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Wayne R. Fagerburg

University of New Hampshire - Main Campus

Jennifer Towle

University of New Hampshire - Main Campus

Clinton J. Dawes

University of South Florida

Anne Boettger

West Chester University of Pennsylvania, sboettger@wcupa.edu

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BIOADHESION IN *CAULERPA MEXICANA* (CHLOROPHYTA): RHIZOID-SUBSTRATE ADHESION¹

Wayne R. Fagerberg², Jennifer Towle

Department of Molecular Cellular and Biomedical Sciences, University of New Hampshire, Durham, New Hampshire 03824, USA

Clinton J. Dawes

Department of Biology, University of South Florida, Tampa, Florida 33620, USA

and Anne Böttger

Department of Biology, West Chester University, West Chester, Pennsylvania 19383, USA

The attachment of the psammophytic alga *Caulerpa mexicana* Sond. ex Kütz., a coenocytic green alga, to crushed CaCO₃ particles was examined utilizing the scanning electron microscope and fluorescently tagged antivitronectin antibodies. Plants attached to the substrate through morphologically variable tubular rhizoidal extensions that grew from the stolon. In this study, we describe two means of attachment: (i) the rhizoid attachment to limestone gravel by thigmoconstriction, where tubular extensions of the rhizoid wrapped tightly around the substrate and changed morphology to fit tightly into crevices in the limestone, and (ii) through adhesion pads that formed in contact with the limestone granules. Flattened rhizoidal pads were observed to secrete a fibrillar material that contained vitronectin-like proteins identified through immunolocalization and that facilitated binding of the rhizoid to the substrate.

Key index words: biofouling; *Caulerpa*; coenocytic green algae; psammophytic alga; Q-dots; rhizoid attachment; thigmotropic; vitronectin-like proteins

Abbreviations: B, blade; DMP, 2,2 dimethoxypropane; FM, fibrillar material; G, gravel; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P, adhesion pad; R, rhizoid; S, stolon-like stipe; Vn, vitronectin; Vn-C, vitronectin-like proteins *Caulerpa*

Bioadhesion is almost universal among prokaryotic and eukaryotic organisms and involves a variety of biocomposites that facilitate cell-to-cell and cell-to-non-cell adhesion (Vreeland and Epstein 1996). In most macroalgae, attachment to the substrate is essential for plants to grow, reproduce, and to invade new aquatic habitats (Norton and Mathieson

1983, Wetherbee et al. 1998). *Caulerpa*, a tropical/subtropical coenocytic, acellular genus of green algae in the phylum Chlorophyta and order Bryopsidales, is one of a number of psammophytic (rhizophytic) green algal genera that can be a significant component of the marine communities it inhabits in Florida (Dawes and Mathieson 2008). The rhizoids of *Caulerpa*, *Udotea*, *Halimeda*, and other rhizophytic green algal genera usually anchor in unconsolidated sediments such as sand and mud (Dawes 1988) and function in nutrient uptake (Williams 1984, Chisholm et al. 1996) similar to the roots of seagrasses (Littler et al. 1988). Little is known about the importance of psammophytic seaweeds in terms of productivity. However, their biomass and that of associated drift algae can reach 85% of the total biomass in subtropical seagrass communities and can equal or surpass the total organic mass of the seagrasses distributed on the west coast of Florida (Dawes et al. 1985, 2004, Mattson 2000). Coenocytic species of *Caulerpa*, *Udotea*, and *Halimeda* also serve in a facultative role in succession of seagrass communities (Williams 1990). One member of the genus *Caulerpa*, *C. taxifolia*, is an invasive plant that has displaced the seagrass *Posidonia oceanica* in the Mediterranean (Boudouresque et al. 1995, de Villele and Verlaque 1995) and is an invasive species in Australia (Millar 2004) and southern California (Jousson et al. 2000).

