Expression of biomineralization-related ion transport genes in *Emiliania huxleyi*

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**Summary**

Biomineralization in the marine phytoplankton *Emiliania huxleyi* is a stringently controlled intracellular process. The molecular basis of coccolith production is still relatively unknown although its importance in global biogeochemical cycles and varying sensitivity to increased pCO2 levels has been well documented. This study looks into the role of several candidate Ca2+, H+ and inorganic carbon transport genes in *E. huxleyi*, using quantitative reverse transcriptase PCR. Differential gene expression analysis was investigated in two isogenic pairs of calcifying and non-calcifying strains of *E. huxleyi* and cultures grown at various Ca2+ concentrations to alter calcite production. We show that calcification correlated to the consistent upregulation of a putative HCO3− transporter belonging to the solute carrier 4 (SLC4) family, a Ca2+/H+ exchanger belonging to the CAX family of exchangers and a vacuolar H+ATPase. We also show that the coccolith-associated protein, GPA is down-regulated in calcifying cells. The data provide strong evidence that these genes play key roles in *E. huxleyi* biomineralization. Based on the gene expression data and the current literature a working model for biomineralization-related ion transport in coccolithophores is presented.

**Introduction**

Coccolithophores, unicellular calcifying marine algae, are a key component of today’s ocean playing an important role in nutrient and carbon cycling, as well as contributing as much as half of current oceanic calcite production (Milliman, 1993). *Emiliania huxleyi* (division Haptophyta class Prymnesiophyceae) is the most abundant coccolithophore species, producing extensive blooms at temperate latitudes (Holligan et al., 1983; Fernandez et al., 1993; Holligan et al., 1993). Despite the obvious importance of coccolithophores, the function of coccoliths and the cellular processes underlying coccolith formation remain largely un-resolved. The intracellular production of coccoliths of a size approaching the diameter of a single cell together with the rapid rate of coccolith production [-1 per hour (Paasche, 1962)] presents unique questions for how Ca2+, H+ and inorganic carbon (C) balance is regulated in the cell. Mass balance equations show that rapid rates of Ca2+ and C uptake must occur at the cell plasma membrane (PM) and at the intracellular biomineralization compartment, the coccolith vesicle (CV). The molecular identities of the transport mechanism for delivery of substrates and removal of products are urgently required for the formulation of cellular models that may help to understand the energetics of calcification and its vulnerability to changes in ocean chemistry associated with ocean acidification.

Calcite crystal growth in the CV is highly regulated and involves an organic baseplate (van der Wal et al., 1983) and possible protein matrix (Corstjens et al., 1998) interacting with polysaccharides (de Jong et al., 1976) to control crystal growth. The mature coccolith is transported to the cell periphery and extruded onto the cellular surface. Since Paasche’s pioneering work on coccolithophore biology (Paasche, 1962), a range of approaches have been applied to understand the mechanisms underlying calcification. Cell physiology studies have mainly investigated; PM transport identifying a novel Cl− inward rectifying current in *Coccolithus pelagicus* (Taylor and Brownlee, 2003), pH homeostasis indicating high PM H+ permeability (Suffrian et al., 2011) and real-time coccolith production showing that coccoliths are extruded across the PM in a single exocytotic event (Taylor et al., 2007). Cell biology and molecular studies have begun to examine the cellular mechanisms of calcification,
including the characterization of two carbonic anhydrases (Soto et al., 2006) and a vacuolar H^+-ATPase (Corstjens et al., 2001), confirming their functions and localizing the vacuolar H^+-ATPase to an endomembrane fraction. A recent quantitative RT-PCR study has analysed putative biomineralization genes from *E. huxleyi* grown at ambient and high pCO_2 showing variation in carbonic anhydrase gene expression but insignificant expression changes in other investigated genes (Richier et al., 2010). Finally, a comparative whole genome transcriptomic study of diploid calcifying cells and isogenic haploid non-calcifying flagellated cells identified many genes that were exclusively expressed in the diploid cells and that may therefore function in biomineralization (von Dassow et al., 2009). The identification of multiple putative biomineralization genes in *E. huxleyi* lays a strong foundation for future investigation, although there are clearly many factors other than the process of biomineralization contributing to differential gene expression between haploid and diploid stages of the life cycle.

In the current study we have applied quantitative reverse transcriptase PCR (qRT-PCR) to analyse transcript levels of eight genes with putative roles in calcification. To examine membrane transport processes related to coccolithophore biomineralization, target genes involved in Ca^{2+}, inorganic carbon (C_i) and H^+ transport and Ca^{2+} binding were selected (Table 1). The majority of these genes were selected from the comparative transcriptomic study of gene expression between coccolithophore life cycle stages (von Dassow et al., 2009), although additional genes implicated in calcification were selected from the current literature. Ca^{2+} transport genes included two putative Ca^{2+/H^+} exchangers (*CAX3* and *CAX4*) and an ER-type Ca^{2+}-ATPase (*ECA2*). Two genes with putative roles in C_i utilization were targeted: a putative HCO_3^- transporter belonging to the SoLute Carrier 4 family (SLC4, termed *AEL1* for anion exchanger like 1) and a gamma carbonic anhydrase (γ-EhCA2), which has previously been cloned and partially characterized with the authors speculating a CV location (Soto et al., 2006). The H^+ transport genes selected for investigation were a voltage gated H^+ channel (*HVCN1*) and subunit c of the V_0 sector of a vacuolar H^+-ATPase (ATPVac/c) previously cloned from *Pleurochrysis carterae* (Corstjens et al., 2001). Of these ion transporters, *CAX3* and *AEL1* are of particular interest as they were both exclusively expressed in the calcifying diploid stage of the life cycle (von Dassow et al., 2009). A Ca^{2+} binding protein shown to be associated with coccolith polysaccharides known as GPA (Corstjens et al., 1998) was also investigated. Here we probe further their expression patterns under different calcification scenarios, identifying key *E. huxleyi* biomineralization-related genes.

