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PASSAGE OF A MARINE BROWN ALGAL DNA VIRUS FROM *ECTOCARPUS FASCICULATUS* (ECTOCARPALES, PHAEOPHYCEAE) TO *MYRIOTRICHIA CLAVAEFORMIS* (DICTYOSIPHONALES, PHAEOPHYCEAE): INFECTION SYMPTOMS AND RECOVERY[1]

Ingo Maier[2], *Elke Rometsch, Susanne Wolf, Markus Kapp, Dieter G. Müller*

Fakultät für Biologie, Universität Konstanz, D-78457 Konstanz, Germany

and

Hiroshi Kawai

Department of Biology, Kobe University, Kobe, 657 Japan

ABSTRACT

A dsDNA virus (EfasV-1) isolated from Ectocarpus fasciculatus *Harvey infected* Myriotrichia clavaeformis *Harvey, a species belonging to a different brown algal order. The virus did not complete its infection cycle in the foreign host but caused infertility due to malformed reproductive structures. After some time in culture, the host's reproductive capacity was sometimes restored with concomitant loss of at least part of the viral genome. This incidence of interordinal virus transfer is discussed in relation to possibilities for virus-mediated horizontal gene transfer in brown algae.*

Key index words: DAPI; DNA virus; Ectocarpus; *electron microscopy; host specificity; infection experiments; intergeneric virus transfer;* Myriotrichia; *PCR; reconstitution*

Marine brown algae belonging to the order Ectocarpales are frequently infected by viruses with relatively large genomes composed of double-stranded DNA (Müller 1996, Müller et al. 1996c). Typically, the vegetative cells of an infected thallus appear morphologically normal, even though the virus genome is latently present in all cells (Kuhlenkamp and Müller 1994). The functions required for mass replication of the viruses are expressed during initiation of host reproduction, and consequently, pathological symptoms become visible only in cells homologous to reproductive structures (i.e. deformed sporangia or gametangia). The viral DNA associates intimately with the host's nuclear genome and passes through mitosis and meiosis as a host gene (Müller 1991a, Bräutigam et al. 1995).

Several virus–host systems have been described and are kept in culture, including EsV-1 infecting *Ectocarpus siliculosus* (Dillwyn) Lyngbye, *E. fasciculatus* virus type 1 (EfasV-1), and *Myriotrichia clavaeformis* virus type 1 (MclaV-1) (Müller et al. 1990, Parodi and Müller 1994, Müller et al. 1996c). Culture experiments suggest a high degree of host specificity, but a few cases of interspecific and intergeneric cross-infection have been discovered (see Discussion). Polymerase chain reaction (PCR) amplification of an EsV-1 gene segment encoding a fragment of structural glycoprotein (gp-1; Klein et al. 1995) can be used as a diagnostic tool for the detection of viral DNA in total nucleic acid extracts from host thalli. Primers specific to gp-1 detect infections by both EsV-1 and EfasV-1 (Bräutigam et al. 1995, Sengco et al. 1996), which are very similar in size and capsid structure (Müller et al. 1996b). Using the same primers, no amplification products could be detected with DNA of MclaV-1 (unpubl.).

We report here on infection experiments and the application of the PCR diagnostic method. These show that EfasV-1 can be transmitted from *Ectocarpus fasciculatus* to *Myriotrichia clavaeformis*.

