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RESEARCH ARTICLE

Reverse genetics demonstrate the role of mucosal C-type lectins in food particle selection in the oyster *Crassostrea virginica*

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ABSTRACT

Prey selection governs species interactions and regulates physiological energetics of individuals and populations. Suspension-feeding bivalves represent key species in coastal and estuarine systems for their ecological and economic value. These animals are able to sort and selectively ingest nutritious microalgae from dilute and composite mixtures of particulate matter. This aptitude was suggested to be mediated by interactions between carbohydrates associated with the surface of microalgae and C-type lectins present in mucus covering the feeding organs, although a direct, unequivocal, role of lectins in food sorting in bivalves remains elusive. This study was designed to identify and characterize mucosal C-type lectins from oysters and manipulate the expression of these proteins in order to obtain decisive information regarding their involvement in food choice. Thus, two mucosal C-type lectins (CvML3912 and CvML3914) were identified based on transcriptomic and proteomic information. Transcripts of these lectins were detected in the feeding organs and their expression was upregulated following starvation. Recombinant lectin (rCvML3912) competitively inhibited the binding of commercial mannose/glucose-specific lectins to microalgae. Short Dicer-substrate small interfering RNA (DsiRNA) targeting these two lectins were designed and used to evaluate the effect of gene silencing on food particle sorting. As a result, the abundance of the two cognate transcripts significantly decreased and food sorting ability was significantly reduced among silenced oysters as compared with control animals. Overall, these findings propose a novel concept establishing the role of carbohydrate–protein interactions to provide efficient food particle sorting, and establish a new dimension for the role of evolutionarily conserved mannose/glucose-binding proteins in metazoans.

KEY WORDS: Suspension feeding, Bivalve, Mucus, RNA interference, Knock down

INTRODUCTION

Diet and prey selection regulates physiological energetics of individuals and populations and governs top-down community-level processes (Cruz-Rivera and Hay, 2000; de Carvalho and Mirth, 2017; Emlen and Emlen, 1975; Holt, 1977; Johnson et al., 2009; Milton, 1979). In marine ecosystems, invertebrates often dominate the benthos and contribute to fundamental ecological functions including the regulation of energy fluxes. This is particularly the case for suspension-feeding organisms, which consume bacterioplankton and phytoplankton, and control energy and nutrient transfer between pelagic and benthic communities. Through their suspension-feeding process, these organisms are confronted with a wide range of suspended material, including a usually high proportion of non-nutritive material and debris, and live plankton of varying nature, size and quality (Alldredge and Silver, 1988). Consequently, and instead of bulk ingestion of suspended material that can quickly saturate their digestive system, many taxa have developed strategies to enhance the nutritive value of ingested material and optimize energy gain. Suspension-feeding bivalves are no exception and their ability to discriminate among particles has been examined for decades (Allen, 1921; Beninger and Decottignies, 2005; Fox, 1936; Pales Espinosa and Allam, 2013; Pastoureaud et al., 1996; Ward and Shumway, 2004). These animals can selectively ingest specific microalga among a mix of several species (Cognie et al., 2001; Shumway et al., 1985), including those with similar sizes and shapes (Pales Espinosa and Allam, 2013). This elaborate ability to select food particles represents an adaptation to various constraints faced by bivalves including a relatively dilute diet with a large proportion of non-nutritive particles that can easily saturate mucociliary transport of food particles, and a discontinuous and energetically costly intracellular digestion. This selective feeding, in conjunction with their astonishing filtration rate, places the group as a main player regulating benthic–pelagic coupling in near-shore waters (Newell, 2004; Prins et al., 1997). This can lead to depletion of phytoplankton populations (Cloern, 1982; Fréchette et al., 1989), stimulation of growth of unselected species (Asmus and Asmus, 1991; Lewin et al., 1975), or more complex effects on the ecosystem (Newell, 1988; Officer et al., 1982).

In bivalves, food particles suspended in the water column are captured by the gills and transported in mucus towards labial palps (small organs surrounding the mouth) that direct particles to the mouth for ingestion or towards the mantle for rejection as pseudofeces. Although some aspects of the selection process in these animals have been elucidated, the actual mechanism(s) by which particles of poor quality are rejected as pseudofeces while those of higher quality are diverted to the mouth and ingested remains unclear. Among hypotheses put forth to explain this ability, several studies offered strong support that biochemical cues of food particles can mediate this selection process (Beninger and Decottignies, 2005; Pales Espinosa et al., 2008, 2016a, 2010c). Specifically, recent studies suggested that food selection is mediated by interactions between carbohydrates covering microalgae cell surface and lectins present in mucus covering the feeding organs (Pales Espinosa et al., 2010c). In this framework, it was hypothesized that particles covered with specific carbohydrates could create strong bonds with feeding mucus, leading to selection, while particles lacking those carbohydrates are not strongly trapped by mucus and, consequently, are less selected. Evidence supporting this scenario comes from the characterization of particles that are

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Received 9 November 2017; Accepted 1 February 2018

Journal of Experimental Biology (2018) 221, jeb174094. doi:10.1242/jeb.174094

preferentially ingested (or, alternatively, rejected). For example, previous studies using either engineered microspheres with tailored surface carbohydrates or microalgae whose cell surface carbohydrates were blocked with specific commercial lectins showed that mussels and oysters (*Mytilus edulis* and *Crassostrea virginica*, which display contrasting architecture of the feeding organs) preferentially ingest particles with accessible mannose/glucose residues (Pales Espinosa et al., 2010b,c). More recently, we showed that food particle preference in mussels and oysters can be predicted based on the relative abundance of mannose/glucose residues on microalgae cell surface, with those enriched in mannose/glucose being preferentially ingested (Pales Espinosa et al., 2016a). In parallel, proteomic analysis of mucosal secretion covering the feeding organs of oysters (Pales Espinosa et al., 2016b) and mussels (E.P.E. and B.A., unpublished) revealed the presence of a large repertoire of mucosal lectins that differentially bind microalga cell surface carbohydrates, possibly triggering selection (Pales Espinosa et al., 2010b).

While our prior studies suggest that carbohydrate–protein interactions may mediate particle selection in suspension-feeding bivalves, a direct, unequivocal, role of lectins in particle sorting remains elusive. This study was designed to test the hypothesis that lectins found in mucus covering the feeding organs of the oyster *Crassostrea virginica* mediate food particle sorting. Mucosal lectins were identified and characterized using a combination of proteomic and bioinformatic tools. Transcriptomic regulation of these molecules in response to starvation was then assessed. Next, a recombinant mucosal lectin was produced and its binding to microalgae was evaluated. Finally, direct investigations using RNA interference (RNAi) experiments were conducted. RNAi uses synthetic double-stranded (ds) RNA to disrupt the translation of target genes (Agrawal et al., 2003; Zamore, 2001) and has been successfully used to assess gene function in several invertebrate taxa, including mollusks (Choi et al., 2013; Fabioux et al., 2009; Huvet et al., 2015). Here, several Dicer-substrate small interfering RNAs (DsiRNAs) were synthesized and used to knockdown the C-type lectins before assessing changes in food selection among silenced oysters.

MATERIALS AND METHODS

Microalgae

Axenic microalgal cultures were obtained from the Northeast Fisheries Science Center Milford Microalgal Culture Collection (Milford, CT, USA; Table 1). For lectin-binding assays, strains were grown in triplicate in F/2-enriched media (Guillard, 1982) at 15°C under a 12 h light/12 h dark cycle. Cultures were harvested in the exponential phase of growth. For feeding experiments, microalgae were produced in 3 l cultures under conditions described above. Growth rate was determined every day, and algae were harvested in the exponential phase of growth and used immediately.

Table 1. List of microalgae used in FITC-labeling and feeding experiments

Species	Strain	Cell length (µm)	Type of experiment
Dunaliella salina	LB200	9	Feeding
Prasinocladus marinus	163/1B	10	Feeding
Amphora coffeaeformis	A-ora	20	Lectin labeling
Chlamydomonas sp.	11/35	10	Lectin labeling
Chlorella autotrophica	580	2	Lectin labeling
Cricosphaera carterae	914	10	Lectin labeling
Nannochloropsis sp.	UTEX-2341	2	Lectin labeling
Pavlova lutheri	MONO	4	Lectin labeling

Strain (Milford Microalgal Culture Collection) and cell size (length) are indicated.

Animals

Adult (80–100 mm in length) eastern oysters, *Crassostrea virginica* Gmelin 1791, were obtained from Frank M. Flower and Sons Oyster Company (Oyster Bay, NY, USA). Bivalve shells were cleaned of all epibionts, then notched at the ventral posterior edge of the shell and acclimated in the laboratory for 1 week (salinity 28, 15°C) and fed daily (15% dry mass) using fresh cultures of *Pavlova lutheri*, *Chamydomonas* sp., *Amphora coffeaeformis* and DT's Live Marine Phytoplankton (Sustainable Aquatics, Jefferson City, TN, USA; Pales Espinosa and Allam, 2006).

