Cold-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations function to support osmoregulation in marine diatoms

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Abstract

Diatoms are a group of microalgae that are important primary producers in a range of open ocean, freshwater, and intertidal environments. The latter can experience substantial long- and short-term variability in temperature, from seasonal variations to rapid temperature shifts caused by tidal immersion and emersion. As temperature is a major determinant in the distribution of diatom species, their temperature sensory and response mechanisms likely have important roles in their ecological success. We examined the mechanisms diatoms use to sense rapid changes in temperature, such as those experienced in the intertidal zone. We found that the diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* exhibit a transient cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) elevation in response to rapid cooling, similar to those observed in plant and animal cells. However, [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations were not observed in response to rapid warming. The kinetics and magnitude of cold-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations corresponded with the rate of temperature decrease. We did not find a role for the [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations in enhancing cold tolerance but showed that cold shock induces a Ca\(^{2+}\)-dependent K\(^{+}\) efflux and reduces mortality of *P. tricornutum* during a simultaneous hypo-osmotic shock. As intertidal diatom species may routinely encounter simultaneous cold and hypo-osmotic shocks during tidal cycles, we propose that cold-induced Ca\(^{2+}\) signaling interacts with osmotic signaling pathways to aid in the regulation of cell volume. Our findings provide insight into the nature of temperature perception in diatoms and highlight that cross-talk between signaling pathways may play an important role in their cellular responses to multiple simultaneous stressors.
Introduction

Diatoms are a group of silicified unicellular algae that represent one of the most important primary producers in modern oceans. They are abundant in diverse marine environments, most notably in polar and temperate upwelling regions, where they play a critical role at the base of the marine food web (Malviya et al., 2016). Diatom communities are abundant across a broad temperature range in the surface ocean from sea ice to tropical oceans. Diatoms are also important primary producers in freshwater and brackish ecosystems, where they likely encounter an even greater range of temperatures (Souffreau et al., 2010).

Global rises in surface temperature due to anthropogenic CO₂ emissions are set to have profound influence on marine ecosystems (Gattuso et al., 2015). These future changes in our climate will also increase the variability of temperature regimes and the prevalence of extreme events, such as marine heat waves, that may co-occur with other stressors such as low pH or deoxygenation (Harley et al., 2006; Smale et al., 2019; Gruber et al., 2021). Understanding the physiological response of diatoms and other marine phytoplankton to changes in global temperature regimes is therefore of the utmost importance. Temperature has an important impact on diatom cell physiology, influencing cell size and formation of the silica frustule (Montagnes and Franklin, 2001; Svensson et al., 2014; Javaheri et al., 2015). Individual species display a thermal niche with distinct temperature growth optima that reflect their natural environment (Liang et al., 2019). The upper and lower thermal tolerance limits, rather than the optima themselves, appear to have the greatest influence on the distribution of individual diatom species (Anderson and Rynearson, 2020), with temperatures in excess of the upper thermal tolerance limits leading to a rapid increase in the rates of cell death (Baker and Geider, 2021).

Many of these studies have focused on the physiological responses of diatoms to longer term changes in temperature. However, diatoms will also experience short-term temperature variations within their natural habitat. This is particularly so for those species that inhabit intertidal rocky shores or estuarine habitats where immersion and emersion is associated with rapid and regular temperature fluctuations. Rapid temperature changes are potentially highly damaging to diatom cells, demonstrated by their much greater vulnerability to abrupt rather than gradual temperature increases (Souffreau et al., 2010). Temperature variability may also have an important influence on the ability of diatoms to adapt to their thermal niche, as Thalassiosira pseudonana exhibited accelerated adaptation to higher temperatures under a fluctuating temperature regime (Schaum et al., 2018). Despite the importance of thermal tolerance in diatom physiology and ecology, relatively little is known about the physiological mechanisms that allow diatoms to perceive and respond to changes in temperature, particularly during short-term fluctuations.

