

## Flower Colour Breaking in Two *Aubrieta* Cultivars Caused by Cucumber Mosaic *Cucumovirus* (CMV) Lacking Satellite RNA-5

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Cucumber mosaic *cucumovirus* (CMV), isolate Aub (CMV-Aub), was the only virus found in plants of *Aubrieta* cvs Blue Emperor and Gloriosa showing symptom of flower colour breaking. CMV-characteristic electrophoretic banding pattern of replicative RNA (dsRNA), lacking satellite RNA-5 (CARNA5), was easily recognized, especially when the RNA was purified from experimentally infected *Nicotiana glutinosa* L. and *N. megalosiphon* Heurck et Muell. plants, but also from original hosts. The pattern was absent in symptom-free *Aubrieta* plants. Virus identification was supported, in addition, by test plant and serological reactions. CMV-Aub is a rather unstable isolate; its particles were detected in purified preparations before and after density gradient centrifugation, however, only in fixed preparations (1% formaldehyde). The virus induced mild leaf symptoms in *Cucumis sativus* L. cv. Delicatess. No difference was found between virus isolates from the two *Aubrieta* cultivars. There were also indications of virus presence in *Aubrieta* cvs Gartenstadt Purpur and Vesuv.

Keywords: *Aubrieta* cultivars, cucumber mosaic *cucumovirus*, lack of RNA-5.

Worldwide, cucumber mosaic *cucumovirus* (CMV) has been found spontaneously to infect several hundred wild and cultivated species belonging to more than 60 plant families (Horváth, 1979, 1980, 1993b; Klinkowski, 1977a, b; Kaper and Waterworth, 1981). The virus has an extremely broad host range, infecting 85 distinct plant families, and up to 1000 species experimentally (Van Regenmortel et al., 2000). Many isolates were demonstrated regarding some specific host range and symptom differences. Two serological groups of strains are known, as well as differences among numerous virus isolates with regard to virus satellite RNA population composition, particularly with respect to the presence of RNA-5 (Francki et al., 1979, 1987; Kaper and Waterworth, 1981; Bos, 1999). Some isolates of CMV on one hand and of tomato aspermy (TAV) and peanut stunt *cucumovirus* (PSV) on the other are somewhat similar in antigenic (in general, at a low degree) and some symptom properties (Kaper and Waterworth, 1981; Brunt et al., 1996; Van Regenmortel et al., 2000).

CMV provokes flower colour-breaking symptom in a series of plants, including *Chrysanthemum*, *Matthiola*, *Petunia*, *Primula*, *Vinca*, *Zinnia*, *Viola*, *Gladiolus*, *Ranunculus* and *Campanula* species, possibly a *Crocus* species (Smith, 1972; Klinkowski, 1977b; Valverde, 1984; Horváth, 1993a, b; Loebenstein et al., 1995; Bos, 1999) and some other species.

In Croatia CMV infections have been recorded in many wild, vegetable and ornamental plants (Šarić and Štefanac, 1988; Juretić, 1994). Sometime we isolated CMV in this region from two *Aubrieta* cultivars that exhibited flower colour breaking. Those cultivars and the cultivars Gartenstadt Purpur and Vesuv (see Material and Methods and Discussion) probably belonged to the species *Aubrieta deltoidea* (L.) DC., however, hybrid plants, e.g. *A. hybrida* Haussknecht (a hybrid between *A. deltoidea* and *A. gracilis* Spruner et Boiss.), or another hybrid, between *A. deltoidea* and *A. erubescens* Griseb., also could be taken into consideration here (Beckett, 1984; Cullen et al., 1995; Tutin et al., 1996). In addition to experiments with test plants and serological experiments within the virus identification procedure, the present paper deals with the analysis of virus RNA profile and stability properties of virus particles.

*Aubrieta* species (*aubrieta*, purple rock-cress), family Brassicaceae (formerly: *Aubrieta*, family Cruciferae) are evergreen trailing or mat-forming perennials native to the mountainous region from Italy to Iran; as horticultural plants they are grown much more widely (Beckett, 1984; Cullen et al., 1995; Tutin et al., 1996). Several viruses have been found to infect about 12 of the species spontaneously, CMV and tomato black ring *nepovirus* (TBRV) being the most common (Klinkowski, 1977a, b, c).