Identification and molecular characterization of mucosal lectins

We used previously published information to identify and molecularly characterize mucosal lectins. A total of 4.1×10^8 raw Illumina GAIIx 108 bp reads generated by McDowell et al. (2014) from whole-oyster tissues were downloaded from NCBI (SRP042090). Reads were filtered and trimmed using the software Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) before being submitted to rRNA cleaning using the program riboPicker (http://ribopicker.sourceforge.net/) against SILVA database v111 (https://www.arb-silva.de/). High-quality filtered reads were then used for *de novo* assembly using Trinity (Haas et al., 2013). The assembled transcripts with sequence length longer than 200 bp, re-mapping FPKM (fragments per kilobase of transcript per million mapped reads) greater than 1 and isoform discovery level greater than 1% were then annotated using Trinotate (http://trinotate.github.io/). This procedure generated a total of 85,472 transcripts (contig N50: 1910 bp, median contig length: 839 bp, average contig length: 1233 bp). This assembly was then used to re-screen proteomics data generated during a previous study on the characterization of mucus covering oyster feeding organs (Pales Espinosa et al., 2016b), which at the time was completed using a combined *C. virginica*/*C. gigas* transcriptomic database because of the scarcity of transcriptomic data from the target species (*C. virginica*). Briefly, mass spectra generated from liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis of mucus collected from gills, mantle and labial palps were extracted from the RAW file and the resulting mzXML data files were searched with the Global Proteome Machine (GPM) X! Tandem search engine against the *de novo* assembled transcriptome. This analysis allowed the identification of two C-type lectins (CvML3912 and CvML3914) that were particularly abundant in mucosal secretions. Validation of each of these two lectins was performed by determining the peptide sequence coverage and manually reviewing the individual peptide-associated spectra and fragmentation table. Further, the amino acid sequences of these lectins were searched for homology against NCBI. Protein motif features were predicted using several bioinformatics resources including ExPASy (www.expasy.org/prosite/) and various tools available at the Center for Biology Sequence Analysis (www.cbs.dtu.dk/services/NetNGlyc, http://www.cbs.dtu.dk/services/NetOGlyc and www.cbs.dtu.dk/services/SignalP). Sequences were aligned using CLUSTAL W2 (www.ebi.ac.uk/Tools/clustalw2/) and BioEdit (version 7.0.5.3).

Localization and regulation of lectin transcripts

To identify the source of mucosal lectins, lectin transcripts were measured in different feeding organs. After acclimation for 1 week

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(see 'Animals', above), eight oysters were dissected and gills, labial palps, mantle and digestive gland tissues were collected and flash-frozen and conserved at −80°C until processing for RNA extraction and gene expression.

The effect of starvation and bacterial challenge on lectin transcription levels was also assessed. Many lectins have immune functions (Allam and Pales Espinosa, 2015) and this experiment evaluated whether the two mucosal lectins contribute to mucosal immunity and respond to bacterial challenge. For this experiment, another batch of acclimated oysters was divided into three equal groups (two replicates per group, n=6 oysters per replicate) and kept under the same conditions of salinity and temperature described in 'Animals' (above). One group was fed the same diet as described above, the other group was unfed and the last group was exposed to a bacterial bath [*Vibrio alginolyticus* grown on marine agar plates for 48 h, scraped, washed with 0.22 µm-filtered artificial seawater (FSW), and resuspended in FSW to 1×10^7 bacteria ml^{-1}]. After 5 days, oysters were killed; gills were dissected, flash-frozen and conserved at −80°C until processing for RNA extraction and gene expression.

Production and binding activity of recombinant lectin

Recombinant protein of one of the two candidate lectins (designated rCvML3912) was produced to allow further functional testing. GenScript Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA) was used to express recombinant lectin. Briefly, the CvML3912 gene was codon optimized (gp67-CvML3912+6His tag) and subcloned into pFastBac1 vector (Invitrogen). The cloned vector was transformed into bacterial DH10Bac competent cells to generate recombinant bacmid DNA that was then extracted and transfected into Sf9 cells using Cellfectin II Reagent (Invitrogen). The P1 viruses were released into culture media and harvested after incubation of the transfected cells at 27°C for 7 days. They were used to infect fresh insect cells (Sf9) and amplify the baculoviral stock (high-titer P2 stock). To improve the secretion efficiency of the target protein, High Five cells (ThermoFisher Scientific, Waltham, MA, USA) were infected with recombinant baculovirus (virus stock P2). Cultures were supplemented with fetal calf serum at 5% final concentration. Seventy-two hours post-infection, the cultures were centrifuged for 30 min at 400 ***g***. Pelleted cells were lysed by sonication in 50 mmol l^{-1} Tris, 500 mmol l^{-1} NaCl, 1% NP-40, 5% glycerol, pH 8.0, denatured by 8 mol l^{-1} urea, and then purified using a Ni-IDA column. The purified target protein was refolded by dialyzing into the storage buffer (50 mmol l^{-1} Tris-HCl, 10% glycerol, 0.5 mol l^{-1} L-arginine, 2 mmol l^{-1} DTT, pH 8.0). Recombinant protein was analyzed by SDS-PAGE and western blot using standard protocols for molecular mass and purity measurements, using a mouse-anti-His monoclonal antibody (GenScript). The concentration of the recombinant protein (i.e. rCvML3912) was determined by Bradford protein assay with BSA as a standard.

The binding of rCvML3912 to microalgae was evaluated by an indirect technique based on competitive binding of commercial fluorescein isothiocyanate (FITC)-conjugated lectins (adapted from Pales Espinosa et al., 2010b). Briefly, microalgal cultures (Table 1) were centrifuged at 400 ***g*** for 10 min, washed once, and resuspended in filtered artificial seawater (FSW, 0.22 µm). rCvML3912 (80 µl, 200 µg ml^{-1} in storage buffer) or storage buffer (control) was added to 1 ml microalgae (10^6 cells, final concentration, in triplicate), incubated for 1 h in the dark at room temperature, centrifuged and washed twice. FITC lectins (EY Laboratories, CA, USA; Table S2) were diluted in FSW to 1 mg ml^{-1}. One 50 µl aliquot of lectin (or FSW control) was added to each replicate and incubated in the same conditions before being washed and resuspended in FSW for flow cytometric analysis. In these experiments, commercial lectins used with each microalgae species were selected based on earlier work establishing lectin affinity profiles for each species (Pales Espinosa et al., 2016a). The effect of carbohydrates on the binding activity of rCvML3912 was also assessed. Aliquots of rCvML3912 (50 µl, 200 µg ml^{-1} in storage buffer) were mixed with 950 µl of a cocktail of D-glucose and D-mannose (150 mmol l^{-1} final concentration for each carbohydrate in 150 mmol l^{-1} NaCl) and incubated for 30 min before the addition of washed microalgae (10^6 cells, final concentration). Only *Chlorella autotrophica* was used in these inhibition assays, based on preliminary data showing high binding of rCvML3912 to this species. Treated algae were then processed for commercial lectin labeling as described above.

Effect of lectin silencing on food particle selection

DsiRNA synthesis

DsiRNAs were designed from the nucleotide sequence of the two candidate mucosal lectins to evaluate changes in particle sorting in silenced oysters. The DsiRNA design tool from IDT (Skokie, IL, USA; https://www.idtdna.com/site/order/designtool/index/DSIRNA_CUSTOM) was used to design DsiRNAs (Table S3). Candidates were then compared (BLAST) against the *Crassostrea gigas* database (NCBI) and *Crassostrea virginica* transcriptome (no genome available at the time) to avoid cross-reactivity by having more than two mismatched bases in the first 19 bases on the plus strand. DsiRNAs were ordered from IDT and kept at −80 until use (within a week). DsiRNAs were dissolved in sterile FSW (2 ml final volume; Table S3). The control DsiRNA (NC5) was prepared separately.

Silencing experiments

Two silencing experiments were performed, one in spring and one in autumn 2016. For each experiment, oysters were randomly divided into three equal groups (two replicates per group, n=10 oysters per replicate). The first group was injected in the adductor muscle with 100 µl of sterile FSW; the second group was injected with DsiRNA-NC5 (negative control purchased from IDT; 4 nmol resuspended in 2000 µl of sterile FSW) and the third group was injected with a cocktail of DsiRNA (4 nmol resuspended in 2000 µl of sterile FSW). In the first experiment, the DsiRNA cocktail was made up of DsiRNA-1 and DsiRNA-3, while in the second experiment, the DsiRNA cocktail was made up of five different DsiRNAs (DsiRNA-2 to DsiRNA-6; see Table S3). Oysters were subsequently placed back in their respective aquaria under standard conditions.