To test the role of putative biomineralization genes in *E. huxleyi*, expression patterns were examined under three different calcification scenarios. Relative gene expression patterns were initially investigated between a diploid (calcifying) and haploid (non-calcifying flagellated form) using the strains RCC1216 and RCC1217, with RCC1217 being a stable naturally occurring haploid isolate of RCC1216 that is thought to play a gametal role in the *E. huxleyi* life cycle. Next to eliminate gene expression variations due to cell ploidy level and life cycle stage, a comparison was made between the calcifying diploid strain CCMP1516 and CCMP1516NC (a stable naturally occurring non-calcifying isolate from CCMP1516). Finally to confirm that expression of the putative calcification genes was directly related to calcification, strain CCMP1516 was cultured in different Ca^{2+} concentrations to observe the effect of inhibiting calcification on gene expression.

**Results**

*RCC1216 vs. RCC1217 and CCMP1516 vs. CCMP1516NC comparisons*

The absence of calcification in the proposed non-calcifying *E. huxleyi* strains RCC1217 and CCMP1516NC, was confirmed by measurement of PIC production rates and from scanning electron microscope (SEM) images. POC production rates were similar between the calcifying strains and the non-calcifying strains. The PIC production rate was nearly twice as high in the highly calcified strain RCC1216, which produces heavily calcified R-type coccoliths, compared with the CCMP1516 strain with A-type coccoliths (Table 2 and Fig. 1). The SEM preparation method used was unsuitable for the haploid strain RCC1217 with cells disintegrating on the filter membrane during sample processing. For the diploid non-calcifying strain, CCMP1516NC SEM preparation resulted in some collapsed cells on the filter membrane (Fig. 1).

To ensure that strain variations played a minimal role in our analysis of gene expression, microsatellite loci lengths were analysed in the strains used to confirm the pairs were isogenic. Primers to five microsatellite loci were used and amplicon lengths analysed (Table 3). CCMP1516NC, a non-calcifying isolate from CCMP1516, had identical loci lengths to CCMP1516. All the loci for RCC1217, a haploid isolate from RCC1216, correlated to RCC1216, although as expected due to its haploid nature some loci were not present.

The expression of eight target genes (Table 1) was analysed using qRT-PCR. Expression was normalized to two endogenous reference genes (ERGs), actin and elongation factor like 1 protein (*EFG1*) that were shown to be the most stable of the three potential ERGs.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Full name</th>
<th>Putative function</th>
<th>Sequence ID</th>
<th>JGI protein ID</th>
<th>Primer name</th>
<th>Primer sequence 5′-3′</th>
<th>Amplicon size</th>
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<td><strong>Actin</strong></td>
<td>Actin</td>
<td>Cytoskeleton protein</td>
<td>S64188.1*</td>
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<td>Actin_F</td>
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<td></td>
<td></td>
<td>S64193.1*</td>
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<td>S64192.1*</td>
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<td>S64191.1*</td>
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<td>S64189.1*</td>
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<tr>
<td><strong>EFG1</strong></td>
<td>Elongation factor 1</td>
<td>Protein elongation during translation. Similar to EFL – elongation factor like protein</td>
<td>GS00217</td>
<td>462457</td>
<td>EFG1_F</td>
<td>GCT GGA AGA AGG ACT TTG TTG</td>
<td>101</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>EFG1_R</td>
<td>TCC ACC AGT CCA TGT TCT TC</td>
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<tr>
<td><strong>PK</strong></td>
<td>Pyruvate kinase</td>
<td>Enzyme involved in glycolysis, catalysing the phosphorylation of ADP to ATP</td>
<td>GS01006</td>
<td>439215</td>
<td>PK_F</td>
<td>ATG GAC GCA AAG GGA ATG</td>
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<td>PK_R</td>
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<td><strong>Cax</strong></td>
<td>Cation/H⁺ exchanger 3</td>
<td>Transports Ca²⁺ against its electrochemical gradient by using a H⁺ gradient</td>
<td>GS00304</td>
<td>416800</td>
<td>CAX3_F2</td>
<td>CTC CTC TGC TGC TTT GCA T</td>
<td>90</td>
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<td>CAX4_R</td>
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<td></td>
<td>ECA2</td>
<td>ER-type Ca²⁺ ATPase 2</td>
<td>GE194135*</td>
<td>522053</td>
<td>ECA2_F</td>
<td>TCC TCA TCA CTC GCA ACA TC</td>
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<td></td>
<td>ECA2_R</td>
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<td><strong>Inorganic carbon transport</strong></td>
<td>AEL1</td>
<td>Anion exchanger like 1</td>
<td>HS05051</td>
<td>198643</td>
<td>AEL1_F</td>
<td>TTC ACG CTC TCA GTC TTG</td>
<td>102</td>
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<td>AEL1_R</td>
<td>GAG GAA GGC GAT GAA GAC</td>
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<tr>
<td></td>
<td>γ-EhCA2</td>
<td>Gamma carbonic anhydrase</td>
<td>DQ644551*</td>
<td>432493</td>
<td>γCA_F</td>
<td>TCT CGC CCT CAG TCA ACC</td>
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<td>γCA_R</td>
<td>AAG TTG TCG ACT GTG CAA CC</td>
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<td><strong>H⁺ transport</strong></td>
<td>ATPVc/ç</td>
<td>Subunit c of the V₀ sector of a Vacuolar H⁺-ATPase</td>
<td>GS03783</td>
<td>359783</td>
<td>ATPV_F</td>
<td>TAC GGC ACT GCA AAG TCT G</td>
<td>83</td>
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<td>ATPV_R</td>
<td>ACG GGG ATG ATG GAT TCC</td>
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<td></td>
<td>HVcn1</td>
<td>H⁺ channel</td>
<td>631975</td>
<td>HVCN1_F</td>
<td>HVCN1_R</td>
<td>CAT GAT CCT CGG GGT CTG</td>
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<td></td>
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<td></td>
<td>CGG CAG CTC CCT CAC TAC</td>
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<tr>
<td><strong>Biomineralization</strong></td>
<td>GPA</td>
<td>Glutamic acid, proline and alanine rich Ca²⁺ binding protein¹</td>
<td>AF012542*</td>
<td>431830</td>
<td>GPA_F</td>
<td>TTC CTC GAC AAG GGT AGA AG</td>
<td>99</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>GPA_R</td>
<td>GCT GCC AAC CTT GGT CTC</td>
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analysed (Actin, EFG1 and pyruvate kinase) and also the two most stable out of all the target and ERGs analysed (geNORM analysis, data not shown (Vandompele et al., 2002).