## Materials and Methods

Within a few comparatively small populations of *Aubrieta* cvs Blue Emperor and Gloriosa plants cultivated in the open some of them potted in the Botanical Garden of the Faculty of Science, University of Zagreb – the seeds having been obtained from the Botanical Garden of the University of Vienna in early 1990s – a small percentage (exceptionally up to 15%) of both cultivars displayed flower colour breaking (observed during April and May and sometimes also during the end of March). A few areas of more or less sharply separated shades of violet or purple, lighter and darker than healthy flower colour – those darker often extending along the petal main veins – were present on some or all of the petals of individual flowers (Fig. 1). Slight petal malformation was present sporadically. Sometimes areas of mild chlorosis and also weak malformation and stunting were present on middle-aged and younger leaves. From such specimens inocula were prepared separately from both cultivars for mechanical inoculation of the test plants *Chenopodium quinoa* Willd., *Nicotiana glutinosa* and *N. megalosiphon*. Leaves of *Aubrieta* plants were triturated without or with the addition of several petals, either in 50 mM pH 7.5 Sørensen's phosphate buffer containing 1 mM NaEDTA or 0.1% thioglycolic acid (TGA), or in tap water alone, at a ratio of ca. 1:15 (w/v). Such a dilution of the inocula was necessary because of a high content of mucilaginous substances in the leaves. Virus isolates were maintained in a glasshouse in the mentioned *Nicotiana* spp., in daylight and at temperatures between 20 and 35 °C. When preparing inocula from leaves of the latter plants using either the buffer or more often water, a ratio of 1:1–2 (w/v) was employed.

Analysis of virus replicative RNA followed the procedure of Valverde et al. (1990) including modifications introduced by Krajačić and Lorković (1992). Specimens



Fig. 1. Colour-breaking symptom in a flower of an *Aubrieta* 'Blue Emperor' plant spontaneously infected with isolate CMV-Aub of cucumber mosaic *cucumovirus* (CMV)

of the two spontaneously infected *Aubrieta* cultivars as well as those of artificially infected *N. glutinosa*, *N. megalosiphon* and *Cucumis sativus* cv. Delicatess were separately processed. Five-gram portions of frozen leaf tissue were ground in 8 ml of STE extraction buffer (0.1 M NaCl, 50 mM tris, 1 mM NaEDTA), pH 6.8. After low speed centrifugation of the homogenate the supernatant was stirred vigorously for 30 min with 2 ml of 2-mercaptoethanol, 1 ml of 10% SDS, 3 ml of neutralized water-saturated phenol and 2 ml of chloroform. Following centrifugation of the emulsion at 4,000g for 15 min the water phase was collected and adjusted to 16% of ethanol (EtOH) content.

From total nucleic acid extracts dsRNA was further purified by chromatography. The samples were poured onto columns of 16% EtOH-equilibrated cellulose CF-11 (0.5 g of dry weight per column). After washing with 40 ml of STE + 16% EtOH dsRNA was released and collected with 6 ml of EtOH-free STE. Precipitation of purified dsRNA was carried out by adding 0.5 ml of 3 M sodium acetate pH 5.5 and 20 ml of 95% EtOH to each sample and storing overnight at  $-20^{\circ}\text{C}$ . dsRNA was pelleted by low speed centrifugation and resuspended in 20  $\mu\text{l}$  of electrophoretic sample buffer (10 mM tris, 1 mM NaEDTA, 0.25% SDS, 0.03% bromphenol blue, 0.03% orange-G, 15% RNase-free sucrose, pH 7.5).

Electrophoresis was running at a constant potential of 100 V for an hour in a horizontal mini slab-gel of 1.2% agarose in TBE buffer (90 mM tris, 90 mM boric acid, 1

mM NaEDTA; pH 8.3). Gel was stained for 10 min in 50 ng/ml of ethidium bromide and visualized on a UV transilluminator at 300 nm wavelength. The reference isolate in these and other experiments was a CMV-Cr isolate from tomato plants with lethal necrosis syndrome (Škoric et al., 1996).

Infected *N. glutinosa* plants were the source for purification of both *Aubrieta* virus isolates. Systemically infected leaves (10 g) were homogenized in an equal volume of 67 mM, pH 7.3 Sörensen's phosphate buffer containing 1 mM NaEDTA and 0.1% TGA. The homogenate was emulsified for 10 min with 5 ml of chloroform, the mixture centrifuged at 4,000g for 10 min and then the supernatant for 90 min at 90,000g. The pellets were resuspended in a small volume of 20 mM phosphate buffer pH 7 and the suspension was centrifuged at a low speed. A portion of the supernatant fluid, i.e. partially purified virus suspension, was fixed by adding to it dropwise an equal volume of 2% formaldehyde.

For density gradient centrifugation, continuous RNase-free 10–40% sucrose gradient was prepared in the virus-resuspending buffer. Running took four hours at 24,000 rpm in a 'Beckman' SW 25.1 rotor. Gradients were analysed at 254 nm wavelength in an ISCO, model 640, density gradient fractionator and UA-5 absorbance monitor.