Food-sorting assays

Particle-sorting assays were conducted 46 and 34 days post-injection for the first and second experiment, respectively, following our previously published protocols (Pales Espinosa and Allam, 2013). Animals were starved in FSW (0.45 µm) for 1 day prior to being used in particle-sorting assays. Preliminary results showed that 1 day starvation does not affect the regulation of CvML3912 and CvML3914. For each assay, a diet made of equal concentrations of two microalgae, *Dunaliella salina* and *Prasinocladus marinus* (ca. 2×10^5 cells ml^{-1} final concentration, exponential phase of growth; Table 1) was delivered to 12 oysters and an empty shell (control), all maintained individually in 4 l tanks. These microalgae were used because they have similar sizes and shapes while still being easy to discriminate using flow cytometry; furthermore, these species were previously shown to be

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differentially selected by oysters and mussels (Pales Espinosa et al., 2016a). Microalgae were kept in suspension by micropipette aspiration every 10 min and, at the same time, water samples were taken to determine possible changes in the ratio between the two algae (possibly from sedimentation or differential retention efficiencies). Pseudofeces, representing particles rejected by oysters before ingestion, were collected from each experimental tank about 15 min after the start of production, vortexed to disrupt particle aggregates, passed through a 50 µm, nylon-mesh sieve, and analyzed using flow cytometry. In this context, particles enriched in pseudofeces represent those preferentially rejected by oysters.

After particle-sorting assays, oysters (n=10) were dissected and gills were flash-frozen and conserved at −80°C until processed for RNA extraction and real-time PCR (qPCR) analysis to ensure a decrease in the transcription of the two targeted lectins among silenced oysters had occurred.

Real-time PCR analysis

RNA was isolated using the NucleoSpin RNA Plus kit (Macherey–Nagel, Düren, Germany) following the manufacturer's protocol. cDNA was generated from extracted mRNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and was used as a template with each set of primers listed in Table S3. Relative quantification was carried out in 10 µl reaction volume with Takyon No Rox SYBR 2× MasterMix blue dTTP (Eurogentec, Liège, Belgium), 100 nmol l^{-1} final primer concentration and 5 ng of RNA-equivalent cDNA. The PCR reactions were performed using a Mastercycler ep realplex PCR machine (Eppendorf, Hamburg, Germany). The correct amplification products were confirmed using gel electrophoresis. Lectin expression levels were normalized to expression of the 18S gene and relative transcript levels were calculated using the ($2^{-\Delta\Delta Ct}$) Ct method (Livak and Schmittgen, 2001).

Flow cytometry analysis

Microalgae were analyzed using a FACSCalibur flow cytometer (BD BioSciences, San Jose, CA, USA). A minimum of 2×10^4 events were analyzed. A 488 nm argon laser was used for excitation, and microalgae were characterized, discriminated and counted based upon one or more of the following parameters: forward (FSC; particle size) and side (SSC; intracellular complexity) light scatter, FITC fluorescence (FL1, 535 nm, for lectin labeling experiments) and photosynthetic pigment auto-fluorescence (FL2, 585 nm, for phycoerythrin-related pigments; FL3, 670 nm, for chlorophyll a) for discrimination of microalgae from the particle-sorting experiments.

Data treatment and statistical analysis

Statistical analyses were conducted using XLSTAT software (XLSTAT 2015.1.02), Statgraphics (v5.1), Sigmastat (v3.1) and R. Results obtained for microalgae treated with rCvML3912 followed by FITC-labeled lectins are presented as a ratio (%) between fluorescence intensities (geometric mean) of treated cells (rCvML3912, FITC-labeled lectins) and baseline (FITC-labeled lectins) in FL1 (control incubated with seawater). Statistical analyses of these data were performed on raw flow cytometric fluorescence histograms using a Kolmogorov–Smirnov (K–S) analysis that is packaged in the CellQuest Pro software in BD FACStation software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The K–S test compares the distributions of fluorescence histograms, calculates a D value that represents the greatest difference between the two curves, and establishes the probability (P) that the two histograms are different.

Data obtained from the feeding experiments were analyzed using goodness-of-fit tests, as described previously (Pales Espinosa et al., 2009). Two series of tests were performed comparing the proportions of each type of particle in samples of the diet and pseudofeces collected from the oysters. The first series of tests ensured that, within each treatment, replicate samples of the diet and pseudofeces were homogeneous. The second series tested the null hypothesis that the proportion of each particle type in the diet or in the pseudofeces was not different between treatments. In addition to the comparison of raw counts, a sorting efficiency (SE) index was calculated to examine particle selection (Iglesias et al., 1992). This index was defined as: SE=1−(P/D), where P and D represent the proportion of the particle of interest in the pseudofeces and diet, respectively. A positive SE for a given particle type indicates that it is preferentially ingested (particle type is depleted in the pseudofeces compared with diet), a negative SE indicates rejection (particle type is enriched in the pseudofeces compared with diet), and zero indicates the absence of active selection. After checking for normality, calculated SE values obtained for each of the two particles in each treatment were compared with zero using a one-sample t-test (two-tailed). The null hypothesis was that the selection efficiencies were equal to zero (i.e. no selection).

Real-time PCR data (ΔCt) were also analyzed using a one-way ANOVA and are presented as relative fold-change in expression ($2^{-\Delta\Delta Ct}$) using the arithmetic mean as a reference.

A non-linear regression (non-linear least squares) was implemented in R in order to evaluate correlation between the SE index for each oyster and gene expression of the two candidate lectins (ΔCt values derived from the qPCR). The Akaike information criterion (AIC) was used to select final models.

RESULTS

Molecular characterization of mucosal lectins

Mucus covering the feeding organs was previously shown to contain a wide range of immune-related molecules (Pales Espinosa et al., 2016b). Results obtained in the current study showed the presence of two C-type lectins that were particularly abundant in mucus covering oyster gills and labial palps. The identification of several peptides displaying credible spectra ($\log_e$<−4; Table S1) confirmed the existence of these two distinct proteins (i.e. CvML3912 and CvML3914; Fig. 1).

The protein sequence analysis of these two lectins showed high similarity (<1E−60) with other C-type lectins from the Pacific oyster *Crassostrea gigas* (XP_019929296.1, XP_019927412.1, EKC30902, XP_011443363.1) including a C-type lectin domain family 4 member, two C-type mannose receptor 2 and a perlucin-like protein. These proteins contain a conserved carbohydrate recognition domain (CRD; conserved domain CLECT_DC-SIGN_like) consisting of 129 residues (Tyr^{26}–Glu^{154}) located in the C-terminus of the protein (Fig. 1). Among the signature features for C-type lectins, CvML3912 and CvML3914 reveal four consensus cysteine residues (Cys^{47}, Cys^{125}, Cys^{145} and Cys^{153}) and two optional cysteine residues (Cys^{19}, Cys^{30}) that are expected to form disulfide bonds (Tasumi et al., 2002). Like most of the other C-type lectins, the two proteins present conserved WGD (Trp^{140}, Gly^{141}, Asp^{142}, for CvML3912) and WSD (Trp^{140}, Ser^{141}, Asp^{142} for CvML3914) residues that are considered to be the principal motifs for calcium binding (Drickamer, 1988, 1993). A secondary calcium-binding motif is also suspected with the conserved residue ENC (Glu^{123}, Asn^{124}, Cys^{125}). Additionally, the two proteins show a QPN (Gln^{115}, Pro^{116}, Asn^{117}) motif, which is determinant for calcium binding and sugar specificity. The first 17 amino acid

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                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
CvML3912         MKTQIIILLA VLTAVLATCP ADWQSYGDKC FFFSRENETF ADALKLCEMI
CvML3914         MKTEIIIFFA ALTAVLATCP ADWNHYRDKC FFLSRENETF ASALKLCEVI

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
CvML3912         GSQYRRVASL ATIDDAGTQK FLANFMRSTG VRAFYFGATD IVHEGTWVWV
CvML3914         GSQYGRVASL ATIDDAGTQN HIANLIKRSG FVTFYIGATD IVHEDTWVWV

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
CvML3912         STGKVFTYTN WGPQQPNNRG GDENCAVLRD SADQVFHMQW GDSPCTTTIN
CvML3914         STGKNATYTN WGPQQPNNRD GAENCAVLNY LPDEGFDMKW SDDPCASYFN

                 ....|....| ....|..
                         160
CvML3912         YICEMAAAEI HSPIVG*
CvML3914         YICEMASAEV HSPLVG*
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	Length (aa)	Mol. mass (Da)	pI	SP
CvML3912	166	18455	5.05	1-17
CvML3914	166	18494	4.71	1-17

Fig. 1. Alignment of the protein sequences of CvML3912 and CvML3914 C-type lectins. Non-matching amino acids are indicated in bold red. Cysteines are indicated in bold green. Conserved ENC triplets and WGD/WSD triplets are boxed in purple. QPN motifs are boxed in red. Signal peptides (SP, blue line) are indicated in the alignment and positions are given in the adjacent table. The table also shows the length (number of amino acids, aa), molecular mass (Da) and isoelectric point (pI) of each protein.

residues from the N-terminus of the two proteins are mostly uncharged, hydrophobic and represent homology to signal peptides known to be present in secreted proteins. One N-glycosylation site (Asn^{37}, Glu^{38}, Thr^{39}, Phe^{40}) and one O-glycolysation site (Thr^{109}) were also predicted to be present in the two proteins.

