M	GM 31857	RSP, fleck	–
Pirovano	RSP, fleck	M	GM 32258	RSP	–
Pinot gris	RSP, fleck	M	Vidal 256	–	–
St. George	RSP	M	White Riesling	–	–
125 AA	RSP, fleck	M	Pinot Chardonnay	–	–
GM 4-46	RSP, fleck	M	SO 4	–	–
Tajoznyt Izumrud	RSP	m	Limberger	fleck	–
Villard noir	RSP, fleck	m	GM 3-46	fleck	–
Maréchal Foch	RSP	m	Limberger	GLR	–
Pinot Chardonnay	RSP	m	Jakaranda	GLR	–
GM 31158	RSP	m	Jubilaeum 75	GLR	–
GM 31253	RSP	m	Golden City	GLR	–
3309 C	RSP	m	Kadarka	GLR	–

M = Major band; m = Minor band; – = Absent.

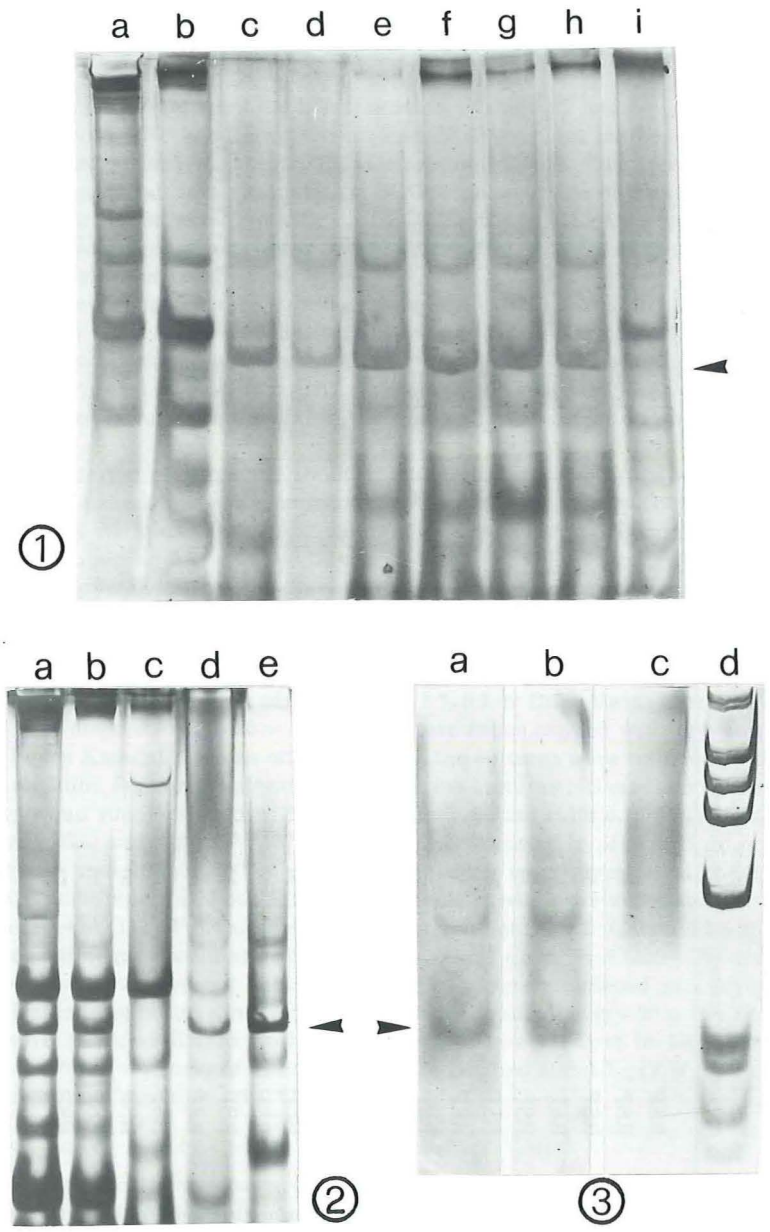

Fig. 1: Polyacrylamide gel electrophoresis of RNA extracted from virus-free (a) White Riesling and (b) Pinot Chardonnay, and RSP-affected (c) Weisser Burgunder, (d) Faberrebe, (e) Muscat d'Alsace, (f) Landot 244, (g) Auxerrois clone 56, (h) Florental and (i) Pinot Chardonnay. Electrophoresis and silver staining procedures are described in 'Materials and methods'. The arrow points to the stem pitting-associated RNA.