Like other species of *Caulerpa*, the acellular *C. mexicana* Sonder ex Kützling (Vroom and Smith 2003, Fagerberg et al. 2010) and *C. prolifera* (Dawes and Rhamstine 1967, Dawes and Barilotti 1969) lack internal cell walls or plasma membranes that separate individual nuclei. The stolons of some *Caulerpa* species can exceed 1 m in length (e.g., *C. ashmeadii*), and most have erect blades, horizontal stolon-like stipes, and downward-growing rhizoids that branch, penetrate the sediments, and bind to particles (Jacobs 1994). To deal with the aquatic environment, various types of substrata and the physical characteristics of water itself (e.g., water motion, density) necessitate rapid attachment of plants to

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²Author for correspondence: e-mail wrf@unh.edu.

the substrate (Dawes 1988). *Caulerpa* and other marine organisms have evolved a variety of bioglues or algal adhesives with unique qualities that function in attachment to deal with these conditions and habitats (Comyn 1981). Many of these bioglues have generated considerable commercial interest because of their role in biofouling and the potential for development of these adhesives into effective glues that could be used in the marine environment (Levi and Friedlander 2004).

In eukaryotes, not only cell-to-substrate but also cell-to-cell adhesion events are mediated by a group of extracellular matrix molecules often consisting of glycoproteins (Diamond and Springer 1994, Yamada and Geiger 1997). These molecules may be anchored directly or indirectly to integrin proteins in the plasma membrane to form the cell-to-substrate attachment and are, in turn, often linked to components of the cytoskeleton (Hay 1991, Wetherbee et al. 1998). Reports of macroalgal adhesion describe a carbohydrate-glycoprotein-containing mucilage present on the adhesive surface of cells or on the attachment structure (Fletcher and Callow 1992). Acidic carbohydrates and glycoproteins have been located in the outermost layer of the extracellular matrix in rhizoids and holdfasts of several macroalgae (Vreeland et al. 1998). The brown algal genus *Fucus* develops adhesion sites between rhizoid and substratum that include the highly sulfated fucan polysaccharide fucoidan (F2) and a protein containing heparin-binding epitopes, similar to those of human vitronectin (Quatrano et al. 1991, Wagner et al. 1992, Shaw and Quatrano 1996). These epitopes appeared to be required for adhesion and are localized at the attachment site (Quatrano et al. 1991, Wagner et al. 1992, Shaw and Quatrano 1996). The F2-bound protein appears to be secreted into the cell wall at the growth site (Wetherbee et al. 1998). Vitronectin proteins are part of the extracellular matrix in animal systems (Diamond and Springer 1994, Yamada and Geiger 1997) and have been identified in a number of plant species (Zhu et al. 1994). These proteins have been implicated in pollen tube movement, bacteria/plant interactions, extracellular adhesion, binding of the plasma membrane to the cell wall (Sanders et al. 1991, Zhu et al. 1994), and substrate adhesion in the coenocytic green alga *Caulerpa* (Levi and Friedlander 2004).

Caulerpa adheres to the substrate through its rhizoidal system. Rhizoids are remarkable because they can arise from all parts of the plant (Friedlander et al. 2006) and attach to a wide variety of wet unconsolidated substrates under constant strong physical forces from currents and wave action. These substrates include clay sand, limestone, and smooth surfaces including glass surfaces (Levi and Friedlander 2004). Levi and Friedlander (2004) reported finding vitronectin-like proteins in *C. prolifera*, which they postulated could act as a bioglu-

attaching the rhizoids to the substrate for this species. Levi and Friedlander (2004) also demonstrated that the rhizoids of *C. prolifera* produce two approximately 60–70 Kd polypeptides with heparin-binding domain epitopes (termed Vn-Cs) similar to human vitronectin.

The current study addresses two questions associated with the presence of vitronectin-like proteins in *C. mexicana*: (i) Are vitronectin-like proteins distributed in both the adhesion pads of attached and nonattached rhizoids? (ii) Are vitronectin-like proteins found elsewhere in the plant other than at sites of attachment?