Localization and regulation of lectin transcripts

For both lectins, transcripts were highest in the digestive gland, followed by the gills, labial palps and finally mantle (Fig. S1). Five days of starvation induced a significant increase in CvML3912 and CvML3914 expression in gills (118- and 18-fold, respectively, ANOVA, $P<0.01$; Fig. 2). Interestingly, the effect of bacterial bath exposure was contrasted for both lectins as it caused a significant increase in the expression of CvML3912 (380.9-fold, ANOVA, $P<0.001$) but a decrease in the expression of CvML3914 (0.2-fold, ANOVA, $P<0.01$).

Recombinant protein activity

rCvML3912 was successfully produced in an insect cell system (Fig. S2) and was tested for its potential carbohydrate-binding activity. In this assay, we determined whether rCvML3912 competitively inhibited the binding of commercial lectins labeled with FITC to microalgae. Results from the assays showed that rCvML3912 significantly altered the binding of the lectin concanavalin A (ConA), pea agglutinin (PEA) and to a lesser extent wheat germ agglutinin (WGA) (K–S test, $P<0.001$; Fig. 3), all of which having a strong affinity for mannose and glucose moieties and related derivatives (e.g. *N*-acetyl-glucosamine; Table S2). Binding of the other lectins [i.e. peanut agglutinin (PNA), *Ulex europaeus* agglutinin (UEA), pokeweed mitogen (PWM) and soybean agglutinin (SBA)] to microalgae was not affected by pre-incubation of the cells with rCvML3912. In parallel, pre-incubating rCvML3912 with a cocktail of D-mannose and D-glucose restored ConA and pea lectin binding to microalgae (Fig. 3). It should be noted that treating microalgae with rCvML3912 did not induce any alteration in the size (FSC), granularity (SSC) or viability of the cells (data not shown).

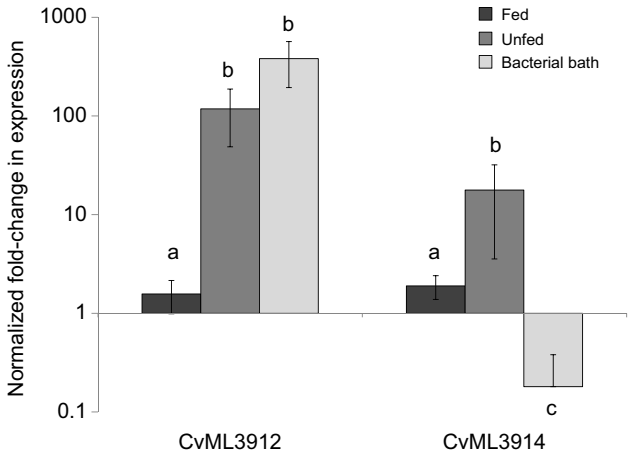

Fig. 2. Expression of CvML3912 and CvML3914 in oyster gills after exposure to starvation or bacterial challenge. Transcript levels were determined by quantitative real-time PCR 5 days after exposure, and were normalized to 18S RNA levels and presented as relative expression (mean ±s.e.m., $n=12$ oysters per data point). Different letters indicate significant differences between treatments (ANOVA, Student–Newman–Keuls, $P<0.05$).

Effect of lectin silencing on particle selection

A series of gene-silencing assays was performed on the oyster *C. virginica* in order to investigate the function of the two mucosal C-type lectins. Our working hypothesis was that these proteins are involved in particle sorting in bivalves and knockdown of the mRNA will significantly decrease this process in oysters. The first set of experiments was primarily done to evaluate the feasibility of the methods and most specifically to determine the efficiency of the DsiRNA in terms of injected dose, possible undesirable effects and the time to obtain a significant response; the second set of experiments explored the effect of the DsiRNAs.

In the first experiment, oysters were injected with a cocktail of two DsiRNAs (Table S3) and subjected to a feeding experiment 46 days post-injection, using a pair of microalgae (*D. salina* and *P. marinus*). As expected based on earlier work (Pales Espinosa et al., 2016a), the prasinophyceae *P. marinus* was significantly rejected in oyster pseudofeces, including among silenced oysters (SEs significantly different from zero, one-sample *t*-test, $P<0.01$). More interestingly, a significant change in the proportion of the two microalgae present in the pseudofeces of animals injected with the two DsiRNAs was observed as compared with oysters injected with FSW or the negative probe control NC5 (goodness of fit test, $n=12$, $P<0.001$). This translated into a significant reduction of the calculated sorting efficiency (Fig. 4). Further, transcription levels of CvML3912 and CvML3914 were measured using qPCR. Even

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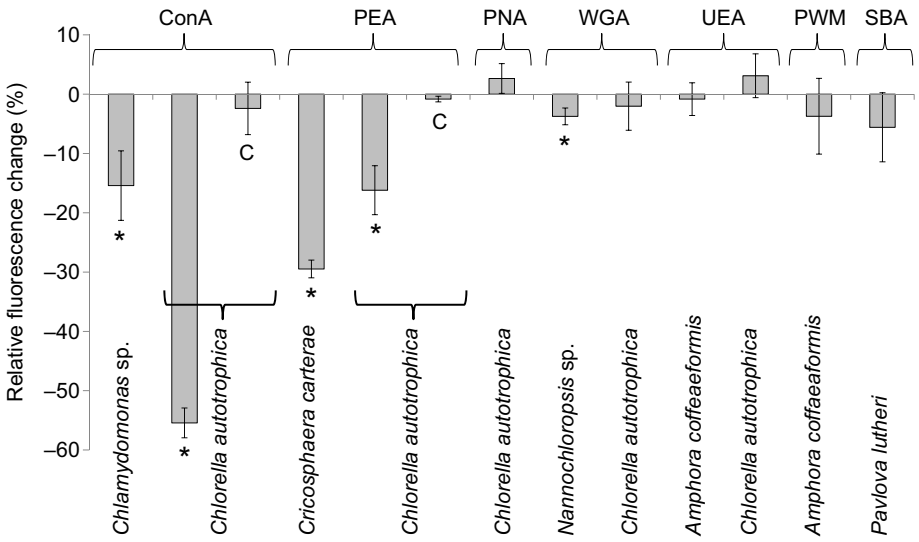

Fig. 3. Effect of recombinant lectin (rCvML3912) on the binding of FITC-labeled lectins to different microalgae species. Data are means±s.d., *n*=3 replicate cultures. Microalgae were labeled with FITC-lectins known to display the highest affinity to each target species (Pales Espinosa et al., 2016a). The letter 'C' indicates the incubation of rCvML3912 with a D-mannose/D-glucose cocktail before exposure to microalgae. Asterisks indicate a significant reduction in relative fluorescence compared with controls not treated with rCvML3912 (represented by the *x*-axis, Kolmogorov–Smirnov test, $P<0.001$). A description of the lectins (ConA, concanavalin A; PEA, *Pisum sativum* lectin; PNA, peanut agglutinin; WGA, wheat germ agglutinin; UEA, *Ulex europaeus* agglutinin; PWM, pokeweed mitogen; SBA, soybean agglutinin) is given in Table S2.

though the expression of the two genes decreased in silenced oysters, this decrease was not significant. The expression of the two genes slightly increased in oysters injected with NC5 (control) treatment, although this change was also not significant.

In the second experiment, treated oysters were injected with a cocktail of five DsiRNAs (Table S3) and subjected to feeding experiments 34 days post-injection, using the same pair of microalgae (*D. salina* and *P. marinus*). *Prasinocladus marinus* was significantly rejected in bivalve pseudofeces as in the first experiment. Similarly, a significant change in the proportion of the two microalgae present in the pseudofeces of oysters injected with the five DsiRNAs was observed as compared with oysters injected with FSW or NC5 (goodness of fit test, $n=12$, $P<0.001$). As a result, the calculated SE significantly decreased among silenced oysters (Fig. 4; Fig. S3). Concomitantly, the expression of both lectins significantly decreased in silenced oysters as compared with FSW and NC5 controls (Fig. 4). As observed in the first experiment, the expression of the two genes slightly increased in oysters injected with NC5 (as compared with FSW controls), but this change was not significant.