Preparations for electron microscopy were stained in 1% phosphotungstate at pH slightly above 7.0 and examined in an Opton I electron microscope.

Serological double-radial immunodiffusion experiments were conducted in 1% agarose gel in distilled water. Phosphate buffer, 20 mM pH 7.3 containing 0.1% TGA, was utilized in preparing plant (*N. megalosiphon*) sap of infected or healthy leaves. Test samples examined included: low speed centrifuged leaf sap of plants systemically infected separately with virus isolates from both *Aubrieta* cultivars and the carnation isolate of CMV (CMV-Cr) as reference isolate, of healthy plants and also unfixed and fixed partially purified *Aubrieta* isolates. Rabbit polyclonal antiserum (titre 1/64) to a CMV-Cr prepared earlier by Dr. E. Luisoni (Istituto di Fitovirologia Applicata, Torino) was used; dilutions of it were prepared in the 67 mM, pH 7.3 buffer without any ingredient.

## Results

### *Test plant reaction*

The infectivities of inocula prepared from Blue Emperor and Gloriosa cultivars in both phosphate buffer and in water were comparable and symptoms in test plants were indistinguishable.

In *C. amaranticolor* Coste et Reyn. and *C. quinoa* symptoms developed only in inoculated leaves: chlorotic lesions with a minute necrotic centre and chlorotic, ochre or necrotic lesions, respectively. Lesions in inoculated leaves, necrotic in *N. glutinosa* and chlorotic, often with a necrotic margin, in *N. megalosiphon*, were accompanied by systemic mosaic and leaf malformation. Sporadic systemic more or less diffuse chlorotic rings developed in *Datura stramonium* L. In *C. sativus* chlorotic lesions arose in inoculated leaves as did vein clearing, vein banding and mosaic of mild to moderate intensity in younger ones.

*dsRNA analysis*

Electrophoretic profiles of dsRNA preparations from naturally infected 'Blue Emperor' and 'Gloriosa' plants contained several nucleic acid bands (Fig. 2). A characteristic banding pattern of the CMV-dsRNA was relatively easily recognized by comparing four more prominent bands to those of CMV-Cr isolate. Some additional dsRNA bands, probably of host plant origin, were situated mainly between virus dsRNA-3 and dsRNA-4 bands. *Aubrieta* plants with flowers of normal appearance yielded no virus electrophoretic bands. As expected, transfer of the isolates to *N. glutinosa* and *N. megalosiphon* significantly intensified CMV-dsRNA bands; contrarily, the intensity of the additional bands mentioned was considerably reduced (Fig. 2). In preparations of both *Aubrieta* and *Nicotiana* plants no trace of satellite dsRNA-5 was detected, in contrast to a very prominent band in the CMV-Cr isolate.

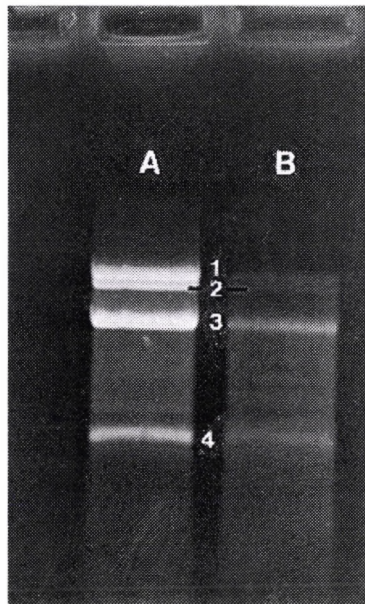


Fig. 2. Agarose gel electrophoresis of dsRNAs from plants infected with isolate CMV-Aub: lane A = experimentally infected *Nicotiana megalosiphon*; lane B = naturally infected *Aubrieta* cv. Blue Emperor; 1, 2, 3, 4 = virus dsRNA-1, dsRNA-2, dsRNA-3, dsRNA-4; Magnification:  $\times 1.6$

Some differences concerning the intensity ratio among particular virus dsRNA bands from different hosts were observed. Both naturally infected *aubrietas* and experimentally infected *Nicotiana* spp. revealed dsRNA-3 band intensity as the highest. In *aubrietas* the intensity of dsRNA-1 and dsRNA-2 was exceeded by that of dsRNA-4, whereas the opposite was true in *Nicotiana* plants (Fig. 2). It was not possible to recover the characteristic CMV-dsRNAs from younger leaves of *C. sativus* that nevertheless showed clear symptoms of infection.