Relative gene expression of RCC1216 (diploid, calcifying) vs. RCC1217 (haploid, non-calcifying) and CCMP1516 (diploid, calcifying) vs. CCMP1516NC (diploid, non-calcifying) is shown in Fig. 2. Of the Ca^{2+} transporters, only CAX3 expression correlated directly with calcification. CAX3 gene expression was diploid specific, with no transcripts detected in the haploid RCC1217 cells, whereas CAX4 and ECA2 genes were expressed more highly in haploid cells. CAX3 and ECA2 genes were significantly upregulated twofold in the calcifying CCMP1516 relative to CCMP1516NC, but CAX4 showed no difference in gene expression.

<table>
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<tr>
<th>Strain</th>
<th>Initial cell conc. × 10^3</th>
<th>Sampling cell conc. × 10^3</th>
<th>Sampling time after inoculation (days)</th>
<th>Growth rate μ (day 2 to harvest)</th>
<th>PIC : POC</th>
<th>POC pg per cell per day</th>
<th>POC pg carbon per cell per day</th>
<th>Calcification rate pg carbon per cell per day</th>
<th>STDEV</th>
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<td>RCC1216</td>
<td>0.5</td>
<td>1.62</td>
<td>0.05</td>
<td>5</td>
<td>0.81</td>
<td>0.03</td>
<td>1.07</td>
<td>8.20</td>
<td>0.19</td>
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<tr>
<td>RCC1217</td>
<td>0.5</td>
<td>1.07</td>
<td>0.16</td>
<td>5</td>
<td>0.66</td>
<td>0.05</td>
<td>0.03</td>
<td>6.28</td>
<td>1.19</td>
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<td>CCMP1516</td>
<td>0.5</td>
<td>1.13</td>
<td>0.11</td>
<td>5</td>
<td>0.67</td>
<td>0.02</td>
<td>0.59</td>
<td>8.14</td>
<td>0.68</td>
</tr>
<tr>
<td>CCMP1516NC</td>
<td>0.1</td>
<td>1.75</td>
<td>0.17</td>
<td>9</td>
<td>0.60</td>
<td>0.01</td>
<td>0.00</td>
<td>6.68</td>
<td>0.14</td>
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</table>

Fig. 1. Particulate organic carbon (POC) and particulate inorganic carbon (PIC) production rates (top), with SEM images of calcifying strains RCC1216 and CCMP1516 and non-calcifying strain CCMP1516NC (bottom). The flagellated haploid cells, strain RCC1217 disintegrated on the filter membrane under the used SEM protocol. Standard deviations are shown.

The putative HCO$_3^-$ transporter *AEL1* exhibited a very similar pattern of gene expression to that of *CAX3*, with specific expression in the diploid phase and strong upregulation (~3.5-fold) in calcifying *CCMP1516* compared with *CCMP1516NC*. Note that both *CAX3* and *AEL1*, which are diploid specific genes, were expressed in *CCMP1516NC* supporting its identification as a non-calcifying diploid. In contrast, the carbonic anhydrase, γ-EhCA2 did not show a significant upregulation in the diploid vs. haploid comparison but did exhibit a significant 1.71-fold upregulation in *CCMP1516* relative to *CCMP1516NC*.

Of the two putative genes involved in H$^+$ transport only the vacuolar H$^+$-ATPase (*ATPVc/c*) showed consistent and significant upregulation correlating with calcification in both of the comparisons. Transcript levels for the voltage-gated H$^+$ channel *HVCN1* were downregulated approximately twofold in diploid *RCC1216* relative to haploid *RCC1217* and were stable in the *CCMP1516* vs. *CCMP1516NC* comparison. Surprisingly, the Ca$^{2+}$ binding protein gene isolated from coccoliths (*GPA*) was down-regulated nearly twofold in diploid *RCC1216* compared with haploid *RCC1217* and exhibited a dramatic 26-fold downregulation in calcifying *CCMP1516* compared with non-calcifying *CCMP1516NC*.

**CCMP1516 at different Ca$^{2+}$ concentrations**

To further examine the role of these putative biominalization genes, the calcifying strain *CCMP1516* was cultured at varying Ca$^{2+}$ concentrations to manipulate calcification rates and relative transcript levels were monitored. The cultures showed maximum growth rates and PIC production rates at ambient (10 mM) Ca$^{2+}$, whereas POC production rates were maximum at 0 mM Ca$^{2+}$. PIC production was almost fully inhibited in the absence of Ca$^{2+}$ and growth rates were significantly reduced. At higher than ambient Ca$^{2+}$ levels growth rates, PIC production rates and POC production rates decreased with increased Ca$^{2+}$ (Fig. 3). SEM images showed no noticeable variation in morphology at higher Ca$^{2+}$ concentrations relative to 10 mM Ca$^{2+}$ and demonstrated complete dissolution of the coccosphere at 0 mM Ca$^{2+}$ resulting in the presence of some collapsed cells on the filter membrane (Fig. 3).

The ERGs Actin and *EFG1* were the most stable throughout the Ca$^{2+}$ treatments. Five target genes (*CAX3, ECA2, AEL1, ATPVc/c* and *GPA*) that showed significant changes in gene expression in the strain comparison analyses described above were examined at four different Ca$^{2+}$ concentrations (Fig. 4). Gene expression is shown relative to 0 mM Ca$^{2+}$ where calcification was nearly completely inhibited. The genes encoding the putative Ca$^{2+}$ transporters, *CAX3* and *ECA2*, showed upregulation at 10, 35 and 60 mM Ca$^{2+}$ when compared with zero Ca$^{2+}$. *CAX3* transcripts were elevated between 2.5- and 3-fold and *ECA2* showed an approximately twofold upregulation. The *AEL1* bicarbonate transporter showed transcript levels between 2.4- and 3.1-fold higher at 10–60 mM Ca$^{2+}$ compared with 0 mM Ca$^{2+}$ and the *ATPVc/c* gene was slightly upregulated 1.6-fold. Therefore, all four of these ion transport genes exhibited increased expression in calcifying *CCMP1516* cultures relative to the 0 mM Ca$^{2+}$ control where calcification was severely inhibited.