MATERIALS AND METHODS

Collection and culture. *C. mexicana* collections from the Florida Keys were purchased from the Gulf Specimen Marine Lab (Panacea, FL, USA) and taken from jetties in south Tampa Bay (27°35'31" N, 82°36'01" W). All collections were shipped overnight to the University of New Hampshire. Samples were grown at 22°C in 57 L culture tanks with a crushed limestone bed (granules, 2–4 mm in diam.) at a salinity of 35–40 ppt (Instant Ocean™, Aquarium Systems Inc., Mentor, OH, USA). All cultures were supplemented with 15 mL of 10 mM sodium nitrate and 50 μM potassium phosphate nutrient solution on a weekly basis. Experimental plants were cultured for ~ 1 month prior to fixation to allow acclimation to the new environment and grown under 14:10 light:dark at ~ 100 μmol photons · m⁻² · s⁻².

Preparation and fixation. Plant regions to be examined were double pinched to form wound plugs (Friedlander et al. 2006, Fagerberg et al. 2010), then bisected between the induced wound plugs producing 15 mm pieces. Rhizoids were removed intact along with the CaCO₃ substrate to which they were attached. Tissues were fixed in Karnovsky's fixative (Dawes 1988) buffered with 0.2 M HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer made up in 16⁰/₀₀ sea water (pH 7.4) for 4 h, then dissected into 5 mm pieces and fixed for an additional 2 h. Tissues were then rinsed with the same buffer and used as whole mount or dehydrated in a graded ethanol series for embedding in polyacrylate resin (Ted Pella™, Redding, CA, USA).

Attached rhizoids were carefully removed from the limestone gravel, and pliers were used to crush the attached limestone gravel into small (1–2 mm) pieces. Dissecting needles were used to gently separate the attached rhizoid/adhesion pad from the gravel. Adhesion pads were then placed in 1% acetic acid solution for 3–4 h to dissolve away the CaCO₃ substrate.

Embedding: Following fixation and dissection, some tissues were embedded in resin for light microscopic study, whereas others were set aside as whole mounts. Embedded tissues were dehydrated through a series of ethanol rinses up to 100%, then placed in acidified 2,2 dimethoxypropane (DMP) overnight. Tissues were rinsed in 100% ethanol twice for 20–30 min, and infiltrated with a 50% ethanol/50% polyacrylic resin mixture for 2 h on a rotator followed by two more changes in 100% polyacrylic resin, (overnight and 2 h, respectively). Tissues were placed into gel capsules with 100% polyacrylic resin and polymerized overnight in a 60°C oven. Tissue blocks were trimmed, sectioned, and mounted on glass microscope slides.

Immunolocalization: Vitronectin-like proteins were localized using anti-vitronectin monoclonal antibodies (Invitrogen, Carlsbad, CA, USA) directly conjugated to quantum dots (Q-dots 655™m Invitrogen). Fixed whole mounts and sections of *C. mexicana* were permeabilized in equal amounts of methanol

and acetone or with PBS containing 0.5% Triton X-100. Undifferentiated background staining was blocked using PBS containing 0.05% Triton X-100 and 2% BSA. Conjugated Q dot-vitronectin antibodies were applied in different concentration levels: 1:50, 1:100, and 1:200 in PBS and incubated in a hydration chamber 24–96 h for whole mounts and slides. Whole mounts and slides were rinsed with PBS and examined on the Zeiss Axioplan II microscope with epifluorescence. Photographs were taken with an AxioCam MR Camera and AxioVision 4.3 software (Carl Zeiss Inc., Thornwood, NJ, USA).

Negative antibody controls. Background staining was reduced by preblocking the tissues with BSA, and autofluorescence was checked in tissues that were not exposed to anti-vitronectin Q dot conjugates. Tissues were also exposed to solutions containing Q-dots but no antibodies against vitronectin and examined with a fluorescence microscope.