Fig. 2: Polyacrylamide gel electrophoresis of RNA extracted from RSP-affected (a) 3309 C, (b) Maréchal Foch, (c) 1616 E, (d) Weisser Burgunder and (e) Auxerrois clone 56. Electrophoresis and silver staining procedures are described in 'Materials and methods'. The arrow points to the stem pitting-associated RNA.

leted again by centrifugation for 10 min at 6,000 *g*. The supernatant was discarded and 2 ml of TSE was added to the pellet. It was resuspended by vortexing for 1 min and the suspension was centrifuged for 10 min at 6,000 *g*. The supernatant was saved and the pellet was washed in another 2 ml of TSE. The supernatants were pooled and 2.5 vol of ethanol added. Nucleic acids were allowed to precipitate overnight at −20 °C and collected by centrifugation for 15 min at 7,000 *g*. The pellet was dried and resuspended in 100 µl TAE (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM EDTA pH 7.8).

Polyacrylamide gel electrophoresis (PAGE) and silver staining

Nucleic acids were analyzed by electrophoresis on vertical polyacrylamide gel slabs (7.3 × 10.2 × 0.075 cm), consisting of a 3 % acrylamide (3.0 : 0.2 acrylamide : bisacrylamide) stacking gel and a 6 % acrylamide (36 :0.33) separating gel. Sample buffer consisting of TAE with 50 % glycerol and 0.05 % bromophenol blue, was added to an equal volume of nucleic acid extract in TAE and 10 µl of the mixture was applied per sample well. Electrophoresis was conducted with 3 W constant power for stacking and 6 W constant power for separation. Gels were soaked in fixative (40 % methanol/10 % acetic acid; v/v) overnight and then stained with a Bio-Rad silver staining kit, according to the manufacturers' instructions. A rough estimate of the molecular weight of the stem pitting-associated RNA was obtained using bacteriophage lambda and ØX-174 restriction enzyme digest (DRIgest III, Pharmacia) fragments as markers.

Nuclease digestion

Nucleic acid extract from RSP-affected Landot 244 was subjected to PAGE in 4 replicates. At the end of the run, the gel was sliced vertically and the replicates placed in 9-cm glass petri dishes with either 100 ml water (control), or 100 ml water containing 5 mg RNase A (low salt), or 100 ml water containing 0.3 M NaCl and 5 mg RNase A (high salt), or 100 ml water containing 30 mM $MgCl_2$ and 1 mg DNase 1. All glassware and instruments had been autoclaved and sterile distilled water was used throughout. The gel slices were incubated at 31 °C for 2 h and the nucleic acids were then visualized by silver staining.

Fig. 3: Polyacrylamide gel electrophoresis of RNA extracted from RSP-affected Landot 244. Lanes (a), (b) and (c) were exposed to DNase 1, RNase A in high salt and RNase A in low salt conditions, respectively, at 31 °C for 2 h prior to silver staining. Lane (d) contains size markers. The arrow points to the stem pitting-associated RNA.

Fig. 1: Electrophorèse d'ARN obtenus de 2 variétés de vigne ne contenant aucun virus, (a) White Riesling et (b) Pinot Chardonnay, et de 7 variétés atteintes de la maladie RSP, (c) Weisser Burgunder, (d) Faberrebe, (e) Muscat d'Alsace, (f) Landot 244, (g) Auxerrois clone 56, (h) Florental et (i) Pinot Chardonnay. Les méthodes utilisées pour l'électrophorèse et la coloration à l'argent sont décrites dans la section «Materials and methods». La flèche indique la location du «stem pitting-associated RNA».

Fig. 2: Electrophorèse d'ARN obtenus de 5 variétés de vigne atteintes de la maladie RSP, (a) 3309 C, (b) Maréchal Foch, (c) 1616 E, (d) Weisser Burgunder et (e) Auxerrois clone 56. Les méthodes utilisées pour l'électrophorèse et la coloration à l'argent sont décrites dans la section 'Materials and methods'. La flèche indique la location du «stem pitting-associated RNA».

Fig. 3: Electrophorèse d'ARN obtenus de 5 variété Landot 144, atteinte du RSP. Les gels (a), (b) et (c) ont été placés à 31 °C pour 2 h avec DNase 1, avec RNase A sous une forte concentration de sels et avec RNase A sous une faible concentration de sels, respectivement, avant d'être colorés à l'argent. Le gel (d) contient des fragments d'acide nucléique à poids definis. La flèche indique la location du »stem pitting-associated RNA«.