In contrast, the gene expression of *GPA* was strongly (~10.8-fold) downregulated at ambient Ca$^{2+}$, relative to 0 mM Ca$^{2+}$. This downregulation in respect to 0 mM Ca$^{2+}$ was less at higher Ca$^{2+}$ concentrations with a 6.6-fold downregulation at 35 mM and 6.1-fold downregulation at 60 mM Ca$^{2+}$. Strikingly, the pattern of gene expression of cells cultured at 0 mM Ca$^{2+}$ vs. ambient Ca$^{2+}$ mirrors very closely that shown in the *CCMP1516NC* vs. *CCMP1516* comparison with all investigated genes up- or downregulated to similar degrees (Figs 2B and 4).

**Phylogenetic analysis**

The gene expression patterns of the ion transport proteins *CAX3* and *AEL1* demonstrate a very clear positive correlation with the calcification process, although we do not know their functional characteristics. Previous phylogenetic analyses of both the Ca$^{2+}$/cation exchange (CaCA) and the SLC4 protein families demonstrate that transporters with different ion specificities within these families can be grouped into distinct clades (Cai and Lytton, 2004;
Pushkin and Kurtz, 2006). The CaCA superfamily includes Na\(^+\)-dependent Ca\(^{2+}\) exchangers (NCX and NCKX), the Ca\(^{2+}\)/H\(^+\) exchangers (CAX), the poorly characterized Ca\(^{2+}\)/cation exchanger family (CCX) and the prokaryote YRBG transporters (Cai and Lytton, 2004). Phylogenetic analysis of *E. huxleyi* CAX3 and CAX4 with characterized Ca\(^{2+}\)/cation antiporter (CaCA) superfamily proteins strongly supports their positioning within the CAX clade (Fig. 5A).

The HCO\(_3^-\) transporters within the SLC4 family form two groups based on their phylogeny or three based on their function (Alper, 2006; Pushkin and Kurtz, 2006). Functionally these consist of Na\(^-\)-independent Cl\(^-\)/HCO\(_3^-\) exchangers [also known as anion exchangers (AE)], Na\(^-\)-HCO\(_3^-\) cotransporters (NBC) and Na\(^-\)-driven Cl\(^-\)/HCO\(_3^-\) exchangers (NDCBE) (Pushkin and Kurtz, 2006). In addition to C\(_i\) transport, a group of SLC4 transporters identified in mammals (Human BTR1/NaBC1), plants (Arabidopsis BOR1) and yeast (BOR1) have been shown to preferentially transport borate against its electrochemical gradient (Takano *et al*., 2002; Park *et al*., 2004). Phylogenetic analysis of known SLC4 proteins indicated AEL1 forms a clade with the putative SLC4 transporter from the picoeukaryote...
karyotic green algae Micromonas sp. RCC299, which was distinct from the plant/yeast clade of borate transporters. Although AEL1 does not group within the characterized vertebrate HCO$_3^-$ transporters, it is distinct from several other E. huxleyi SLC4 transporters identified by Richier et al. (2010), which fall into the plant/yeast borate transporter clade (data not shown).

**Discussion**

We used qRT-PCR to study a select number of genes with putative roles in calcification in a quantifiable and repeatable manner. As calcification influences the ion fluxes and energy demands of the cell, the transcription of genes involved in processes that support calcification such as increased ATP requirements or increased membrane cycling may also exhibit transcriptional regulation in addition to those directly involved in calcification. By comparing two independent pairs of calcifying vs. non-calcifying strains, in addition to altering calcification rates via manipulation of Ca$^{2+}$ concentrations, we can identify genes that have a strong likelihood of a role in calcification or a calcification related process.

**Ca$^{2+}$ transport and homeostasis**

Ca$^{2+}$ transport from seawater to the intracellular coccolith forming compartment represents one of the highest transcellular net transport rates for Ca$^{2+}$ with a calcifying cell transporting around five million Ca$^{2+}$ ions every second (Mackinder et al., 2010). Fig. 3 indicates that ambient Ca$^{2+}$ (10 mM) is optimal for both calcification and growth.
Elevated Ca²⁺ levels resulted in a decline in PIC production, POC production and growth rate, which is in agreement with a study by Herfort and colleagues (2004) where a decline in calcification and photosynthesis was shown at 50 mM Ca²⁺. The physiological effects of above optimal levels of Ca²⁺ is probably linked to the increased energetic costs associated with Ca²⁺ homeostasis at higher Ca²⁺ concentrations. In the absence of Ca²⁺ the decline in growth rate and a severe inhibition of PIC production concurs with previous studies which investigated lowered Ca²⁺ concentrations (Herfort et al., 2004; Trimborn et al., 2007; Leonardos et al., 2009). The associated decrease in growth rate and PIC production with the removal of Ca²⁺ can be respectively attributed to the limited Ca²⁺ impacting essential cellular processes necessary for cell division and the lack of substrate for calcification. The removal of Ca²⁺ has a slight positive impact on POC production on a per cell basis most likely due to reduced cell division allowing the reallocation of energy to cellular growth.