### *Properties of virus particles*

Virus particles could be detected by electron microscope only in fixed partially purified preparations. It was similar with the detection of virus sedimenting zones in density gradients. In addition to the upper zone located just below the gradient meniscus, only the mentioned preparations revealed a lower (virus particle) zone which, however, seemed to be heterogeneous, as followed from the shape of UV absorption peak.

### *Serological experiments*

Weak and somewhat diffuse precipitation lines were obtained with both clarified and purified (in the latter ones only those formaldehyde-fixed) *Aubrieta* isolates. They were slightly more intense with purified preparations, but still weaker than those with the CMV-Cr isolate. Low intensity precipitation of normal plant proteins was obtained with clarified healthy and infectious sap, and undiluted or 1/2 diluted antiserum.

## Discussion

Several *Aubrieta* species, among them *A. deltoidea* and *A. deltoidea* var. *graeca* (Griseb.) Regel, infected with CMV and possibly all of them found as showing the symptom of breaking of flower colour have been recorded (e.g. Blatný and Schmelzer, 1976; Fletcher, 1987; reviewed by Klinkowski, 1977a). In brassicaceous plants such virus-induced symptom is known to be caused mainly by turnip mosaic *potyvirus* (Tomlinson, 1970; Klinkowski, 1977b; Cooper, 1993; Loebenstein et al., 1995). At least 30–40 other viruses are known to induce in various plants the symptom (flower colour-break, flower-breaking or breaking of petal colour), i.e. mosaic or variegation of flower petals (Bos, 1970, 1999; Smith, 1972; Horváth et al., 1975; Klinkowski, 1977a, b, c; Cooper, 1993; Horváth, 1993a; Loebenstein et al., 1995; Chen et al. 2000, etc.).

After virus replicative RNA analysis placed our two isolates from 'Blue Emperor' and 'Gloriosa' plants in the *Cucumovirus* genus, serological reactions together with test plant response (cf. Gibbs and Harrison, 1970; Kaper and Waterworth, 1981) – especially *C. sativus*, *D. stramonium*, *Nicotiana* spp. – confirmed their CMV identity. No significant difference was found between our two isolates, so for both we will use a common designation: isolate CMV-Aub (simply: Aub). Recently, we detected flower colour breaking in several specimens of *Aubrieta* cvs Gartenstadt Purpur and Vesuv growing within a comparatively bigger and a small, respectively, population in the mentioned Botanical Garden in Zagreb. The seeds of the plants were obtained as described in Materials and Methods ('Gartenstadt Purpur'), and that of 'Vesuv' originated from a Vienna park, Wien-Belvedere (in 1996). Preliminary investigations indicated that a symptom, of similar appearance to that in cvs Blue Emperor and Gloriosa, was also caused by CMV without associated RNA-5. We do not know whether the virus (CMV) was contained in the obtained *Aubrieta* seeds, or the plants were infected in Zagreb with some CMV local isolate.

As in our previous work (Krajačić and Juretić, 1993; Škorić et al., 1996; Hrženjak et al., 1999), the present results proved virus replicative RNA analysis to be a powerful

tool for detection and starting identification of CMV. A lower dsRNA concentration, as expected, in naturally infected *Aubrieta* plants was sufficient for virus detection dealing with an increased but still practical amount of tissue. The production of virus dsRNA reached in case of CMV-infected *Nicotiana* plants an extremely high level. As could be estimated from the electrophorogram, a starting amount of 1 g or even less of infected tissue would yield a recognizable dsRNA pattern.

Differences in the intensity ratio of virus dsRNA bands from *Aubrieta* and *Nicotiana* plants revealed what was probably a different strategy in virus genome expression in different hosts. The relative proportions of the encapsidated RNA components have been found to vary considerably with virus strain, host plant and several other conditions (Kaper and Waterworth, 1981). How these factors influence the RNA-component ratio in CMV particles is not completely understood. The amount of replicated nucleic acid species available – represented by quantity of double-stranded form – may be the only or the main factor governing the amount of encapsidated RNA.

According to Kaper and Waterworth (1981), 'the procedures used to purify various strains of *cucumoviruses* are nearly as varied as the strains involved', factors that affect CMV yield having been reviewed by Yarwood (1971). In our attempt we combined some steps and conditions from protocols which proved satisfactory for most CMV strains (Francki et al., 1979). The absence of a virus sedimenting zone in unfixed preparations after density gradient centrifugation suggested that we were dealing with a rather unstable isolate. The heterogeneous virus zone obtained with fixed preparations could be due to particle damage and aggregation. This and the abundant material included in surface zone close to meniscus, which originated probably not only from the host protein but also from constituents of disrupted virus particles, speaks in favour of the isolate Aub particle instability. The low intensity and especially the appearance of isolate Aub serological reactions could be taken, at least partly, as a result and also indicator of that instability.

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