Many of the cellular mechanisms involved in temperature sensing in eukaryotes involve temperature-induced changes in the structure of nucleic acids, proteins, or biological membranes that lead to a range of downstream physiological responses (Sengupta and Garrity, 2013). Ca²⁺-dependent signaling mechanisms play an important role in these temperature sensing pathways. In animal cells, heat stress is associated with Ca²⁺ influx into the cytosol via the transient receptor potential (TRP) cation channel family of temperature-sensitive ion channels (Xu et al., 2002; Clapham and Miller, 2011). Ca²⁺ signaling also plays a role in sensing low temperature in animals, for example, underpinning the rapid cold hardening response of insects (Teets et al., 2013). Land plants also employ Ca²⁺ signaling mechanisms in their response to both low and high temperatures. Rapid cooling of plants induces a transient cytosolic Ca²⁺ ([Ca²⁺]₉₅) elevation, which leads to changes in gene expression and the establishment of cold tolerance (Knight et al., 1996; Tahtiharju et al., 1997; Knight and Knight, 2012). Some plants, such as the moss Physcomitrium, also display [Ca²⁺]₉₅ elevations in response to heat shock (Saidi et al., 2009). In other plants, such as Arabidopsis (Arabidopsis thaliana), support for the role of high temperatures in inducing [Ca²⁺]₉₅ elevations is mixed, although Ca²⁺ elevations are observed within the chloroplast (Lenzoni and Knight, 2019). However, a recent study demonstrated that elevated temperature-induced [Ca²⁺]₉₅ elevations in Arabidopsis leaves, but not in pollen tubes (Weigand et al., 2021). Potential temperature sensors in plants include the cold-sensitive regulator of G-protein signaling (COLD1/ RGA1) complex in rice (Oryza sativa), which is proposed to either function as a Ca²⁺ channel or to activate other Ca²⁺ channels (Ma et al., 2015). Specific cyclic nucleotide-gated ion channels and annexins may also play a role in temperature sensing pathways, with mutant strains in Physcomitrium, O. sativa, and Arabidopsis exhibiting diminished [Ca²⁺]₉₅ elevations in response to cold and heat shock (Cui et al., 2020; Liu et al., 2021). However, it is currently unclear whether these ion channels sense temperature directly or are activated indirectly, for example, through changes in membrane rigidity (Plieth et al., 1999) or the cytoskeleton (Pokorna et al., 2004).

Our understanding of Ca²⁺ signaling in diatoms remains in its infancy, although Ca²⁺-dependent signaling mechanisms have been identified in response to a range of environmental stimuli, such as the supply of nutrients (phosphate and iron), hypo-osmotic shock, and the detection of toxic aldehydes (Falcioleti et al., 2000; Vardi et al., 2006; Helliwell et al., 2021a, 2021b). Initial experiments using Phaeodactylum tricornutum cells expressing the bioluminescent Ca²⁺ reporter aequorin did not detect [Ca²⁺]₉₅ elevations in response to low (4°C) or high (37°C) temperature (Falcioleti et al., 2000). More recently, genetically encoded fluorescent Ca²⁺ reporters have been successfully expressed in P. tricornutum and T. pseudonana, enabling high-resolution imaging of [Ca²⁺]₉₅ elevations in single diatom cells (Helliwell et al., 2021a, 2021b). These advances will now...
allow detailed examination of diatom signaling in response to range of stimuli, including temperature.

In this study, we set out to examine the ability of diatoms to sense short-term changes in temperature. In particular, we examined whether the well-characterized \([Ca^{2+}]_{cyt}\) elevations observed in animal and plant cells in response to rapid changes in temperature were conserved in diatoms. Using the model species \textit{P. tricornutum} and \textit{T. pseudonana}, which can both inhabit coastal environments that experience variable temperature regimes (De Martino et al., 2007; Alverson et al., 2011), we found that diatoms consistently exhibit a \([Ca^{2+}]_{cyt}\) elevation in response to cold shock, but do not exhibit \([Ca^{2+}]_{cyt}\) elevations in response to elevated temperature. We did not find a requirement for cold shock-induced Ca\(^{2+}\) signaling in increasing tolerance to low temperatures, but found that cold shock increases tolerance to simultaneous hypo-osmotic shocks, suggesting that integration of multiple signaling inputs may contribute to an enhanced ability to respond to these environmental stimuli.

**Materials and results**

**Rapid changes in temperature in intertidal environments**

\textit{Phaeodactylum tricornutum} was first isolated from a tidal pool in the UK and has since been identified in a range of coastal and brackish habitats (De Martino et al., 2007). To assess the dynamic temperature regimes potentially experienced by intertidal diatoms, we measured the temperature of a tidal pool located on the upper region of a rocky shore (South Cornwall, UK) over a 7-day period during July (UK summer). Temperatures within the pool were very stable around 15°C during immersion at high tide (Figure 1). However, at low tides temperatures in the exposed tidal pool rose substantially during the day (up to 30°C) and decreased at night (to 12°C), before being rapidly restored to the bulk seawater temperature by the immersion of the pool at high tide. These data illustrate that diatoms inhabiting intertidal environments in temperate regions will regularly experience periods of substantial warming followed by rapid cooling. The fluctuations in temperature are likely to be even greater in smaller volumes of water, such as the surface of estuarine mudflats or very shallow pools.