Ca²⁺ for calcification most probably enters the cell down its electrochemical gradient via Ca²⁺-permeable PM channels. Following Ca²⁺ entry a number of routes for Ca²⁺ transport to the CV are possible, the most likely pathway appears to be via the endomembrane system (Brownlee and Taylor, 2005; Mackinder et al., 2010). Loading of endomembrane compartments with Ca²⁺ would require either ATP-dependent pumping or ion exchange using the electrochemical gradient of another ion species. The upregulation of CAX3 in all the calcifying scenarios studied here supports a potential role in calcification for Ca²⁺ loading into endomembrane compartments via a H⁺ gradient. Here we confirm diploid-specific expression of CAX3, with levels below detection limits in haploid cells (von Dassow et al., 2009). As CAX4 is not upregulated in calcifying cells, it may play a more homeostatic house-keeping role in Ca²⁺ transport, unrelated to calcification. Phylogenetic analysis of the two CAX proteins investigated in the present study indicates that they are closely related, although their expression is very different. CAX proteins are found in plants, fungi, bacteria and lower invertebrates (Shigaki et al., 2006) and have been shown to export cytosolic cations to maintain optimal chemical and ionic levels for the cell. The first CAX proteins to be functionally characterized were Arabidopsis CAX1 and CAX2 (Hirschi et al., 1996). Expression of Arabidopsis CAX1 and CAX2 in a yeast mutant with Ca²⁺ hypersensitivity rescued the original phenotype with pH-dependent Ca²⁺/H⁺ exchange (Hirschi et al., 1996). Further investigation of other CAX proteins from plants and Chlamydomonas reinhardtii has shown that transport is not limited to Ca²⁺, with CAX proteins transporting other cations including Mn²⁺, Na⁺, Cd²⁺, Ba²⁺ and Co²⁺ (Kamiya and Mae-shima, 2004; Kamiya et al., 2005; Edmond et al., 2009; Liu et al., 2009; Pittman et al., 2009). Regulation of CAX activity has been shown to be varied and complex with N-terminal auto-inhibition (Pittman and Hirschi, 2001) and interacting proteins (Cheng et al., 2004a,b) playing key roles in some CAXs. To date all plant CAXs have been shown to be tonoplast localized (Shigaki and Hirschi, 2006).
2006) although bacterial CAXs are expected to be PM localized (Waditee et al., 2001).

Together with the Ca\(^{2+}\)-ATPases, Ca\(^{2+}\) exchangers play three key housekeeping roles in eukaryote cells; (i) they maintain a strong Ca\(^{2+}\) gradient between intracellular compartments and the cytosol (ii) they terminate Ca\(^{2+}\) signals by restoring cytosolic [Ca\(^{2+}\)] and (iii) they maintain organelle [Ca\(^{2+}\)] essential for biochemical functions (Sanders et al., 2002). In general, Ca\(^{2+}\)-ATPases have a higher affinity for Ca\(^{2+}\) than Ca\(^{2+}\) cation exchangers but
Dissolved inorganic carbon transport

Calcification and photosynthesis require large and sustained C\textsubscript{i} fluxes. It has been hypothesized that calcification and photosynthesis are intrinsically linked with calcification increasing \(\text{CO}_2\) availability for ribulose bisphosphate carboxylase oxygenase (Rubisco) but a recent accumulation of contrasting evidence has shown no dependence of photosynthesis on calcification. The almost complete absence of PIC fixation in cells grown at 0 mM Ca\textsuperscript{2+} in the present study, in conjunction with a minimal effect on POC production rate is in agreement with previous studies (Herfort et al., 2002; Trimborn et al., 2007; Leonardos et al., 2009) and further demonstrates that photosynthesis can be completely decoupled from calcification. At current seawater pH 8.2, the dissolved C composition is approximately 91% HCO\textsubscript{3}\textsuperscript{-}, 8% CO\textsubscript{2}\textsuperscript{(g)} and 1% \(\text{CO}_3\textsuperscript{2-}\). There is strong evidence that coccolithophores utilize both \(\text{CO}_2\) and HCO\textsubscript{3}\textsuperscript{-} as their external C source for photosynthesis (Sekino and Shiraiwa, 1994; Buitenhuis et al., 1999; Herfort et al., 2002; Rost et al., 2003; Trimborn et al., 2007) and mainly HCO\textsubscript{3}\textsuperscript{-} for calcification (Sikes et al., 1980; Nimer et al., 1997; Buitenhuis et al., 1999; Herfort et al., 2002). With \(\text{CO}_2\) as the substrate for Rubisco, conversion of internalized HCO\textsubscript{3}\textsuperscript{-} to \(\text{CO}_2\) would be necessary to support \(\text{CO}_2\) supply. The transport routes of the C components, their associated transport proteins and the C speciation in cellular compartments is relatively unknown. Here we report the gene expression levels of a \(\gamma\) carbonic anhydrase (\(\gamma\)-EhCA2) and a member of the SLC4 family (AEL1). \(\gamma\)-EhCA2 has been functionally characterized \textit{in vitro} (Soto et al., 2006) and its gene expression appears to be responsive to pCO\textsubscript{2}/pH, with \(\gamma\)-EhCA2 transcripts significantly reduced (3.8-fold) in RCC1216 cells grown at an elevated pCO\textsubscript{2} (770 ppm relative to cells grown at 440 ppm) (Richier et al., 2010). In that study, no significant change in calcification rates were observed between the treatments, indicating \(\gamma\)-EhCA2 gene expression is possibly more dependent on C availability and speciation or pH than cellular calcification. Our data show increased \(\gamma\)-EhCA2 gene expression under calcifying conditions for CCMP1516 compared with CCMP1516NC, but there was no significant upregulation in the diploid vs. haploid comparison. Cellular localization of CAs is key to understanding C fluxes and speciation, Soto and colleagues (2006) speculate on a CV location of \(\gamma\)-EhCA2 and external localization of \(\delta\)-EhCA1. External CA activity has been recorded previously in coccolithophores (Nimer et al., 1996; Herfort et al., 2002), but isofrom specific localization studies are essential for completely understanding their role in C uptake and calcification.