Finally, the results showed a significant positive correlation between SE and the gene expression levels of CvML3912 and CvML3914. A non-linear regression (NLS) approach was applied and the best fit was obtained using an exponential function combining expression levels of both lectins (AIC=−28.59, $r^2=0.42$).

DISCUSSION

This study provides the first direct evidence for the involvement of lectins in food particle recognition and sorting in metazoans. Retrospectively, a role for carbohydrate–lectin interactions in food sorting in suspension-feeding bivalves is not surprising. Carbohydrates covering the cell surface are information-rich ligands for carbohydrate-binding receptors that function as recognition molecules in a wide range of biological systems (Sharon and Lis, 2004), and the use of such ligands would easily allow the discrimination between living (nutrient-rich) and non-living particles. Thousands of lectins have been identified in animals (Vasta et al., 2004; Zelensky and Gready, 2005), among which C-type lectins represent a remarkable family because of their structural and functional diversity (Drickamer and Taylor, 1993; Zelensky and Gready, 2005). These proteins act as signaling receptors and play a major role in many biological functions, particularly non-self-recognition and host–microbe interactions (Sharon and Lis, 2004; Weiss et al., 1998). In particular, C-type lectins have been shown or suggested to mediate recognition and acquisition of symbiotic microbes in several marine invertebrate species (Bulgheresi et al., 2006; Gourdine et al., 2007; Kvennefors et al., 2008). In parallel, the role of C-type lectins in predator–prey interactions has been demonstrated among planktonic microbes, such as the protozoan *Actinophrys sol* which produces a calcium-dependent lectin-like molecule that mediates prey capture (Kakuta and Suzaki, 2008; Sakaguchi et al., 2001). Similarly, Wootton et al. (2007) demonstrated the involvement of a Ca^{2+}-dependent mannose-binding lectin as a feeding receptor for recognizing prey in the marine dinoflagellate *Oxyrrhis marina*. Our findings expand the role of carbohydrate–protein interactions in particle sorting to suspension-feeding bivalves.

We focused this work on the two most abundant lectins found in pallial mucus, annotated as C-type mannose receptor 2 (Pales Espinosa et al., 2016b). Among the four Ca^{2+}-binding sites identified in CvML3912 and CvML3914, site 2 is well conserved and is known to be the only one involved in carbohydrate-binding activity (Zelensky and Gready, 2005). This specific site contains a conserved ENC residue and another triplet motif, either WGD (CvML3912) or WSD (CvML3914), both of which are essential for calcium-binding activity (Drickamer, 1988, 1993). Although this last motif is highly conserved in vertebrates (always WND), it is more versatile in mollusks and several other triplets have been identified (reviewed by Wang et al., 2011). This motif is thought to promote the affinity and the specificity of carbohydrate binding. Finally, the two identified lectins present a QPN motif, which is also involved in calcium-binding activity and determines carbohydrate specificity. Typically, EPN and QPD motifs have been found in vertebrate C-type lectin CRDs, but in mollusks, this motif is very variable, including, for example, QPS (Pales Espinosa et al., 2010a), YPD (Jing et al., 2011) and EPD (Huang et al., 2015). Although EPN and QPD have been found to bind mannose and galactose residues, respectively (Drickamer, 1992), it is more difficult to assign carbohydrate specificity of the other triplets. Nevertheless, the elegant study by Yang et al. (2015) demonstrated that the proline (P) residue in the triplet was indispensable for ligand binding and that the asparagine (N) was involved in mannan binding. Based on these observations, it could be hypothesized that the QPN motif

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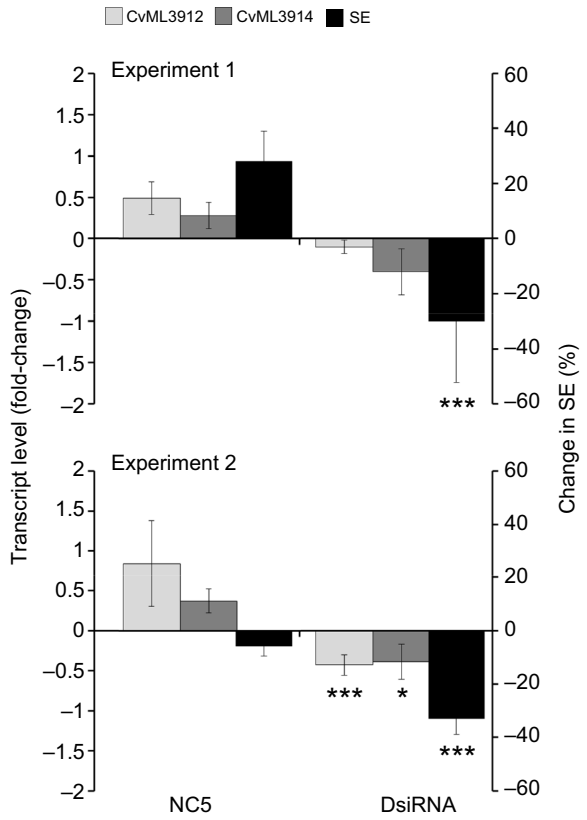

Fig. 4. Relative change in gene expression and sorting efficiency of *Dunalliela salina* in silenced (DsiRNA) and control (NC5) oysters compared with oysters injected with seawater. Data are means±s.e.m. In experiment 1 (46 days post-injection, two complementary strands of DsiRNA; see Table S3 for details), a significant decrease of sorting efficiency (SE) was measured but changes in CvML3912 and CvML3914 transcript levels were not significant. In experiment 2 (34 days post-injection, five complementary strands of DsiRNA; see Table S3 for details), a significant decrease was measured in SE as well as gene transcript levels. For both experiments, no significant differences were noted between oysters injected with NC5 and seawater (represented by the *x*-axis). Asterisks denote differences between silenced oysters and those injected with seawater (**P*<0.05 or ****P*<0.001).

found in the two C-type lectins displays specificity for mannose residues. This is further supported by the binding activity of the recombinant CvML3912 to microalgae cell surface that competitively inhibited the binding of ConA and pea agglutinin. These findings strongly suggest that these molecules (i.e. recombinant CvML3912, ConA and pea agglutinin) bind to the same active sites on microalgae cell surface and have similar specificity for carbohydrates. ConA (from *Canavalia ensiformis*) is known to specifically bind α-D-mannosyl and α-D-glucosyl residues (Loris et al., 1998). PEA (lectin from *Pisum sativum*) is also known to be specific to D-mannose and D-glucose residues as well as, but to a lesser extent, D-fructose and L-sorbose (Kornfeld et al., 1981; Van Wauwe et al., 1975). The fact that pre-treating rCvML3912 with D-mannose and D-glucose inhibits its binding to microalgae further supports the specificity of rCvML3912 to these carbohydrates.

While the food-sorting ability of oysters was positively correlated with the level of CvML3912 and CvML3914 transcripts, it was not completely inhibited in silenced oysters. Our previous investigations demonstrated the presence of several other, less abundant, mucosal lectins in oysters (Pales Espinosa et al., 2016b), some of which were suggested to play a role in feeding (Jing et al., 2011). It is therefore possible that such lectins (e.g. CvML, Jing et al., 2011) may take over and functionally replace the ones that were knocked down to help maintain a certain level of food-sorting ability. In addition, inter-individual variability was observed among silenced oysters, with some being very responsive and some not. This variability may in part be attributed to the injection protocol. Indeed, it was not possible to inject the DsiRNA cocktail directly into the gills and palps because of the fragile nature of these organs and consequently the DsiRNAs were injected into the circulatory system (adductor muscle), on the assumption that the hemolymph would efficiently transport the DsiRNA to the target feeding organs. Thus, it is possible that in some individuals, the DsiRNAs did not fully reach their target. The overall physiological state of individual oysters may also contribute to some of the variability we measured between oysters even though the results showed similar trends during two different periods of the year (spring and autumn).