**Calcium signaling in response to changes in temperature**

\textit{Phaeodactylum tricornutum} cells expressing the R-GECO1 Ca\(^{2+}\) biosensor were perfused with seawater at high or low target temperatures (30°C or 12°C). Note that actual temperatures in the perfusion dish differed by ±2°C from these target temperatures due to equilibration of the small volume of warm or cold perfusate with room temperature (RT). Actual dish temperatures were therefore recorded and are displayed for all experiments. We routinely observed a single transient \([Ca^{2+}]_{cyt}\) elevation in cells exposed to a cold shock from 30°C to 12°C (97% cells, \(n = 63\)) (Figure 2A). In contrast, cells exposed to a rapid rise in temperature from 12°C to 30°C did not show \([Ca^{2+}]_{cyt}\) elevations (Figure 2A). No \([Ca^{2+}]_{cyt}\) elevations were observed in cells perfused with these solutions after they had been equilibrated to RT, indicating that the act of switching between the perfusion solutions does not contribute to the signaling responses (Figure 2A). Analysis of the spatial characteristics of cold shock-induced \([Ca^{2+}]_{cyt}\) elevations indicated that many initiate at the apex of the cell and propagate to the central region (Figure 2B), in a manner similar to those induced by mild hypo-osmotic shock (Helliwell et al., 2021b). This suggests that the apices of the cell may play an important role in sensing the temperature changes. Cells exposed to a second cold shock 2 min after a previous cold shock demonstrated \([Ca^{2+}]_{cyt}\) elevations with no substantial attenuation in amplitude, although the percentage of cells responding was slightly lower (97%–81% of cells, \(n = 63\)) (Supplementary Figure S1).

The \([Ca^{2+}]_{cyt}\) elevations observed during cold shock were represented by a >10-fold increase in R-GECO1 fluorescence. Assuming a \(K_d\) of 480 nM for R-GECO1 and comparison with published maximum \(F/F_0\) values (Zhao et al., 2011), we estimate that \([Ca^{2+}]_{cyt}\) elevations reach concentrations in the micromolar range. In addition to these large increases in fluorescence that are attributed to \([Ca^{2+}]_{cyt}\) elevations, much smaller changes in the baseline fluorescence of each cell could be observed following changes in temperature.
Cold signalling in diatoms

PLANT PHYSIOLOGY 2022: 190; 1384–1399 | 1387

Figure 2 Phaeodactylum tricornutum exhibits cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{\text{cyt}}\)) elevations in response to rapid cooling. A, Eight representative fluorescence ratio traces (F/F\(_0\), blue lines) of P. tricornutum cells expressing R-GECO1 representing changes in cytosolic Ca\(^{2+}\). Cells were perfused with ASW of different temperatures to cause rapid temperature shifts (black line). Cold shock 30°C–12°C, heat shock 12°C–30°C or control 22°C–22°C. B, False color images of a PtR1 cell exhibiting a [Ca\(^{2+}\)\(_{\text{cyt}}\) elevation in response to cold shock. The temperature decrease begins at t = 0 s. Note that the [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations initiate at the tips of the cell and spread toward the central region. Left part indicates a differential interference contrast (DIC) image overlaid with chlorophyll autofluorescence. Bar represents 10 μm.

Rapid cooling is required to elicit a [Ca\(^{2+}\)\(_{\text{cyt}}\) elevation

We therefore examined the nature of the temperature change required to elicit [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations, by manipulating the flow rate of the perfusion to vary the rate of cooling. Rapid cooling (2.5°C s\(^{-1}\)) resulted in [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations in 100% of cells examined (n = 45), whereas only 7% of cells exhibited a [Ca\(^{2+}\)\(_{\text{cyt}}\) elevation at a cooling rate of 0.4°C s\(^{-1}\) (n = 45) (Figure 3A and B). The amplitude of the [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations in responding cells closely corresponded with the cooling rate, with much larger [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations observed at rapid cooling rates (Figure 3C). Examination of a broader range of cooling rates indicated that a cooling rate > 1°C s\(^{-1}\) was required to elicit [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations in 50% of the population (Figure 3D). These data suggest that the cold shock-induced [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations can therefore relay information relating to the nature of the stimulus both in terms of the number of cells responding and the nature of the [Ca\(^{2+}\)\(_{\text{cyt}}\) elevation itself.