MATERIALS AND METHODS

Cultures. A viral-infected clonal culture of *E. fasciculatus* isolated from Britanny, France (Parodi and Müller 1994), served as a source for EfasV-1. Species designation was substantiated by the presence of the betaine lipid diacetylglycerylhydroxymethyltrimethyl-β-alanine as a chemotaxonomic marker (Müller and Eichenberger 1995, Eichenberger, pers. comm.). *Myriotrichia clavaeformis* infected by MclaV-1 originated from Las Grutas, Argentina (Müller et al. 1996c). A healthy (i.e. virus-free) sporophyte of *M. clavaeformis* isolated from the same locality was the recipient strain for EfasV-1. Suspensions of EsV-1 were obtained from *E. siliculosus* strain NZ-Vic-Z14 (Lanka et al. 1993). All viral-infected strains and *Myriotrichia* from infection experiments were cultivated at 12° C, whereas the healthy sporophyte of *Myriotrichia* was kept at 18° C under white fluorescent light at an irradiance of ~20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a 14 h light period, respectively. The culture medium was based on a commercial salt mixture (hw Meersalz professional, Wiegandt GmbH, Krefeld, Germany), autoclaved and supplemented with Provasoli's ES enrichment (Starr and Zeikus 1993). The release of viruses from *E. fasciculatus* was induced in the same manner as described by Müller (1991b) for *E. siliculosus*. Infection experiments were carried out by coincubation of freshly released Efas-viruses and swimming spores from plurilocular sporangia of *Myriotrichia* in about 400 μL of culture medium on a polystyrene petri dish for 1 h at 18° C, followed by flooding with culture medium. Two independent infection experiments, each with several replicates, were carried out. The developing progeny were grown to maturity and after 2–3 weeks examined for morphological abnormalities under 63× magnification. Sterile thalli with lateral vesicular appendages were tentatively classified as infected. Of these, 26 were used to establish clonal subisolates and investigated further. They were propagated by fragmentation.

Light microscopy. For fluorescence DNA staining, the algae were fixed with 2.5% glutaraldehyde in 70% culture medium for 30

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[2] Author for reprint requests; e-mail Ingo.Maier@uni-konstanz.de.

min. Afterwards, they were washed twice with distilled water, stained with 4',6-diamidino-2-phenylindole (DAPI, 2 $\mu g \cdot mL^{-1}$ in distilled water) for 30 min, and examined immediately. Observations were made on a Zeiss epifluorescence microscope with UV excitation at 365 nm equipped with a Zeiss 100× Neofluar 1.30 PH objective. A KP 500 filter (Zeiss, Germany) was used to filter out red chlorophyll fluorescence. Light micrographs were taken on 25 ASA Agfa Ortho (brightfield), 400 ASA Ilford HP5 Plus (fluorescence), or 50 ASA Kodak Technical Pan film (phase contrast).

Electron microscopy. The algal material was fixed with 2.5% glutaraldehyde in fixation buffer (70% culture medium, 50 mM sodium cacodylate, pH 7.5) containing 0.2% caffeine for 2 h at room temperature. After washing with buffer (3 times, 10 min each) postfixation was carried out with 1% osmium tetroxide in buffer for 2 h at room temperature. This was followed by washing with buffer, 50% buffer, and distilled water (10 min each), *en bloc* staining with 1% uranyl acetate in distilled water for 1 h, and another washing step with distilled water (3 times, 10 min each). For dehydration, the samples were carried through an acetone series in 10% steps (15 min each at room temperature) to 70%. At this step, they were kept in a refrigerator overnight. Dehydration was continued the following day to 100% acetone and was completed by three changes of dried 100% acetone (30 min each). The material was infiltrated with Spurr's resin (Spurr 1969) by dropwise addition of resin to 25% within 2 h, another 3 h at 25%, 16 h at 50%, 4 h at 75%, and a total of about 24 h at 100% with three changes. The whole infiltration process was carried out on a slowly rotating mixing device. Thallus fragments were flat-embedded between two sheets of Aclar embedding film (Plano, Marburg, Germany) sandwiched between glass slides, after polymerization examined under a light microscope, excised, and mounted onto prepolymerized resin blocks. They were sectioned parallel to the longitudinal axis of the thallus on a Reichert Om U3 ultramicrotome using a diamond knife. Sections were stained with uranyl acetate (10 min) and lead citrate (5 min). The preparations were examined with a Zeiss EM 900 electron microscope and photographed on Agfa Scientia plates.

PCR analyses. Viral DNA was detected by amplification of a genome sequence of EfasV-1, which is homologous to a 692-bp fragment of a gene encoding glycoprotein gp-1 of EsV-1 (Klein et al. 1995). Total DNA extraction and PCR protocols, including those for control experiments, followed Bräutigam et al. (1995) and Sengco et al. (1996). PCR analyses of clonal isolates recovered from infection symptoms and photographic documentation of their habit (Fig. 6) were made simultaneously.