Despite the high sequence similarity between CvML3912 and CvML3914, the regulation of the two lectins differed. For instance, starvation caused an upregulation of both CvML3912 and CvML3914, suggesting that oysters may increase lectin production to enhance the capture and selection of scarce food particles (Jing et al., 2011). In contrast, bacterial challenge induced an upregulation in CvML3912 and a downregulation in CvML3914, suggesting that these two lectins may have different roles, or at least different kinetics, during immune response. These results are not surprising as C-type lectins have been found to be involved in various immunological functions in mollusks, in particular as pattern recognition receptors where they can mediate microbe opsonization and encapsulation (Bulgakov et al., 2004; Kim et al., 2006; Takahashi et al., 2008; Yang et al., 2011), sometimes displaying bacteriostatic or bactericidal activity (Takahashi et al., 2008; Wang et al., 2007). The specific function of CvML3912 and CvML3914 in immune processes requires further investigation, but it should be noted that we did not find any systemic effect of DsiRNA injection in oysters as internal immune parameters (total and differential blood cell counts, hemocyte phagocytic activity and reactive oxygen production) were unchanged among silenced oysters (data not shown).

Overall, the results presented here are in agreement with previous data demonstrating that oysters (and mussels) preferentially ingest microalgae species with a cell surface enriched with mannose/glucose residues (Pales Espinosa et al., 2016a). Altogether, this information demonstrates that interactions between microalgae cell surface mannose/glucose residues and mucosal carbohydrate-binding proteins are a major driver of particle selection, at least in *C. virginica*. The preference of oysters for microalgae with cell surfaces enriched with mannose-related residues has important ecological implications. For example, it is now conceivable to develop predictive models to forecast the selective predation pressure exerted by oysters on a phytoplankton community based on the surface carbohydrate profiles of each species within the community (Pales Espinosa et al., 2016a). A quantitative assessment of the sorting behavior, based on carbohydrate and mucosal lectin profiles, could be developed and would be of major interest for ecophysiological models of bivalve growth and reproduction that currently overlook pre-ingestive selection processes (Barillé et al., 2011).

Beyond benthic–pelagic coupling, the significance of our findings can be extended to the uptake and ingestion of abiotic particles present in seawater, including anthropogenic contaminants such as microplastics. For instance, the aging of microplastics in the natural environment involves the formation of a biofilm that could provide the carbohydrate ligands recognized by suspension-feeding bivalves, triggering selective sorting and ingestion. In this framework, the recently reported enhanced uptake of aged

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microplastics by zooplankton (Vroom et al., 2017) may involve similar processes. In contrast, most investigations evaluating the concentration and behavior of microplastics in bivalve mollusks during laboratory studies have used 'clean' engineered particles void from any surface ligands, possibly underestimating particle ingestion and concentration in bivalve tissues. With that regard, our prior work showed that coating polystyrene beads with mannose residues enhances ingestion in mussels (Pales Espinosa et al., 2010c). Obviously, other factors (e.g. the nature and shape of the particles) affect the pre-ingestive fate of abiotic particles but a better consideration of surface carbohydrate profiles of these particles during field and laboratory studies is warranted if the aim is to provide an ecologically relevant assessment of exposure risk.

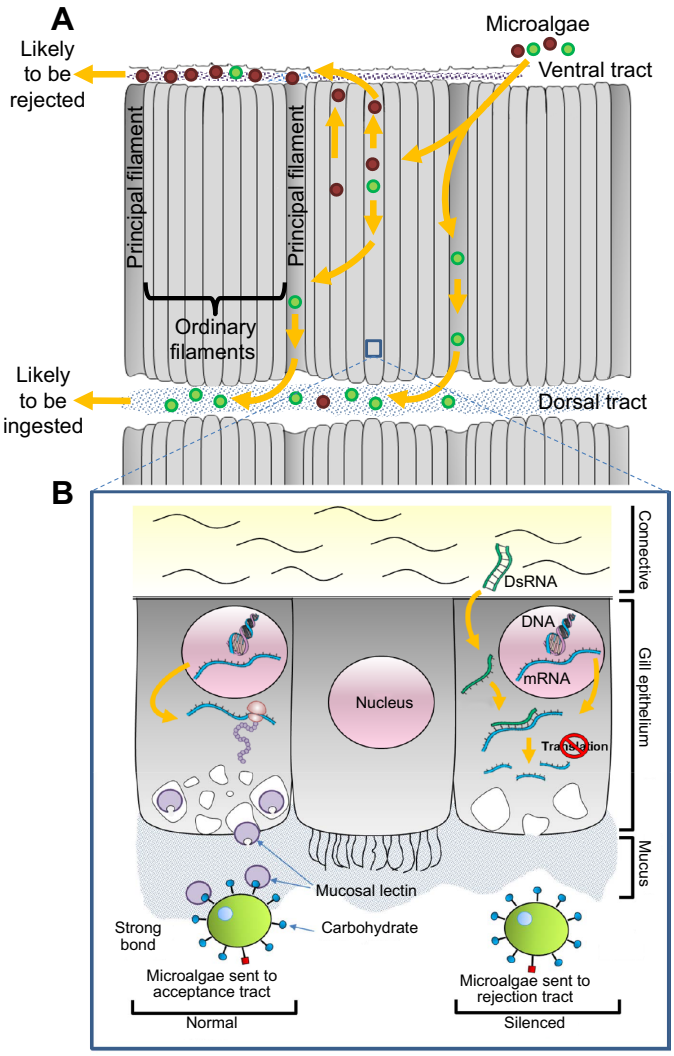

Fig. 5. Simplified schematic representation of particle selection in the oyster *Crassostrea virginica* including proposed roles of lectin–carbohydrate interactions. (A) Selective capture and transport of particles on oyster gill. Particles entering the principal filament are directed to the dorsal tract while those on ordinary filaments could be transported either to the ventral tract or to the dorsal tract via the principal filaments. It is hypothesized that the assignment of particles to the dorsal tract involves mannose/glucose-binding lectins present in mucus covering the gills (B) and inhibition of these proteins following dsRNA injection reduces particle selection. The precise, sub-organ-level mechanisms directing particle transport remain to be defined. Not to scale. A is based on data from Ribelin and Collier (1977) and Ward et al. (1994).

In conclusion, we provide direct evidence for the involvement of mucosal C-type, mannose-binding lectins in prey recognition and capture in the eastern oyster. These findings validate a novel concept establishing the role of carbohydrate–protein interactions to provide efficient food particle sorting, and establish a new dimension for the role of evolutionarily conserved mannose/glucose-binding proteins in metazoans. Suspension-feeding bivalves are able to regulate food uptake and sorting based on their physiological demands (Bayne and Svensson, 2006; Pales Espinosa and Allam, 2013). In this context, the regulation of particle uptake and sorting may be operated via modulation of lectin expression, therefore providing bivalves with a flexible means to qualitatively and quantitatively control particle uptake to satisfy internal requirements (Pales Espinosa and Allam, 2013). Assuming that the energetic cost of lectin production is lower than that of behavioral changes such as increasing filtration rate, the regulation of lectins would represent an efficient strategy for the capture of nutritious particles when food is restricted (Pales Espinosa and Allam, 2013). This cost-efficient energetic strategy perfectly fits the general functional model of bivalve feeding proposed by Willows (1992).

Prior work in *C. virginica* showed that particles intended for ingestion or rejection are transported via 'mucus conveyor belts' along the dorsal (typically for ingestion) or ventral (typically for rejection) tracts of the gills (Ribelin and Collier, 1977; Ward et al., 1994). Therefore, the most plausible scenario is that carbohydrate–lectin interactions likely create a molecular bond sufficiently robust to allow carbohydrate-rich food particles (typical of live microalgae) to be transported to the food acceptance tract (Fig. 5) and directed to the mouth, while particles lacking these ligands (typical of mineral and detrital particles) are preferentially directed to food rejection pathways and eliminated as pseudofeces. Interestingly, the chemical and physical characteristics of mucosal secretions in the two tracts was suggested to be different (Beninger and Dufour, 1996; Beninger and St-Jean, 1997), and our preliminary proteomics analysis of mucus from each of these tracts shows enrichment of mannose-binding proteins in the dorsal tract of *C. virginica*, supporting our scenario. Nevertheless, the precise, sub-organ level, role of mucus in the processing of particles on the feeding organs needs to be defined. It also remains to be determined whether carbohydrate–protein interactions represent a main driver for food particle selection among other suspension-feeding organisms.

Acknowledgements
We would like to thank Frank M. Flower and Sons Oyster Company (Oyster Bay, NY, USA) for providing oysters, and Prof. Robert Cerrato, Dr Dwight Martin and Dr Erwan Corre for valuable help during this study. We also thank Drs C. Fabioux (UBO, France) and C. Corporeau (Ifremer, France) for valuable discussions regarding gene silencing strategies.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.P.E., B.A.; Methodology: E.P.E., B.A.; Validation: E.P.E.; Formal analysis: E.P.E.; Investigation: E.P.E., B.A.; Resources: E.P.E.; Writing - original draft: E.P.E.; Writing - review & editing: E.P.E., B.A.; Visualization: E.P.E., B.A.; Supervision: E.P.E.; Project administration: E.P.E., B.A.; Funding acquisition: E.P.E., B.A.