As very low perfusion rates also resulted in a lower overall decrease in temperature (due to equilibration of the perfusate with RT), we next examined the absolute temperature decrease required to initiate signaling. Cells were perfused at 30°C for 1 min and then perfused at a constant flow rate with cold artificial seawater (ASW) (4°C) for different durations to vary the decrease in temperature whilst maintaining similar rates of cooling. A very brief perfusion (4 s) lowered the temperature by 2.4 ± 0.6°C but did not induce [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations (Figure 3, E and F). However, the rate of cooling in this treatment was considerably lower than the other treatments, due to buffering of the temperature by the residual volume within the perfusion dish (1 mL). Perfusions of a longer duration (7–26 s) resulted in a consistent cooling rate of 2.1–2.4°C s\(^{-1}\). A temperature decrease of 8.8 ± 0.4°C induced a [Ca\(^{2+}\)\(_{\text{cyt}}\) elevation in 38.4% of cells (n = 126), whereas greater decreases in temperature resulted in [Ca\(^{2+}\)\(_{\text{cyt}}\) elevations in nearly all cells (Figure 3, E
The amplitude and duration of \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations increased with the greater duration of the temperature decrease (Figure 3, G and H). A cooling duration of 26 s did not increase the amplitude of the \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation beyond those observed at 9 s, but greatly increased the duration of the \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation (Figure 3, G and H). Taken together, our results show that the cooling rate and the duration of the cold shock influence the amplitude and duration of the \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation and the percentage of cells responding.

The cold shock response is conserved in the centric diatom *Thalassiosira pseudonana* but displays different characteristics. *Thalassiosira pseudonana* is a planktonic centric diatom found in marine, estuarine, and freshwater environments (Alverson et al., 2011), where it is also likely to be exposed to substantial changes in temperature. We found that *T. pseudonana* cells expressing the R-GECO1 biosensor exhibited \([\text{Ca}^{2+}]_{\text{cyt}}\) elevations in response to cold shock, with the amplitude of these elevations also dependent on...
the rate of temperature decrease (Figure 4, A and B). As in *P. tricornutum*, a control perfusion using ASW media equilibrated to RT did not induce 

\[ Ca^{2+}_{\text{cyt}} \]

elevations (Figure 4C). The cold-induced \( \text{Ca}^{2+}_{\text{cyt}} \) elevations in *T. pseudonana* were of a longer duration than those observed in *P. tricornutum*, with a slower rise and fall in \( \text{Ca}^{2+}_{\text{cyt}} \). The percentage of *T. pseudonana* cells responding to cold shock was also considerably lower than *P. tricornutum* (19% versus 81%, respectively), although variable levels of expression of R-GECO1 in *T. pseudonana* likely prevented detection of \( \text{Ca}^{2+}_{\text{cyt}} \) elevations in all cells within a field of view (Helliwell et al., 2021a) (Figure 4D–F). Taken together, these findings suggest that cold shock-induced \( \text{Ca}^{2+}_{\text{cyt}} \) elevations are exhibited by both pennate and centric diatom lineages and may therefore represent a conserved mechanism in many diatom species.

**Cellular mechanisms underlying the cold shock response**

We next examined the cellular mechanisms responsible for cold shock \( \text{Ca}^{2+} \) signaling in *P. tricornutum*. Removal of external \( \text{Ca}^{2+} \) by perfusion of PtR1 cells with cold \( \text{Ca}^{2+} \)-free ASW completely abolished the \( \text{Ca}^{2+}_{\text{cyt}} \) elevations (Figure 5A). Restoration of external \( \text{Ca}^{2+} \) to cooled cells did not induce a \( \text{Ca}^{2+}_{\text{cyt}} \) elevation. However, when these cells were subsequently warmed to 30°C and then cooled, \( \text{Ca}^{2+}_{\text{cyt}} \) elevations were observed in the majority of cells. Thus, the generation of cold-induced \( \text{Ca}^{2+}_{\text{cyt}} \) elevation depends on the presence of external \( \text{Ca}^{2+} \), and the \( \text{Ca}^{2+}_{\text{cyt}} \) elevation is triggered by the rapid drop in temperature rather than low absolute temperature itself.