RESULTS

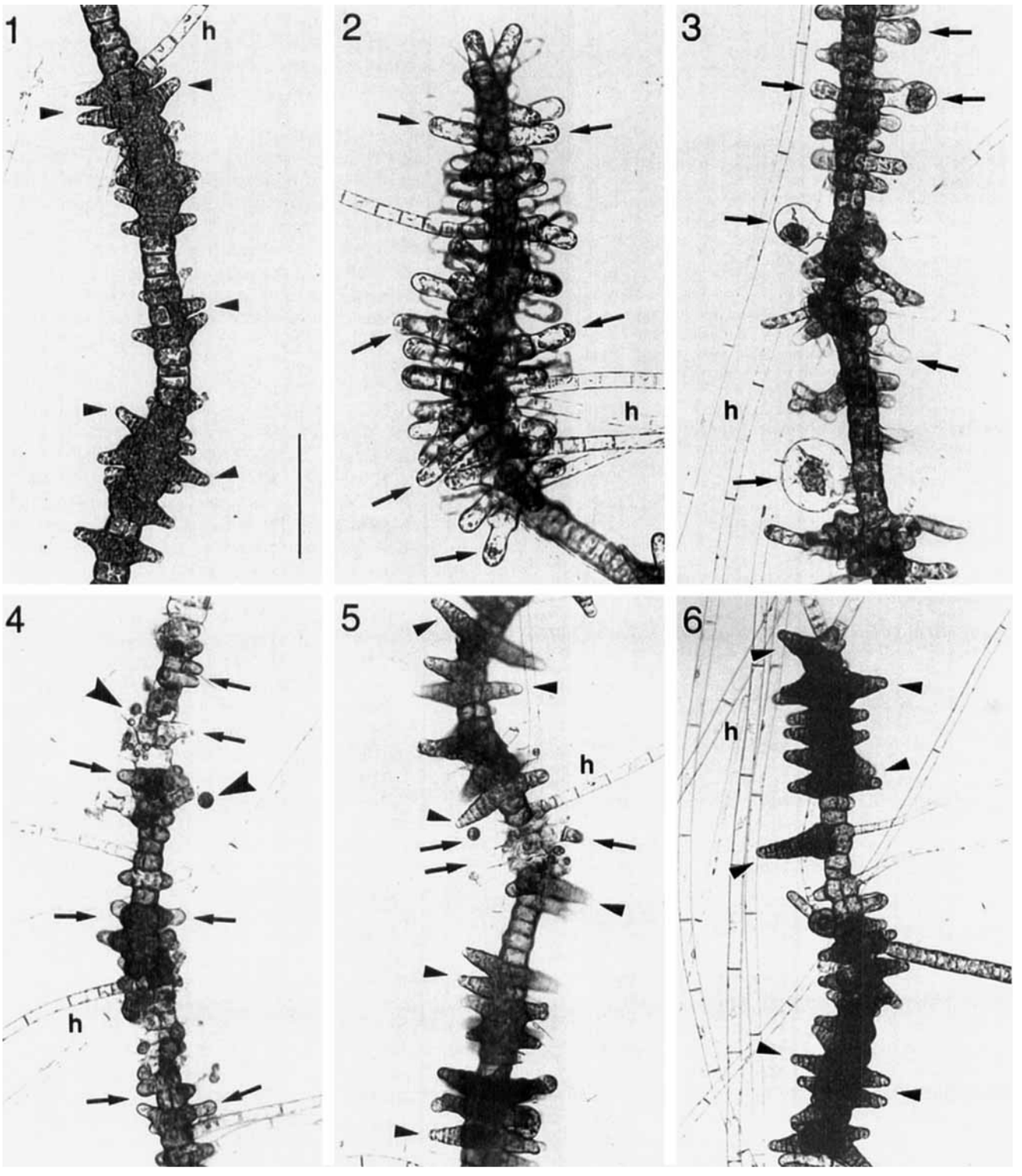
From 3% to 10% of *M. clavaeformis* spores exposed to EfasV-1 developed into well-growing, but sterile, thalli in various experiments with a total of ~1000 thalli determined over all experiments. In contrast to the parent strain which forms plurilocular (Fig. 1) but no unilocular sporangia, they produced lateral sacciform (sac-like) cells with pale contents. Superficially, they were similar to virus-forming cells in *Myriotrichia* infected with the genuine *Myriotrichia* virus (Fig. 2), but they often appeared swollen and degenerate at later stages (Fig. 3). Infection experiments with EsV-1 and *Myriotrichia* zoospores were unsuccessful and produced only normal fertile *Myriotrichia* progeny.