Results and discussion

Of the 31 grapevine cultivars diagnosed as RSP-affected, 21 (68 %) contained an RNA which was absent from the 11 RSP-free controls (Table). In 14 of the 21 cultivars, this RNA was present as a major component (Fig. 1, lanes c—h; Fig. 2, lanes d and e) and in the remaining 7, it appeared as a minor component (Fig. 1, lane i; Fig. 2, lanes a and b). The number and intensity of the bands which presumably represent host material varied considerably from one cultivar to the next. Surprisingly few bands were found in those extracts which contained the stem pitting-associated RNA as a major component. The different banding patterns, which were reproducible for each cultivar tested, may simply reflect cultivar differences. None of the RSP-free controls contained a band which migrated similarly to the stem pitting-associated RNA (Fig. 1, lanes a and b).

The RNA detected in this investigation was shown to be dsRNA, as it was not digestible by DNAse 1, but was digestible by RNAse A at low, but not high, salt conditions (Fig. 3). The dsRNA had an apparent size of 359 ± 12 base-pairs, or approximately 237,000 ± 8,000 Da (MW ± SE, n = 8).

All of the 31 grapevine cultivars diagnosed as having RSP disease produced pitting symptoms on St. George and no CB symptoms on LN-33. The pitting on St. George was observed on all parts of the stem and was not limited to the area below the inoculation bud (PRUDENCIO 1985). The observation that 10 of the 31 vines diagnosed as RSP-positive did not contain the dsRNA suggests that there may be more than one 'RSP-like' disease, distinct from CB. Thus, the dsRNA detected in this investigation is referred to as a 'stem pitting-associated RNA' (SP-A RNA) rather than as an 'RSP-associated RNA'. On the basis of its apparent size, the SP-A RNA is not a viroid. Although viroids contain extensive intramolecular double-stranded regions, they typically consist of only 300—400 nucleotides. The SP-A RNA is clearly not the replicative form (RF) of a closterovirus either, as these would have a much greater size, e.g. 4—13 · 10^6 Da (DODDS and BAR-JOSEPH 1983). dsRNAs of sizes comparable to the one reported here have been detected by these authors in plants infected with the closteroviruses carnation necrotic fleck virus, beet yellows virus and citrus tristeza virus. The absolute mobilities of these dsRNA segments were consistently similar for a single virus and their relative mobilities were different and diagnostic for each closterovirus. One interesting possibility is that the SP-A RNA reported here may represent a subgenomic component of a larger virus. Should this be the case, the absence of the SP-A RNA in 10 of the 31 RSP-affected samples might reflect virus strain differences (DODDS *et al.* 1987). Alternatively, the SP-A RNA may be the RF of an unidentified agent which causes stem pitting.

It was most interesting to observe that, of the 10 RSP-affected grapevines which did not contain the SP-A RNA, 6 contained RNA bands which migrated similarly to nucleic acids extracted from *in vitro* shoot tip cultures of GLR-affected vines (e.g. Fig. 2, lane c; manuscript in preparation). The implications of this observation could be important in view of the fact that closterovirus-like particles have been detected in both GLR-diseased and stem pitting-affected vines (MILNE *et al.* 1984; CORBETT and WIID 1985; MOSSOP *et al.* 1985; ROSCIGLIONE and GUGERLI 1986).

Since 1961, numerous reports have been published of diseased grapevines showing stem pitting symptoms. The diseases have variously been termed 'legno riccio' (GRANITI and CICCARONE 1961), 'stem pitting' (AGRIOS 1971), 'bark and wood pitting' (HEWITT and NEJA 1971), and 'stem grooving' (ENGELBRECHT and NEL 1971). The relationships between these diseases and RSP remain unclear for the moment. dsRNA extraction

from grapevine shoot tip cultures and analysis by PAGE might be useful for clarifying these relationships. The techniques also have the potential of reducing the indexing tests to a rapid laboratory procedure, but first the causative agent(s) of stem pitting disease(s) must be positively identified and the link between the stem pitting-associated dsRNA and the causal agent(s) must be firmly established.

Summary

Nucleic acids were extracted from *in vitro* shoot tip cultures of 31 grapevine cultivars affected with rupestris stem pitting (RSP) disease and from cultures of 11 RSP-free cultivars, 4 of which were disease-free, 2 of which were fleck-affected and 5 of which were grapevine leafroll disease-affected. Analysis of the extracts by polyacrylamide slab gel electrophoresis showed that 21 of the 31 RSP-affected cultivars contained a previously unreported nucleic acid which was absent from the RSP-free controls. Nuclease digestions showed that the nucleic acid was double-stranded RNA (dsRNA). The apparent size of the dsRNA was inconsistent with that expected for either a viroid or a closterovirus. The observation that 10 of the 31 RSP-affected cultivars lacked this dsRNA is consistent with the view that there may be more than one 'RSP-like' disease. The dsRNA detected in this investigation may be associated with one of these diseases.

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