Funding
This work was funded by a grant from the National Science Foundation (IOS-1656753 to E.P.E. and B.A.).

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.174094.supplemental

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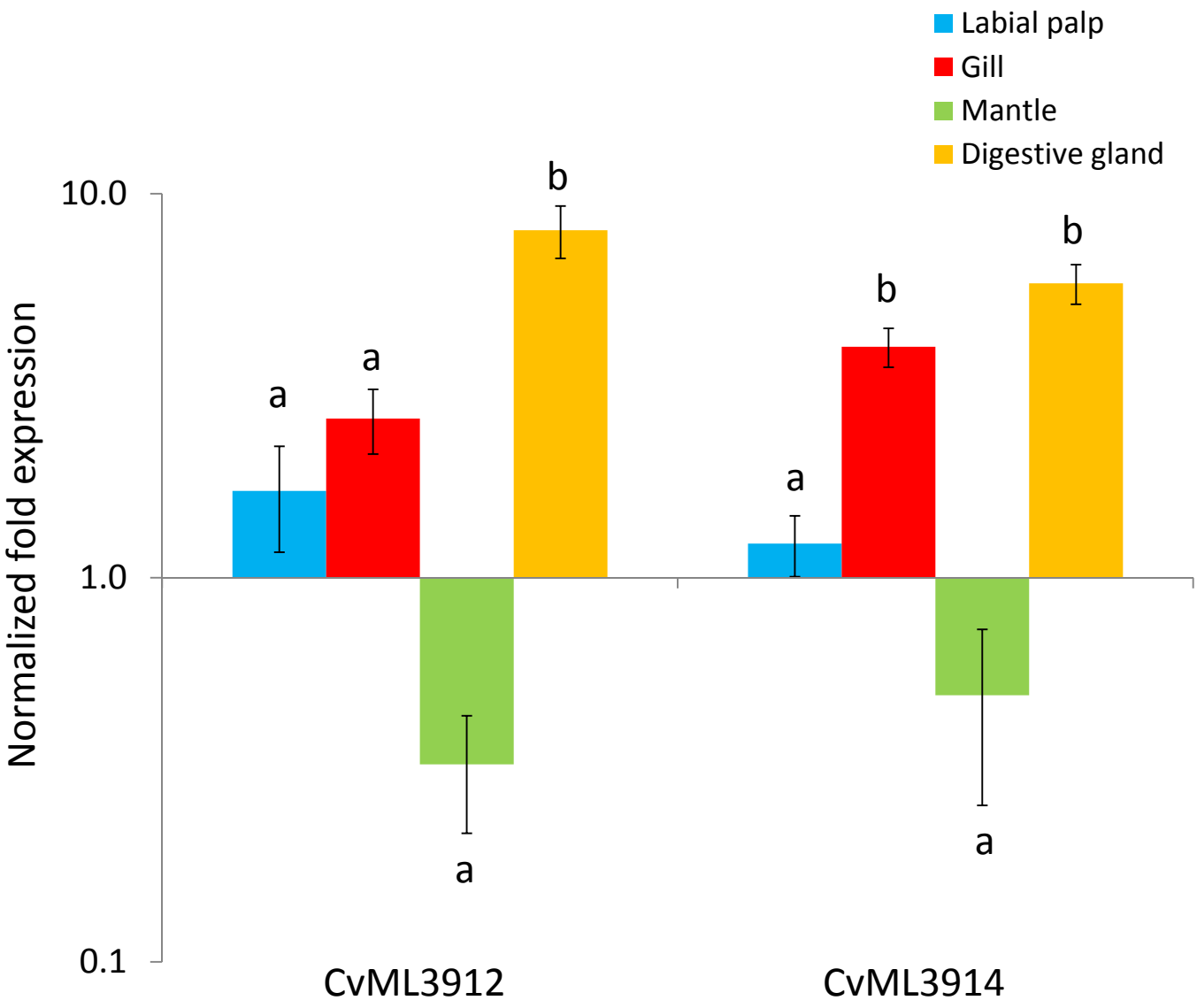

Figure S1. Relative expression of CvML3912 and CvML3914 in oyster tissues determined by quantitative real-time PCR. Expression levels were normalized to 18S RNA and are presented as relative expression (mean ± SEM, n=8 oysters). Different letters indicate significant differences between tissues (ANOVA, SNK, $p < 0.05$).

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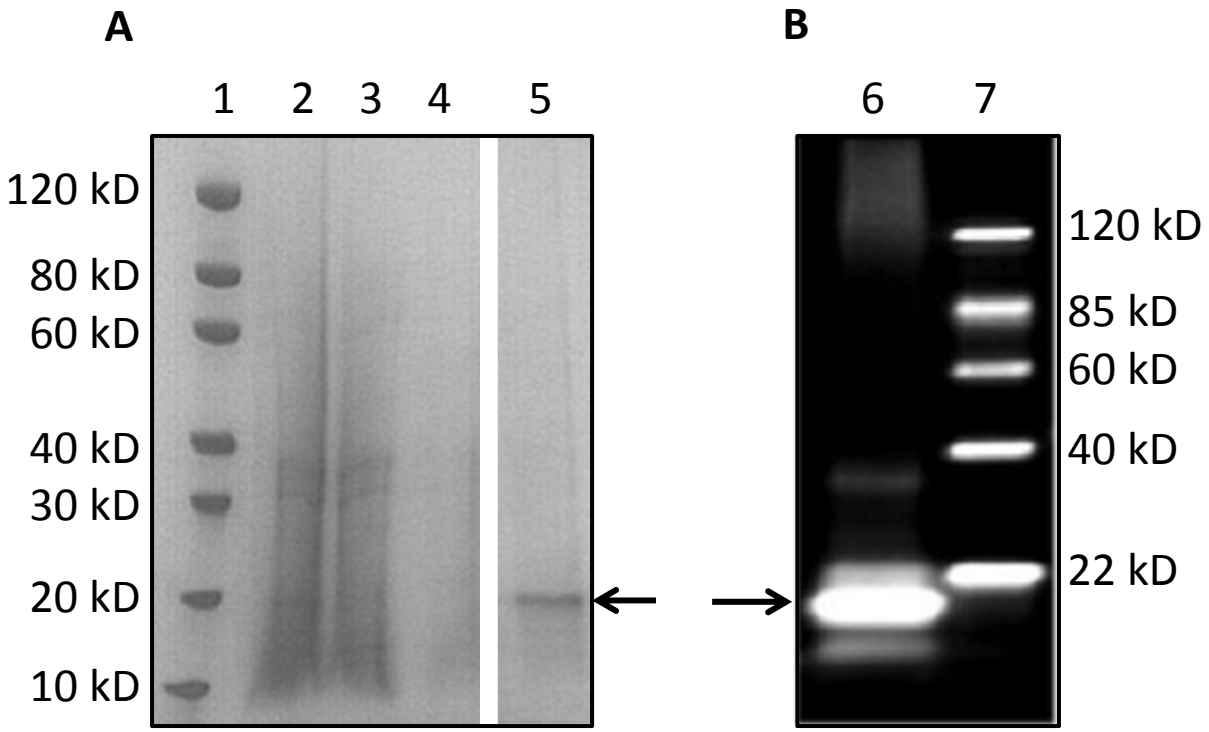

Figure S2. SDS-page (A) and Western Blot (B) analysis of the recombinant CvML3912 protein (rCvML3912). The SDS-PAGE was run on 4%~20% gradient gel, followed by Coomassie Blue staining. Lane 1: Protein marker (M00516, GenScript), Lane 2: Load, Lane 3: Flow through, Lane 4: Final wash, Lane 5: Elution with urea buffer and 300 mM imidazole, Lane 6: Western blot using anti-His antibody (A00186, GenScript), Lane 7: Protein marker (MM0908, GenScript). Arrows indicate rCvML3912.