After several weeks or months some of the cultures infected with EfasV-1 gradually became fertile and formed plurilocular sporangia. These were often reduced in overall size, and in addition to normal spores they produced comparatively large motile aggregates (Figs. 4, 5). With phase-contrast optics they could be seen to be multiflagellated (not shown). Sporelings isolated from these cultures developed into thalli showing the mixed and partly normalized character of the parent thalli. Some infected *Myriotrichia* isolates had a stronger tendency to suppress the infection symptoms. They became fully fertile and formed erect thallus portions with plurilocular sporangia within 3 months after infection. Two such filaments were used to establish subclones, which maintained their normal phenotype (Fig. 6). Typical for *Myriotrichia*, all isolates had multiseriate thalli and formed colorless phaeophycean hairs, irrespective of viral infection (Figs. 1–6).

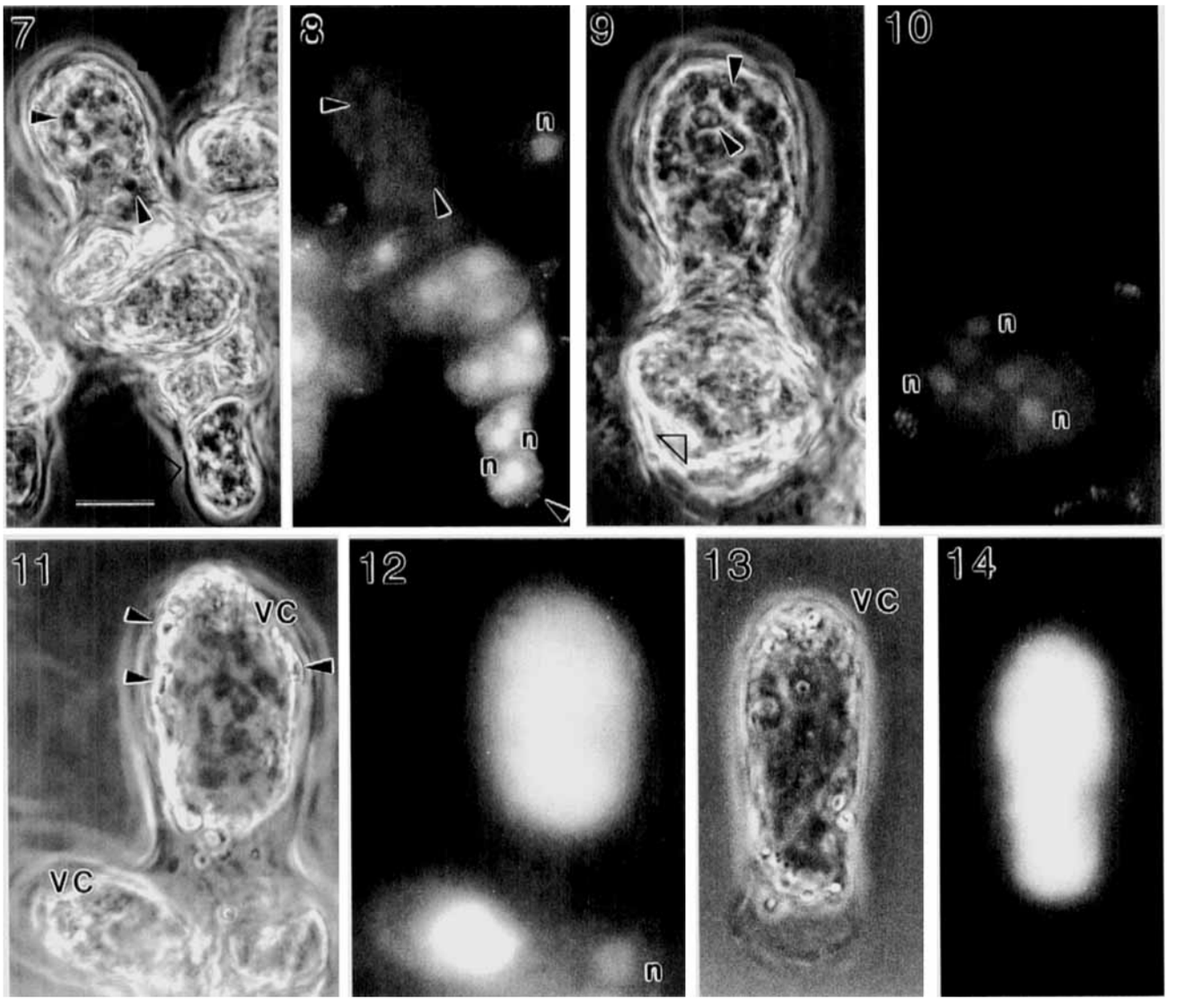
In their early development, sacciform cells of *Myriotrichia* showing symptoms of EfasV-1-infection became multinucleate. DAPI staining revealed two, four, and eight nuclei in such cells, as well as circular chloroplast genomes. In contrast, neither nuclear nor plastid DNA was found in cells at a later developmental stage (Figs. 7–10). Chloroplasts could still be identified by phase contrast microscopy (Figs. 7, 9), but they lacked pigmentation and no chlorophyll autofluorescence was detected (not shown). Occasionally, a few particles of approximately the same size and similar appearance as virus cores have been observed in these cells, but never complete virions. In *Myriotrichia* infected by MclaV-1 most of the volume of mature virus-forming cells was strongly stained by DAPI and thus occupied by DNA, presumably of viral origin (Figs. 11–14). A comparative investigation showed that the virus-forming cells in *Myriotrichia* infected with MclaV-1 were uninucleate throughout their development, with the single nucleus enlarging dramatically during viral DNA replication (Fig. 12, unpubl.). In the *E. fasciculatus*–virus donor strain, however, the development of virus-forming cells involves multinucleate stages very much like those observed in *Myriotrichia* infected with EfasV-1 (not shown).