The use of HCO\textsubscript{3}\textsuperscript{-} as the primary external and internal C\textsubscript{i} species used for calcification requires facilitated transport at the PM and CV. Mammalian, fish and squid SLC4 HCO\textsubscript{3}\textsuperscript{-} transport proteins characterized to date have been shown to play key roles in PM carbon transport and pH homeostasis (Shmukler et al., 2005; Pushkin and Kurtz, 2006; Piermarini et al., 2007). Phylogenetic analysis did not group AEL1 with these well-characterized animal HCO\textsubscript{3}\textsuperscript{-} transporters, although AEL1 appears in a distinct group from the plant/yeast borate transporter group. We found that expression of the \(\text{AEL1}\) gene was diploid specific, supporting data from von Dassow and colleagues (2009). The upregulation of \(\text{AEL1}\) gene expression in all the calcifying scenarios strongly links this gene to a role in calcification, potentially through the provision of C\textsubscript{i}. This distinctive gene expression pattern strongly supports further functional characterization of AEL1 in order to determine more clearly its cellular roles. A PM location for AEL1 could potentially allow an inward Na\textsuperscript{+} gradient and/or an outward Cl\textsuperscript{-} gradient to drive C\textsubscript{i} uptake. Interestingly, the expression of five \textit{E. huxleyi} SLC4 genes (including \(\text{AEL1}\)) was shown to be unresponsive to changes in \(\text{CO}_2\) (Richier et al., 2010), although only a small CO\textsubscript{2} range was tested.

H\textsuperscript{+} transport and pH homeostasis

pH homeostasis is a fundamental process for all eukaryotic cells. H\textsuperscript{+} gradients are maintained across endomembranes and the PM, with many enzymatic and transport processes being dependent on the maintenance of localized pH in different cellular compartments. In animals, ion transport across the PM is commonly energized by a Na\textsuperscript{+} gradient, whereas land plants use primarily H\textsuperscript{+} electrochemical gradients to drive uphill transport of solutes (Sze et al., 1999). In coccolithophores, H\textsuperscript{+} production via calcification may potentially exert significant influence on pH...
homeostasis (Brownlee and Taylor, 2005; Suffrian et al., 2011). The use of HCO₃⁻ as the source of inorganic carbon for calcification (Sikes et al., 1980; Nimer et al., 1997; Buiteenhuis et al., 1999; Herfort et al., 2002) produces 1 mol H+ for each mol of calcite, which have to be removed from the cytosol or rapidly buffered. Coccolithophores possess a PM localized voltage-gated H⁺ channel that plays an important role in regulating intracellular pH (Taylor, et al., 2011). The HVCN1 gene encoding the voltage-gated H⁺ channel was not transcriptionally regulated by calcification, suggesting that it may serve a general role related to H⁺ transport in haploid and diploid cells. Ion channels have a very high capacity relative to other ion transport mechanisms and their activity can be effectively regulated at the level of channel gating without the requirement for transcriptional regulation.

In contrast, ATPVc/c′ encoding the c/c′ subunit of the vacuolar H⁺-ATPase (V-ATPase) was consistently upregulated between 1.5- and 2-fold in all of the calcifying scenarios. V-ATPases are localized to both endosomal membranes and the PM and generate H⁺ gradients and membrane voltage, which drive numerous ion and other transport processes (Beyenbach and Wieczorek, 2006). A Ca²⁺-stimulated V-ATPase activity was identified in the CV containing fraction of P. carterae (Araki and González, 1998) and the c subunit was cloned and immuno-localized to the coccolith producing fraction (Corstjens et al., 2001). Our results implicate V-ATPase activity in calcification in E. huxleyi, supporting this earlier work in P. carterae.

Biomineralization

The gene expression of the coccolith-associated Ca²⁺-binding protein GPA showed an unexpected trend. GPA gene transcripts were approximately twice as abundant in haploid than diploid cells, suggesting a role for GPA in the absence of calcification. von Dassow and colleagues (2009) confirmed the presence of haploid expression of GPA in RCC1217 via non-quantitative RT-PCR and Richier and colleagues (2009) found GPA expression to be relatively stable between the diploid RCC1216 and haploid RCC1217 strains. The discrepancy between our GPA expression data and the data presented in Richier and colleagues (2009) may relate to important differences in the normalization procedures used for gene expression, with Richier and colleagues (2009) normalizing to total RNA rather than ERGs. Remarkably, GPA transcripts were strongly upregulated in calcifying diploid cells with a ~26-fold downregulation relative to non-calculifying diploid cells and a ~10.8 downregulation relative to cells in 0 mM Ca²⁺. GPA was initially isolated from coccoliths and has been shown to have strong Ca²⁺ binding properties possibly related to the presence of 2 EF hand-like domains (Corstjens et al., 1998). There is also a link between gene sequence and coccolith morphology among different E. huxleyi morphotypes, with a genetic marker termed the coccolith morphology motif within the 3' untranslated region (3' UTR) of GPA that distinguishes between A and B morphotypes (Schroeder et al., 2005). The association of GPA with coccoliths (Corstjens et al., 1998) suggests a close relationship with calcification but the strong downregulation of GPA when the cell is calcifying is counter-intuitive. Three possible explanations for this downregulation need to be investigated further: (i) High concentrations of GPA may act as an inhibitor of calcification. It is possible that at low levels GPA plays a role in calcite nucleation and determining coccolith geometry, but increased cellular concentrations of GPA may result in inhibition of calcite precipitation. This explanation would seem plausible for the RCC1216 vs. RCC1217 and CCMP1516 vs. CCMP1516NC comparisons but in the low Ca²⁺ treatment when the cell cannot calcify due to lack of substrate, an upregulation in a calcite inhibitor protein does not fit. (ii) GPA protein or GPA mRNA may play a role in gene regulation. A possible epigenetic role could be carried out by GPA transcripts or their protein products acting as regulating elements on calcification related genes. However, no conserved DNA binding motif is present in the GPA peptide. The presence of the coccolith morphology motif in the 3’ UTR is intriguing indicating a potentially complex and mRNA related influence of GPA on coccolith morphology. (iii) There may be a translational block. GPA transcripts are not translated to protein resulting in accumulation of mRNA. Understanding this unexpected result is clearly very important in determining the regulation of calcification in coccolithophores.