RESULTS

C. mexicana attached to the substrate by branched tubular outgrowths from the stipe called rhizoids (Fig. 1). Rhizoids were observed attached to the substrate through two distinct processes: (i) Thigmoconstriction involved the rhizoid segment wrapping tightly around irregularities and crevices in the substrate particles (Fig. 2a). The constricting elements of the rhizoid were tightly appressed to the contours and irregularities of the gravel particles (G), and often their morphology was distorted to match the shape of the indentation in the substrate particle (Fig. 2a). (ii) The rhizoid tips formed an adhesion pad with associated fibrous adhesion material (Fig. 2, b–d). Adhesion pads formed when rhizoid tips came into contact with the substrate and the tubular rhizoid flattened to form a pad. These pads were amorphously shaped and appeared to provide increased surface area for adhesion (Fig. 2b).

Large amounts of fibrous material occurred at the tips of attached adhesive pads (Fig. 2, c and d) and bound readily to antibodies against human vitronectin (Vn) conjugated with Q-dots[®] (Fig. 3, a–d). This material corresponded to the fibrous material

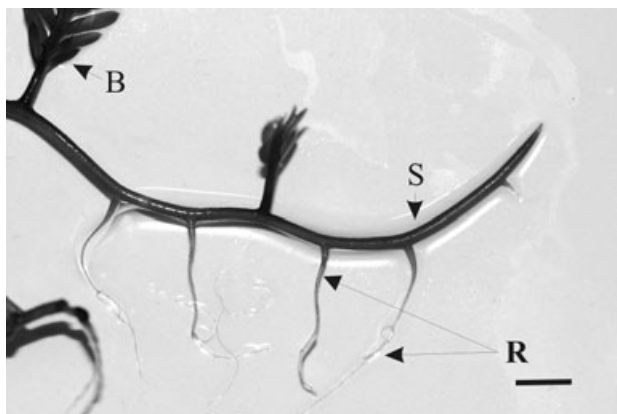


FIG. 1. Habit photograph of *Caulerpa mexicana* showing blades (B), stolon-like stipe (S), and downward-growing rhizoids (R). Scale bar = 5.0 mm.

observed with SEM (Fig. 2, c and d). Compared to rhizoids that had developed adhesion pads, nonattached rhizoids and thigmoconstricted rhizoids did not contain significant amounts of Vn-like protein based on the low level of Vn antibody binding (Fig. 4a). Blades and other parts of the plant also showed no external Vn antibody binding (Fig. 4b).

DISCUSSION

Psammophytic (rhizophytic) green algae in the coenocytic green algal order Bryopsidales anchor in soft sediments by means of individual rhizoidal clusters as in species of *Caulerpa* or by a massive rhizoidal holdfast as found in the genera *Avrainvillea*, *Cladocephalus*, *Halimeda*, *Penicillus*, *Rhipocephalus*, or *Udotea* in Florida (Dawes and Mathieson 2008). In both groups of coenocytic seaweeds, the rhizoidal system forms densely branched holdfasts that bind to large amounts of particulate sediment (Multer and Votava 1992, Bedinger and Bell 2006). The fine, colorless rhizoids are siphonous extensions of the thallus that not only anchor the plant and stabilize sediments, but also function in nutrient uptake, as shown for *C. cupressoides* (Williams 1984) and *C. taxifolia* (Chisholm et al. 1996), and thus are similar to the function of marine angiosperm roots (Littler et al. 1988).

The highly branched, morphologically variable rhizoids of *Caulerpa* sp. (Fig. 1) penetrate the substrate, attach the plant to the rocks and sand, and are the least studied part of the plant (Friedlander et al. 2006, Fagerberg et al. 2010). Considerable progress has recently been made in our understanding of substrate attachment in several animal and plant systems, although no attachment process has been fully characterized (Kamino et al. 2000, Wiegmann 2005). Two methods of attachment by *C. mexicana* rhizoids were described in this study: thigmoconstriction (Fig. 2a) and pad adhesion (Fig. 2, b–d).