Electron microscopical observations corroborated the light microscopical results, confirming the occurrence of multinucleated stages as well as the degeneration of nuclei and chloroplasts in diseased cells (Figs. 15–17). In the chloroplasts, the thylakoid stacks became loose and irregularly arranged, and pyrenoids disappeared (Fig. 17). Mitochondria were seen in different stages of degeneration. Golgi and other cellular organelles could not be identified in degenerated sacciform cells. Occasionally, remnants of internal cell walls were observed (Fig. 15). No evidence for the formation of virus particles or viral coats could be obtained. On the other hand, virus-forming cells in *Myriotrichia* infected by MclaV-1 were densely packed with virus particles (Fig. 18).

PCR amplification of a virus-specific DNA sequence confirmed that *Myriotrichia* thalli showing morphological abnormalities after exposure to EfasV-1 (Fig. 3) indeed had incorporated viral DNA (Fig. 19). The corresponding genomic sequence could not be detected in DNA obtained from the



FIGS. 1–6. Healthy and virus-infected sporophytes of *Myriotrichia clavaeformis*. FIG. 1. Part of a healthy thallus bearing plurilocular sporangia (arrowheads). FIG. 2. A cluster of numerous virus-producing cells (some are marked by an arrow) formed on a thallus infected by MclaV-1. FIG. 3. Habitus of an isolate infected by EfasV-1 with sacciform cells (arrows) in different stages of development and degeneration. FIG. 4. Another isolate of *Myriotrichia* infected by EfasV-1 at an early stage of recovery. Relatively small, laterally formed sacciform cells (arrows) release portions of cytoplasm, sometimes large motile aggregates (arrowheads). FIG. 5. A partially recovered sporophyte still showing some infection symptoms (arrows) but also producing functional plurilocular sporangia (arrowheads). FIG. 6. A fully recovered and gp-1-negative (PCR) thallus forming normal plurilocular sporangia (arrowheads). Hyaline phaeophycean hairs (h) are visible on all isolates. Scale bar = 100 μm.