A conceptual model

To stimulate further investigation, Fig. 6 provides a conceptual cellular model of calcification with possible routes for Ca²⁺, Ca²⁺ and H⁺ transport and the possible location of transporters investigated in this study. The model undoubtedly simplifies the transport processes involved in calcification and has not taken into account the processes of calcite nucleation and crystallization and coccolith transport involving membrane cycling. Assuming AEL1 can act as a HCO₃⁻ transporter, it could play a role in the uptake of C for calcification across the PM using either Na⁺ cotransport and/or Cl⁻ exchange to drive HCO₃⁻ uptake. Alternatively, AEL1 could function intracellularly, facilitating HCO₃⁻ uptake into the CV. The action of CAAs, such as γ-EhCA2, may aid in maintaining HCO₃⁻ concentrations within the cytosol and generating gradients across membranes, although the localization of the individual CA isoforms in E. huxleyi remain unknown. The close apposition of the peripheral ER to the PM in
E. huxleyi suggests this endomembrane system may play an important role in ion transport processes. Ca$^{2+}$ entry via PM channels would generate a micro-localized Ca$^{2+}$ elevation between the PM and peripheral ER, facilitating Ca$^{2+}$ uptake into the endomembrane network via Ca$^{2+}$ exchangers. CAX3, which is specifically expressed in diploid cells, may therefore play a role in this process, presumably exchanging Ca$^{2+}$ for H$^+$. This would require a H$^+$ gradient across the peripheral ER relative to the cytosol, most probably generated by a V-type ATPase, such as ATPVc/c'. The action of Ca$^{2+}$-ATPases could also aid Ca$^{2+}$ uptake into the peripheral ER, although the expression pattern of ECA2 suggests it most probably contributes more generally to Ca$^{2+}$ homeostasis. From the peripheral ER, Ca$^{2+}$ could then be transported into the maturing CV, avoiding transport across the cytosol. Within the CV and associated endomembrane network, CaCO$_3$ formation from Ca$^{2+}$ and HCO$_3^-$, possibly via an amorphous pre-cursor (Mackinder et al., 2010), would generate H$^+$. To maintain suitable conditions for calcite precipitation, H$^+$ must be removed from the CV, presumably into the cytosol, where excess H$^+$ may be released via the action of the PM localized voltage gated H$^+$ channel (Hv1) or may be pumped into endomembrane compartments (i.e. via an V-ATPase) to regulate cytosolic pH. Calcite nucleation and crystal growth is thought to be primarily controlled via organic molecules such as polysaccharides (de Jong et al., 1976; Marsh et al., 1992) and proteins (such as GPA) as well as through substrate supply and H$^+$ removal. Our data suggest an important role for GPA in regulating the calcification process. Elucidation of this function should provide much needed insight into the cellular mechanisms of calcification in coccolithophores.

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Experimental procedures

Culturing

Four previously isolated clonal strains of *E. huxleyi* (CCMP1516, CCMP1516NC, RCC1216, RCC1217) were batch cultured. CCMP1516 and RCC1216 were isolated separately from the South Pacific as calcifying diploid strains. RCC1217 was subsequently isolated from RCC1216 as a naturally occurring non-calcifying haploid. Both of these strains were obtained from the Roscoff Culture Collection (RCC; Roscoff, France). The CCMP1516 strain maintained at the Centre for the Culture of Marine Phytoplankton (CCMP; Bigelow, ME) has lost the ability to calcify and is referred to in this manuscript as CCMP1516NC. However, before CCMP1516 lost the ability to calcify a subculture was sent to the Plymouth Culture Collection (PCC; Plymouth, UK) where it is identified as strain M217. The culture maintained at the PCC has retained the ability to calcify and is referred to in this manuscript as CCMP1516. All strains were cultured in sterile filtered artificial seawater containing vitamins and metals at f/4 concentration (Guillard, 1975), 88 μmol kg⁻¹ nitrate and 5.2 μmol kg⁻¹ phosphate and 10 nmol kg⁻¹ selenium and 2 ml of sterile North Sea natural sea water kg⁻¹. CO₂ fugacity (fCO₂) was set to 400 ppm by the addition of Na₂CO₃ and removing excess alkalinity using certified HCl to give a total alkalinity of ~2325 μmol kg⁻¹. Cells were grown at 15°C under a 12:12 h light:dark cycle at photon flux density 150 μmol m⁻² s⁻¹. Culture concentration was determined as the mean of three measurements obtained with a Z series Beckman Coulter counter. Growth rates (μ) were calculated as

\[
\mu = \frac{\ln C_t - \ln C_0}{t_f - t_0}
\]

where \(C_t\) and \(C_0\) are concentrations at time (in days) of \(t_f\) and \(t_0\) respectively. Gene expression was further investigated in CCMP1516 cultured in 0, 10, 35 and 60 mM Ca⁴⁺.

Sampling

Pre-cultures maintained in their exponential growth phase (for > 10 generations) were inoculated in triplicate in fresh media to give a starting cell concentration of 1 × 10⁵ or 5 × 10⁵ cells ml⁻¹. Cell concentration was monitored until sampling at 1–2 × 10⁵ cells ml⁻¹. All sampling and cell counts were conducted within a 3 h time period between hours 5 and 8 of the light phase to minimize temporal variations.

**TPC/POC/PIC**

Duplicate samples for total particulate carbon (TPC, 100 ml) and particulate organic carbon (POC, 200 ml) were filtered onto pre-combusted (450°C, 6 h) GF/F filters and frozen at −20°C until analysis. POC filters were fumed in a desiccator with 37% HCl for 2–2.5 h to remove all inorganic carbon before the drying of filters (POC and TPC) for 15 h at 60°C. All filters were measured on a Euro EA Elemental Analyser (Ehhardt and Koeve, 1999). Particular inorganic carbon (PIC) was determined by the difference between TPC and POC. POC and PIC production rates were calculated by multiplying growth rate (μ) with POC and PIC cellular content respectively.

**SEM**

Approximately 2.5 × 10⁶ cells of each strain were gently filtered onto polycarbonate filters (0.2 μm), dried for 1 h at 60°C and sputter-coated with gold-palladium. SEM images were obtained with a CamScan-CS-44 SEM.