Thigmotropic responses have been studied and described for land plants (Esmon et al. 2005). These responses involve membrane-bound touch receptors, Ca^{2+} signal cascades, unequal growth responses, and changes in cell wall elasticity (Esmon et al. 2005). It is likely that many of those facets are involved in the thigmotropic response of *Caulerpa*. However, the response mechanisms have not yet been described in this plant. In the thigmotropic response of *Caulerpa*, there is morphological remodeling of the rhizoid to fit more precisely the surface of the object to which the plant is attaching (Fig. 2a), which involves a localized change in cell wall plasticity. The mechanism(s) for localized increases in cell wall plasticity have not been investigated for *Caulerpa* sp. and whether they follow models described for vascular plant wall softening (VanVolkenburgh 1999, Cosgrove et al. 2002) is not known.

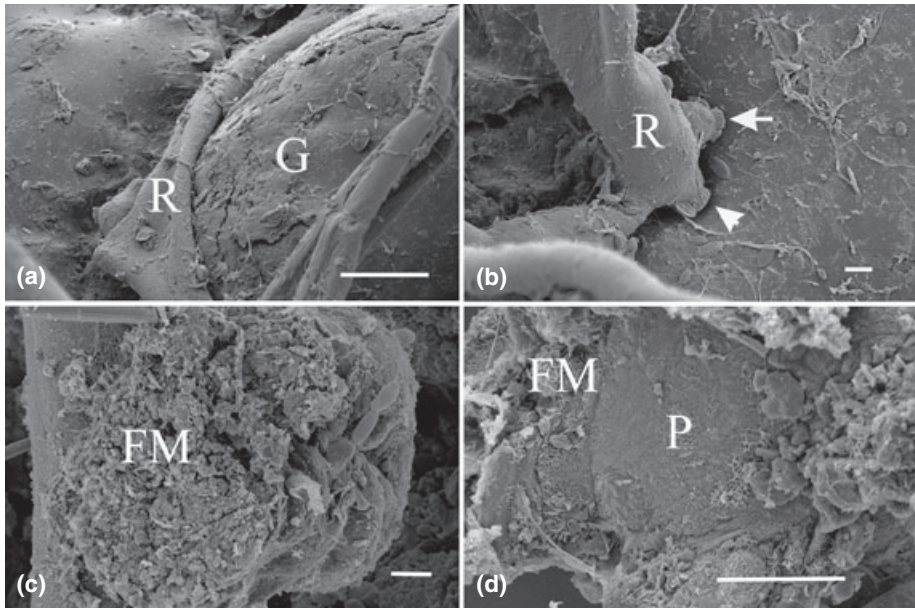


FIG. 2. Scanning electron micrographs of rhizoids of *Caulerpa mexicana*. (a) A thigmotropic attachment by a rhizoid (R) to a gravel particle (G) shows tightly appressed rhizoid filaments (R) following the contours in the particle. Scale bar = 100 μm . (b) Sites of adhesion pad formation (arrows) on gravel particle. R = Rhizoid. Scale bar = 10 μm . (c) The base of an adhesion pad consists of filamentous material (FM), which adheres to the substrate surface. Scale bar = 10 μm . (d) The adhesion pad base has a central clear area (P) and a peripheral area of filamentous material (FM). Scale bar = 10 μm .