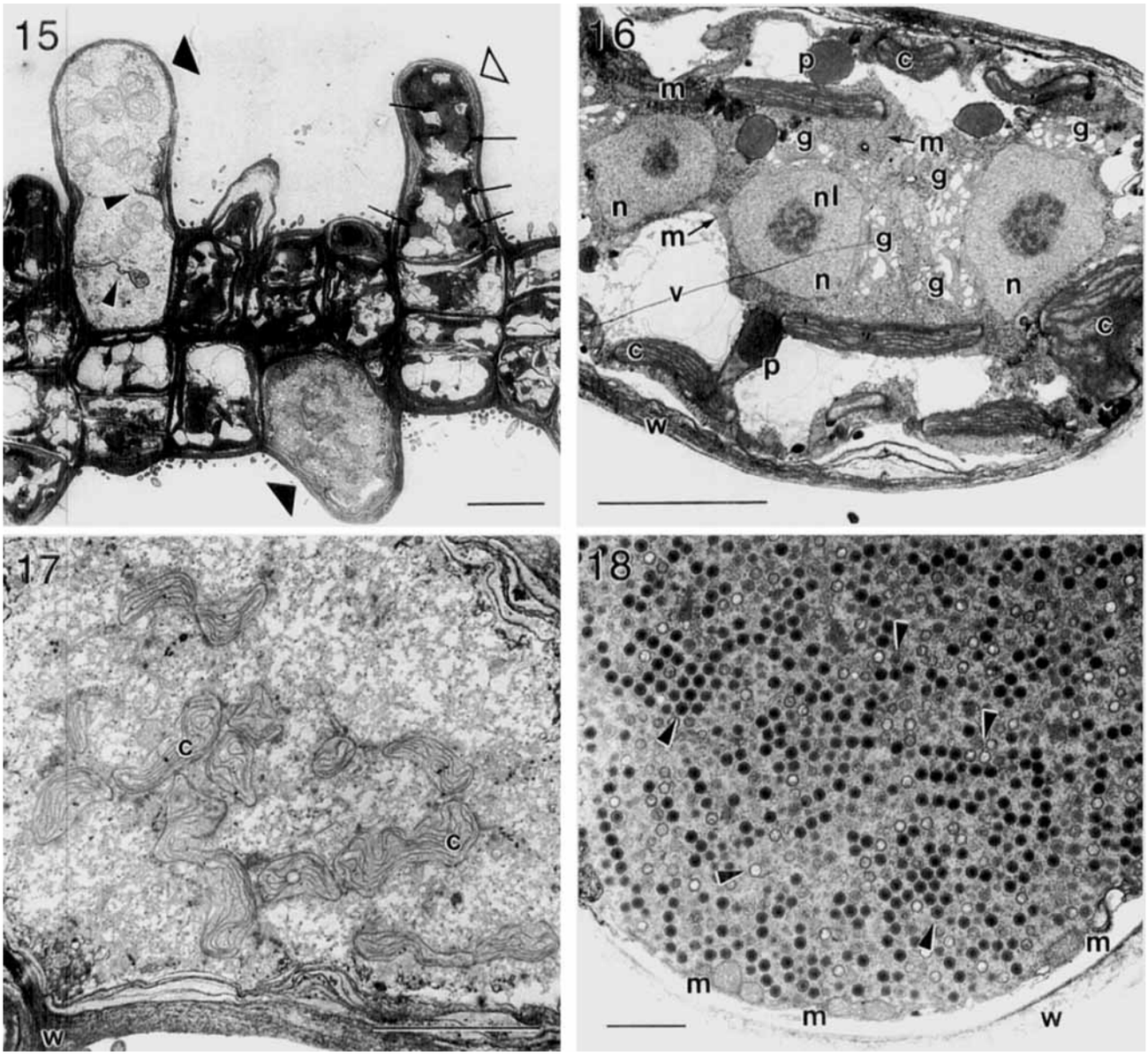


FIGS. 7–14. Fluorescence and phase-contrast micrographs of DAPI-stained *Myriotrichia* infected by EfasV-1 or MclaV-1. FIGS. 7, 8. Two lateral sacciform cells on a thallus infected by EfasV-1. In the upper cell (filled triangle) no nuclear, chloroplast, or viral DNA is evident, but chloroplasts (arrowheads) are present. The lower, smaller cell (open triangle) contains four nuclei (n) and chloroplast DNA is stained by DAPI (arrowhead). FIGS. 9, 10. Similar to Figures 7 and 8. In the upper cell (filled triangle) no DNA can be detected by DAPI staining. Remnants of chloroplasts can be recognized (arrowheads). In the lower cell (open triangle) eight nuclei (six in focus) were stained. FIGS. 11, 12. Virus-forming cells (VC) on a thallus of *Myriotrichia* infected with MclaV-1 in different stages of development. Apart from a peripheral cytoplasmic layer containing chloroplasts (arrowheads), most of the upper cell contains viruses strongly stained by DAPI. The lower cell is in an earlier stage of development. Compared to the nucleus of a vegetative cell, that of the virus-forming cell is dramatically enlarged and strongly fluorescent due to commencing viral DNA replication. FIGS. 13, 14. Similar to Figures 11 and 12. A mature, isolated virus-forming cell showing strong flourescence of DAPI-stained viral DNA. Scale bar = 10 μm.