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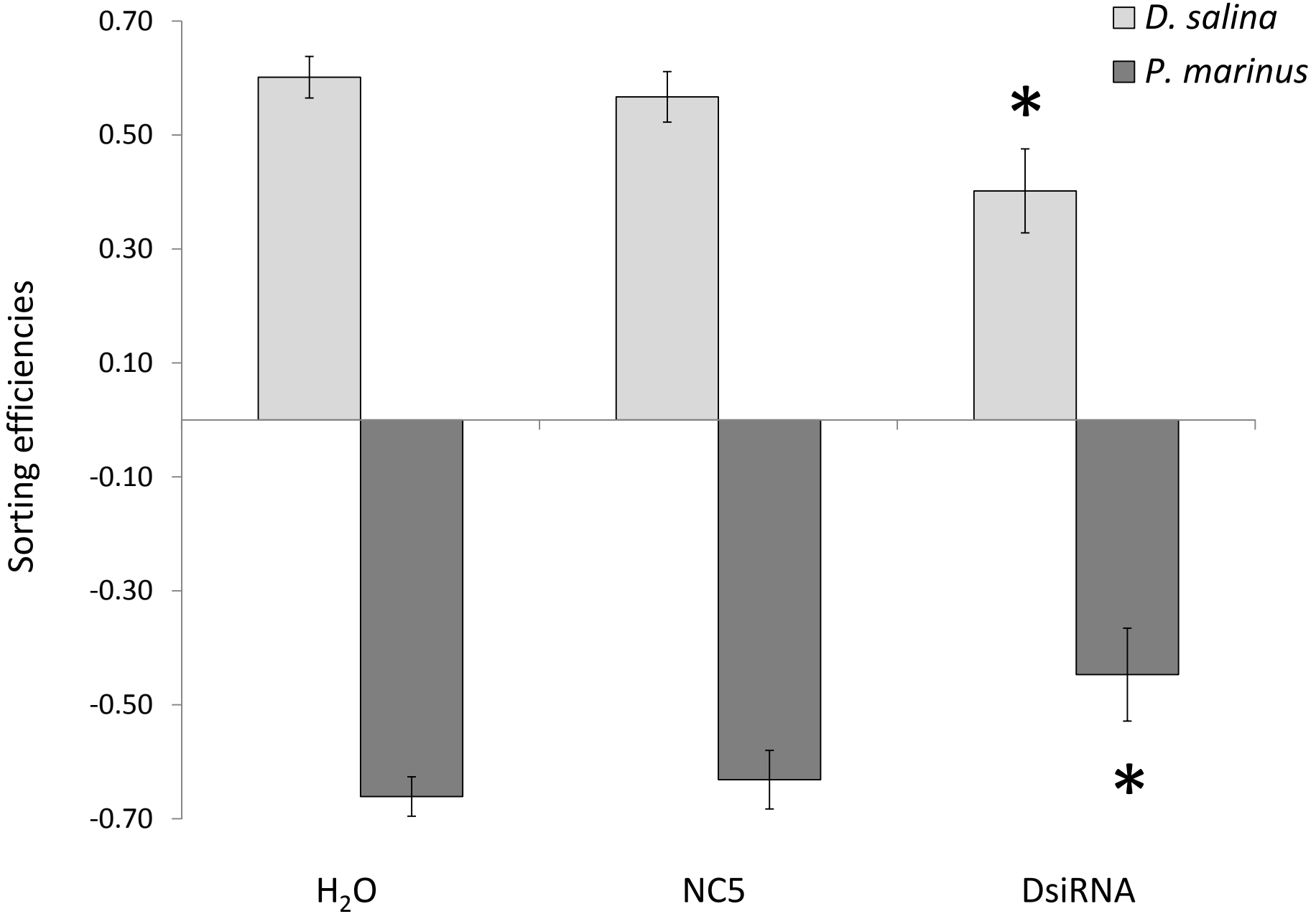

Figure S3.

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Figure S3. Sorting efficiencies (SE, mean ± SEM) of *C. virginica* injected (34 days post-injection, experiment 2) with either seawater (H2O), NC5 or DsiRNAs and fed a diet made with equal proportions of the *D. salina* and *P. marinus*. SE are significantly different from 0 for all treatments (one sample *t*-test, n =12, $p<0.001$). Asterisks indicate a significant change in SE (G-test, n=12, $p<0.001$) among silenced oysters (DsiRNAs) as compared to oysters from the two other treatments. No difference was observed between the two controls (H2O and NC5).

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Table S1. LC-MS/MS detection of specific peptides from the two proteins CvML3912 and CvML3914. Position (starting and ending) of each peptide on the corresponding protein sequences, number of spectral count and Log(e) values (subscript) are indicated.

		c102634_g1_i1_3912		c102634_g1_i2_3914	
PEPTIDES	**Position**	**Assay1**	**Assay2**	**Assay1**	**Assay2**
MKTQIIILLAVLTAVLATCPADWQSYGD	1-28		1 (-1.9)		
TCPADWQSYGDK	18-29	2 (-5.7)	2 (-4.1)		
TCPADWNHYR	18-27			7(-3.5)	
DKCFFLSR	28-35			2 (-2.8)	
LCEVIGSQYGR	46-66			2 (-6.9)	4 (-6.3)
LCEMIGSQYR	46-65	6 (-5.6)	14 (-6.6)		
RVASLATIDDAGTQK	66-70	3 (-4.7)			
VASLATIDDAGTQNHIANLIK	67-77			21 (-14.7)	14 (-11.3)
VASLATIDDAGTQK	67-70	2 (-13.2)	2 (-11.0)		
TIDDAGTQNHIANLIK	62-70			2 (-5.0)	2 (-6.5)
VFTYTNWGPQQPNNR	105-119	7 (-5.5)	4 (-8.5)		
DGAENCAVLNYLPDEGFDMK	120-139			4 (-13.3)	6 (-4.2)
GGDENCAVLR	120-129	2 (-3.8)	3 (-2.7)		

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Table S2. FITC lectins used to characterize microalgae cell surface carbohydrates. Origin and carbohydrate specificity (provided by the manufacturer EYLabs) of each lectin are given.

Name	Origin	Carbohydrate specificity
ConA	*Canavalia ensiformis*	α-D-Mannose, α-D-Glucose, branched mannose
PEA	*Pisum sativum*	Methyl-D-Mannopyranoside, D-Mannose
PNA	*Arachis hypogaea*	Terminal β-Galactose
PWM	*Phytolacca americana*	Oligomers of β(1,4)-linked N-Acetylglucosamine
SBA	*Glycine maxima*	α and β- N-Acetylgalactosamine, α and β- Galactose
UEA	*Ulex europaeus*	α-L-Fucose
WGA	*Triticum vulgaris*	Chitobiose, N-Acetyl-glucosamine

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Table S3. Primers and DsiRNA used in this study. NC5 represents the negative control DsiRNA and was used in both experiments.

Name	Sequence (5’-3’)
qRT-PCR primers	
18S-F	CGCCGGCGACGTATCTTTCAA
18S-R	CTGATTCCCCGTTACCCGTTA
CvML3912-F	GTTCCTGGCAAATTTTATGCGAA
CvML3912-R	AATGAAAGCCGCAGAATCGG
CvML3914-F	CCACATAGCAAACCTCATTAAAC
CvML3914-R	AATCTGAAGCACATGGGTC

Name	Sequence (5’-3’)	Exp1	Exp2
DsiRNA			
DsiRNA-1_Sense	rCrArGrCrArGrArUrCrArArGrUrArUrUrCrCrArCrArUrGCA	✓	
DsiRNA-1_AntiSense	rUrGrCrArUrGrUrGrGrArArUrArCrUrUrGrArUrCrUrGrCrUrGrArG	✓	
DsiRNA-2_Sense	rCrUrGrArCrArGrCrArGrUrUrCrUrUrGrCrArArCrArUrGCC		✓
DsiRNA-2_AntiSense	rGrGrCrArUrGrUrUrGrCrArArGrArArCrUrGrCrUrGrUrCrArGrGrA		✓
DsiRNA-3_Sense	rGrUrGrArGrArUrGrArUrUrGrGrUrUrCrArCrArArUrArCCG	✓	✓
DsiRNA-3_AntiSense	rCrGrGrUrArUrUrGrUrGrArArCrCrArArUrCrArUrCrUrCrArCrArG	✓	✓
DsiRNA-4_Sense	rCrCrArUrGrUrGrCrUrUrCrArGrArUrUrUrCrArArUrUrATA		✓
DsiRNA-4_AntiSense	rUrArUrArArUrUrGrArArArUrCrUrGrArArGrCrArCrArUrGrGrGrU		✓
DsiRNA-5_Sense	rGrCrCrArCrCrGrArCrArUrCrGrUrCrCrArUrGrArArGrATA		✓
DsiRNA-5_AntiSense	rUrArUrCrUrUrCrArUrGrGrArCrGrArUrGrUrCrGrGrUrGrGrCrUrC		✓
DsiRNA-6_Sense	rGrUrGrCrCrGrUrGrCrUrUrArArUrUrArCrUrUrArCrCrAGA		✓
DsiRNA-6_AntiSense	rUrCrUrGrGrUrArArGrUrArArUrUrArArGrCrArCrGrGrCrArCrArG		✓
NC5_Sense	rCrArUrArUrUrGrCrGrCrGrUrArUrArGrUrCrGrCrGrUrUrArG	C	C
NC5_AntiSense	rUrGrGrUrArUrArArCrGrCrGrCrArUrArUrCrArGrCrGrCrArArUrC	C	C