**Microsatellite data**

To test for strain identity five primer pairs designed to microsatellite loci (EHMS37, P01F08, P02B12, P02F11, P02E09 (Iglesias-Rodríguez et al., 2002; 2006) were ran on genomic DNA (gDNA) extracted from the four strains under study. Approximately 5 × 10⁵ cells were filtered onto 0.45 μm cellulose acetate filters. gDNA was extracted using an Invitrogen DNA extraction kit (Invitex). Briefly, 400 μl of lysis buffer and 40 μl of proteinase K were added to the filter in a 1.5 ml centrifuge tube, vortexed for 20 s followed by a 10 min water bath sonification at 52°C, then shaking (950 r.p.m.) for 50 min at 52°C. The filter was removed and discarded. DNA was bound to the column as in the supplied protocol and eluted in 100 μl of elution buffer. DNA concentration was determined using a NanoDrop spectrophotometer. The above forward primers end-labelled with fluorescent phosphoramidite dyes (HEX or 6-FAM) were used to amplify (30 cycles) 2 ng of gDNA using pro Taq DNA polymerase (Promega). One microlitre of PCR sample was analysed on an AB3130 MSAT Genetic Analyser. Microsatellite electropherogram data were scored using the software GeneMarker (Soft Genetics, State College, PA, USA).

**qRT-PCR**

**RNA extraction and reverse transcription.** 100 ml of culture was filtered onto 0.8 μm polycarbonate filters, immediately washed off with 1 ml of RNA later (QIAGEN) and kept on ice until frozen at −20°C. RNA was extracted using the RNaseasy mini kit (QIAGEN) as follows: the supernatant was directly removed if the cells had settled out during storage (due to the density of RNA later some cells settled out at −20°C), if this was not the case cells were centrifuged (10,000 r.p.m., 3 min) and the supernatant removed. After the removal of supernatant lysis buffer containing β-mercaptoethanol was added as in the protocol, cell lysis was achieved by a 30 s vortex, 3 min bath sonification, 30 s vortex all at room temperature. RNA was bound to columns and eluted in 40 μl of RNase free H₂O as in the provided protocol and frozen at −80°C. RNA concentration, quality and integrity was analysed on a Bio-Rad Experion. All samples had a RNA quality indicator (RQI) > 7 (except sample TQ26 1N biological replicate 2, RQI 4.5). 625 ng of RNA per sample was DNase treated then reverse-transcribed using a Quantitect kit (QIAGEN) primed with a mixture of oligo-dT and random primers. Samples for no reverse transcription control (NRTC) were removed post DNase treatment but before the RT step. The resulting cDNA and NRTCs were stored at −20°C and −80°C respectively.
**Primer design.** Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) Primers to the genes EFG1, PK, CAX3, CAX4, AEL1 and ATPVc/c were designed to EST clusters from von Dassow and colleagues (2009), while primers to ECA2, γ-EhCA2 and GPA were designed to EST or complete gene sequences deposited in GenBank (Table 1). Primers to HVCN1 correspond to sequence data (JGI protein ID 631975) provided with permission from the E. huxleyi Genome Project (http://genome.jgi-psf.org/Emih1/Emih1.home.html). The primers to the multi-copy gene Actin amplify identical amplicons from six different gene copies (Bhat- charya et al., 1993). For clarity, gene names have been used, which correspond to the annotation in the E. huxleyi Genome, although these were not available for all genes (Table 1). Primers were tested on qDNA and cDNA and products ran on 1.5% agarose gel to check for single amplicons and primer dimers.

**Efficiency curves.** Equal amounts of cDNA from all samples were pooled and serially diluted to generate efficiency curves from five or more cDNA concentrations. All efficiency curves had a R² greater than 0.99 and efficiencies between 90–105%.

**ERG analysis.** To test for the most stable ERGs equal amounts of cDNA (2 ng of reverse transcribed RNA) were analysed with the primers for three ERGs (Actin, EFG1 and pyruvate kinase). Threshold cycle (Ct) values were plotted per sample to show expression variation between samples. ERG stability was also tested using the program geNorm, which generates a gene expression stability value and allows ranking of genes according to their expression stability (Vandesompele et al., 2002).

**qPCR chemistry.** 20 μl reaction volumes were set up consisting of 10 μl 2 × Fast SYBR Green Master Mix (Applied Biosystems), 0.4 μl forward and reverse primers (10 pmol μl⁻¹), 4 μl of sample (cDNA 2 ng of reverse transcribed RNA), NRTC (2 ng of RNA) or no template control (NTC) and 5.2 μl of HPLC grade H₂O. All samples were ran in technical triplicates with NRTCs ran for each sample and NTCs (2 ng of RNA) or no template control (NTC) and 5.2 μl of HPLC grade H₂O. All samples were ran in technical triplicates with NRTCs ran for each sample and primer dimers ran on every plate and NTCs for each primer pair per plate. Samples were ran using the fast cycling program on an Applied Biosystems Step One Plus qPCR machine.

**qRT-PCR data analysis.** Before data analysis checks were carried out on the raw qPCR data. The baseline was checked and adjusted if necessary, the Ct threshold was adjusted to 0.2 and checked to ensure the crossing point was in the log phase. The standard deviations of triplicates were checked and if >0.3 outliers were removed. Sample Ct’s were checked and in all cases were at least five Ct’s lower than the NRTC and NTC and fell within the range covered by the efficiency plots. Melt curves were checked to ensure there was no non-specific priming in samples and specific priming in NTCs.

**qRT-PCR data** were analysed using a efficiency corrected ΔΔCt method, normalizing to the geometric mean of two ERGs (Vandesompele et al., 2002). P-values were obtained using a pairwise fixed reallocation randomization test using the relative expression software tool (REST) (Pfaffl et al., 2002).

**Phylogenetic analysis**

Amino acid sequences of characterized and uncharacterized proteins belonging to the Ca²⁺/cation antiporter (CaCA) superfamily and SoLute Carrier 4 (SLC4) family respectively were aligned using ClustalW multiple sequence alignment software. Alignments were manually checked to ensure only unambiguous residues were compared. For CaCA proteins, a truncated alignment of amino acids comprising of the conserved α1 and α2 domains was used for tree construction. An alignment of amino acids encompassing the transmembrane domains was used for SLC4 phylogenetic analysis. Minimum evolution phylogenetic analysis was conducted using MEGA version 4 (Tamura et al., 2007).

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