healthy sporophyte that was used as a source of zoospores in infection experiments. Also no amplification product was obtained with two clonal isolates which, 3 months after infection, had lost the symptoms of infection and were reproductive (Figs. 6, 19).

DISCUSSION

Our infection experiments showed that the *E. fasciculatus* virus type 1 can enter zoospores of *M. clavaeformis*, is propagated to all cells of the developing host thallus, and induces pathological symptoms in reproductive structures. The virus does not, however, complete its infection cycle in the foreign species. *Myriotrichia* thalli infected by EfasV-1 form multinucleate, sacciform cells which degenerate instead of producing virus particles. Homology with virus-producing cells in other virus–host systems can be deduced from the following features. They are morphologically very similar to virus-forming cells in *Myriotrichia* infected with the genuine *Myriotrichia* virus (MclaV-1, Müller et al. 1996c). In addition, the oc-



FIGS. 15–18. Electron microscopy of *Myriotrichia* infected by EfasV-1 or MclaV-1. FIG. 15. Overview of part of a thallus infected by EfasV-1 in longitudinal section. The multiseriate structure of the thallus and lateral sacciform cells in different stages of development are shown. Two larger cells (filled triangles) are largely degenerate; in one of them remnants of internal cell walls are present (arrowheads). In a younger cell (open triangle) the profiles of five nuclei (arrows) are visible. Scale bar = 10 μm. FIG. 16. Detail of a multinucleate cell as shown in Figure 15. p = pyrenoid, w = cell wall, g = Golgi body, v = vacuole, n = nucleus, c = chloroplast, m = mitochondrion, nl = nucleolus. Scale bar = 5 μm. FIG. 17. Detail of a sacciform cell like those in Figure 15 with degenerated chloroplasts and cytoplasm. Scale bar = 5 μm. FIG. 18. Cross section of a virus-forming cell of *Myriotrichia* infected by MclaV-1 containing densely packed virus particles at different stages of maturation (arrowheads). Scale bar = 1 μm.

currence of multinucleate stages during their development corresponds to observations on virus-forming cells in *E. fasciculatus* infected by EfasV-1 (Maier, unpubl.) and *E. siliculosus* infected by EsV-1 (Müller et al. 1990). There, viral DNA synthesis and virion formation are also initiated after a series of synchronous nuclear divisions in a syncytium. In contrast to the formation of multinucleate cells in *Myriotrichia* infected with EfasV-1, no nuclear divisions were observed in *Myriotrichia* cells producing *Myriotrichia* viruses. The occurrence of syncytia in infected *Myriotrichia* is thus indicative for a partial expression or regulatory function of the foreign virus genome, even if no virions are produced. Cell wall rudiments like those occasionally observed in diseased multinucleate cells have also been observed during EsV-1 formation (Müller et al. 1990) and could be the result of interrupted cytokinesis or of an autolytic process. The latter may also apply to the degradation of nuclear and plastid DNA.

The molecular basis of incompatibility between EfasV-1 and *M. clavaeformis* remains unknown but is