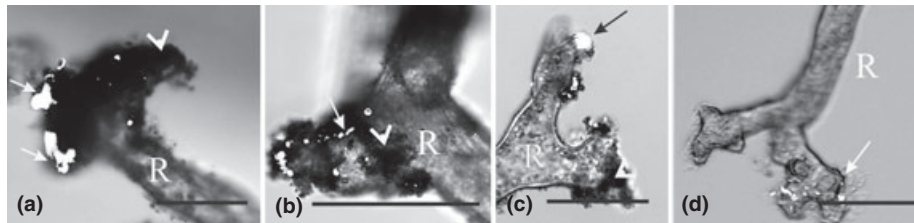


FIG. 3. (a–d) Light micrographs consisting of an overlay of a bright-field and fluorescent micrographs of adhesion pads of rhizoids (R) showing fibrillar material (dark material, arrowheads) and location of clusters (arrows) of vitronectin (Vn)-Qdot binding. Scale bars = 100 μm .

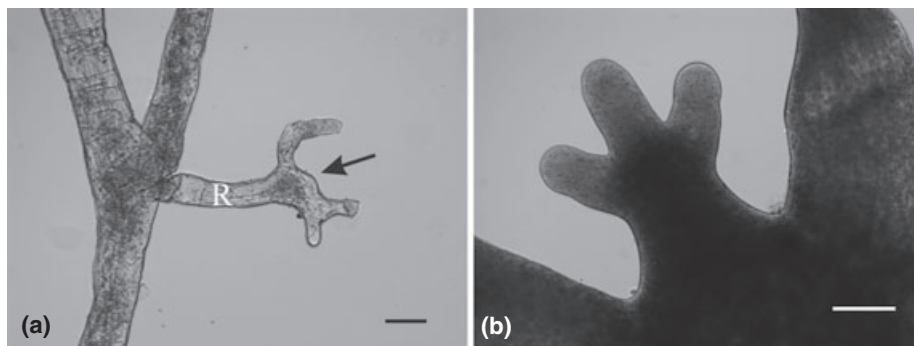


FIG. 4. (a) Bright-field micrograph and fluorescent overlay of a rhizoid (R) showing a thigmoconstrictive attachment site (arrow). Note no detectable Vn-Qdot binding. Scale bar = 100 μm . (b) The bright-field micrograph and fluorescent overlay of a blade tip (B) showing no detectable binding of Vn-Qdot antibodies. Scale bar = 1 mm.

The formation of adhesion pads also involves localized morphological remodeling (Fig. 2b), which likely follows similar pathways to that of the thigmotropic remodeling. Fibrous material produced by the adhesion pads bound vitronectin antibodies, indicating that vitronectin-like proteins were part of the adhesion material, as surmised by Levi

and Friedlander (2004). Other components are likely present as well, such as those described in other algal adhesion systems (Quatrano et al. 1991, Fletcher and Callow 1992, Wagner et al. 1992, Shaw and Quatrano 1996, Vreeland and Epstein 1996). Most reports of macroalgal adhesion describe a mucilage-containing carbohydrate/glycoprotein adhesive

present on the surface of cells of the attachment structure (Fletcher and Callow 1992). Acidic carbohydrates and glycoproteins were located in the outermost layer of the extracellular matrix in rhizoids and holdfasts of several macroalgae (Vreeland et al. 1998). Following initial adhesion, algae may eventually become tightly bound to the substrate by a range of processes involving the complex extracellular polymeric substances or extracellular matrix, including cross-linking mechanisms that have been proposed for brown algae (Wang et al. 1997, Wustman et al. 1997, Vreeland et al. 1998). Secondary adhesion may enhance the initial binding through a number of common processes, including a fiber-phenolic-catalyst mechanism that cross-links extracellular matrix components, including those of the adhesion complex (Vreeland and Epstein 1996, Wang et al. 1997, Wustman et al. 1997, Vreeland et al. 1998). It is not clear whether or not those secondary binding reactions are features of rhizoid adhesion in *C. mexicana*.

In summary, we describe two types of attachment by the rhizoids of *C. mexicana*, thigmotropic binding and production of a vitronectin-like carbohydrate/glycoprotein adhesive. In a number of cases, we observed both types of attachment on the same rhizoidal filament as well as single filaments utilizing only one or the other type. We did not find vitronectin present in regions of the plant where attachment did not occur.

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