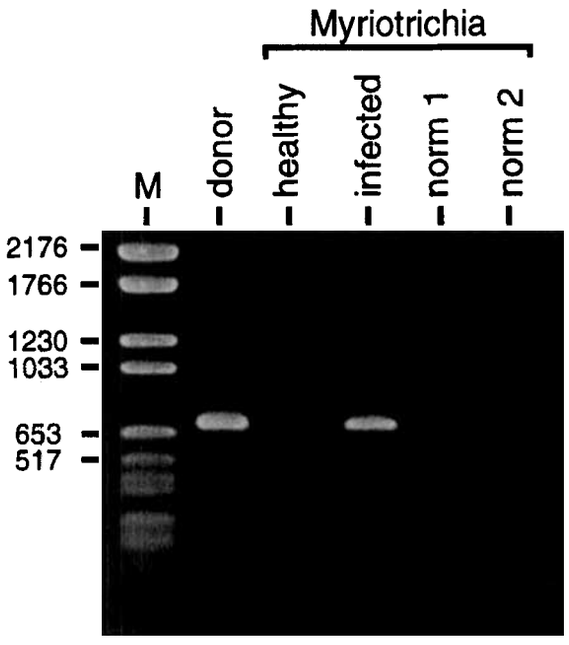


FIG. 19. PCR amplification of gp-1 gene sequences. M, markers with sizes in base pairs as indicated; donor: *E. fasciculatus* infected with EfasV-1; healthy: *M. clavaeformis* sporophyte without symptoms of virus infection, recipient of EfasV-1 in the infection experiment; infected: *Myriotrichia* infected with EfasV-1; norm 1, norm 2: two clonal isolates of *Myriotrichia* recovered from infection symptoms.

probably related to the genetic distance between the two host genera, *Ectocarpus* and *Myriotrichia,* which are assigned to different orders of brown algae. The results of the present study in combination with previous observations discussed below support the view that the viruses described from different brown algal species (Müller 1996, Müller et al. 1996c) are discrete entities. However, species specificity is not absolute. It is not known to what extent a general relationship between virulence and taxonomic affiliation of the hosts exists. In previous experiments EfasV-1 could be transferred from *E. fasciculatus* to the sister species *E. siliculosus.* In this host it caused aberrant symptoms of infection, and the virus particles formed were incomplete and noninfectious. Subisolates, which after some weeks in culture had lost the infection symptoms, still contained at least part of the viral genome, the gp-1 sequence. Conversely, EsV-1 is not able to infect *E. fasciculatus* (Müller et al. 1996b). On the other hand, it can pass to the closely related genus *Kuckuckia* (Müller 1992, Müller and Schmid 1996) and to *Feldmannia simplex* (Crouan) Hamel (Müller and Parodi 1993, Müller et al. 1996a), another ectocarpalean alga. In both cases, the EsV-1 genome persists in the foreign host. Although the virus is fully expressed in *Kuckuckia,* it does not multiply in *F. simplex,* but causes malformations similar to those observed in *M. clavaeformis,* followed by immediate reconstitution of the host to normal morphology and fertility (Müller and Parodi 1993). The loss of the gp-1 viral DNA sequence during recovery in EfasV-1-infected *M. clavaeformis* points to a mechanism for the elimination of viral DNA that has not been observed before in brown algae. It is possible, however, that only parts of the EfasV-1 genome have been lost or modified. Our PCR diagnosis gave information on the presence or absence of the gp-1 gene fragment only; "normalized" *M. clavaeformis* may still contain other parts of the viral DNA.

Ectocarpus fasciculatus and *M. clavaeformis* are both found in the same habitats on many temperate coasts, and a substantial proportion of *Ectocarpus* individuals on the coasts of all parts of the world is affected by virus infections (Sengco et al. 1996). Thus, the passage of viruses from one host to another as observed in our experiments may also occur in nature. Horizontal gene transfer between different viruses appears likely, provided that coinfection of a host organism by two different viral genomes may occur. In brown algae, it is not yet known whether the viral DNA integrates into the host genome, although this is indicated by its Mendelian segregation during meiosis of the host (Müller 1991a). Assuming this to be the case, excision of a viral genome from the host genome could be incomplete, leaving sequences that could either be integrated into another viral genome during a successive infection or remain integrated in the host genome and propagated in sexual reproduction. This way, horizontal gene transfer, not only between viruses but also between the virus and host, appears feasible. Similarly, retroviruses are regarded as mediators of gene flux in vertebrates (Amábile-Cuevas and Chicurel 1993) and, as an example of horizontal transfer of genetic information between viruses, a genomic region coding for a 130 amino acid protein sequence has probably been exchanged between quite distantly related mammalian retroviruses (McClure et al. 1987). In brown algae, the occurrence of horizontal gene transfer, a possible key mechanism of viral and host evolution, has not yet been demonstrated. The different virus–host systems now in culture, however, offer a chance to explore this aspect experimentally.

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