*Phaeodactylum tricornutum* lacks cyclic-gated nucleotide channels, which are important for thermal sensing in plants, although it does possess multiple TRP channels (Verret et al., 2010). The temperature-sensitive TRP cation channel subfamily M 8 (TRPM8) in animal cells is responsible for cold-induced \( \text{Ca}^{2+}_{\text{cyt}} \) elevations and can be activated directly by the plant secondary metabolite, menthol, at micromolar concentrations (Peier et al., 2002; Yin et al., 2018). Perfusion of PtR1 cells with 1-mM menthol did not elicit \( \text{Ca}^{2+}_{\text{cyt}} \) elevations, indicating that this ligand is likely specific to the ion channels involved in animal cold signaling (Figure 5B). In plant and fungal cells, cold shock-induced \( \text{Ca}^{2+}_{\text{cyt}} \) elevations have been studied through the application of dimethyl sulfoxide (DMSO), which is proposed to mimic cold-induced membrane rigidification (Furuya et al., 2014). DMSO elicited \( \text{Ca}^{2+}_{\text{cyt}} \) elevations in a dose-dependent manner in *P. tricornutum*, with 8% and 50% of cells exhibiting \( \text{Ca}^{2+} \) elevation in response to addition of 1% and 5% DMSO, respectively \( (n = 24, 25) \) (Figure 5, C and D). Ruthenium red (RR) is a nonselective \( \text{Ca}^{2+} \) channel blocker shown to affect numerous TRP channels including the cold-sensitive TRPA1 channel (Andrade et al., 2008; Silva et al., 2015; Christensen et al., 2016). RR also inhibits \( \text{Ca}^{2+}_{\text{cyt}} \) elevations in *P. tricornutum* induced by the resupply of phosphate to phosphate-limited cells, but does not

![Figure 4](https://example.com/figure4)

**Figure 4** *Thalassiosira pseudonana* also shows cold-induced \( \text{Ca}^{2+}_{\text{cyt}} \) elevations. A, Fluorescence ratio of *T. pseudonana* cells expressing cytosolic R-GECO1 in response to a cold shock (from 30°C to 10°C). For these experiments the temperature in the dish was not monitored, so perfusion flow rate is shown to indicate rate of cold shock. Arrow indicates onset of cold stimulus. Four representative traces are shown. B, As in (A) but at a slower flow rate. C, Treatment control using perfusion of ASW at RT. D, Fluorescence image of *T. pseudonana* cells expressing R-GECO1 overlaid with chlorophyll autofluorescence. Scale bar represents 20 µm. E, *Phaeodactylum tricornutum* cold shock response under identical treatment as in A for comparison. F, Percentage of cells exhibiting \( \text{Ca}^{2+}_{\text{cyt}} \) elevations. Values in parentheses denote \( n \).
inhibit [Ca$^{2+}$]$_{cyt}$ elevations caused by hypo-osmotic shock (Helliwell et al., 2021a, 2021b). Pretreatment of PtR1 cells for 5 min with 5–10 μM RR did not significantly reduce the amplitude of cold-induced Ca$^{2+}$ elevations (Figure 5, E and F). However, RR-treated cells exhibited a significantly slower response time than nontreated control cells (defined as
Cold signalling in diatoms

**Interaction between cold and hypo-osmotic shock**

Diatoms inhabiting intertidal regions may regularly experience a cold shock during tidal cycles (Figure 1), but this is unlikely to represent an isolated stressor. In particular, warming of shallow tidal pools can greatly increase their salinity due to evaporation (Firth and Williams, 2009), leading to a substantial hypo-osmotic shock when the incoming tide reaches the tidal pool. *Phaeodactylum tricornutum* is highly perceptive to hypo-osmotic shock, exhibiting a large transient \([Ca^{2+}]_\text{cyt}\) elevation similar to those induced by cold shock (Falciatore et al., 2000; Helliwell et al., 2021b). Since cold and hypo-osmotic shocks are likely to regularly co-occur in intertidal environments, we examined cellular \([Ca^{2+}]_\text{cyt}\) signaling when these stressors were applied simultaneously.

A relatively mild hypo-osmotic shock (100% ASW to 95% ASW) administered to cells at 25°C resulted in a single \([Ca^{2+}]_\text{cyt}\) elevation, as observed previously (Helliwell et al., 2021b) (Figure 7A). When the same hypo-osmotic shock was applied simultaneously with a cold shock (25°C–10°C), both the amplitude and duration of the \([Ca^{2+}]_\text{cyt}\) elevations was substantially increased, although the number of cells exhibiting \([Ca^{2+}]_\text{cyt}\) elevations did not change (Figure 7, A–C). Hypo-osmotic shocks cause an increase in cell volume in *P. tricornutum*, which likely initiates \([Ca^{2+}]_\text{cyt}\) elevations through the activation of mechanosensitive ion channels (Helliwell et al., 2021b). However, cell volume did not increase during cold shock (Supplemental Figure S3), indicating that the rapid cooling does not simply elicit \([Ca^{2+}]_\text{cyt}\) elevations by mimicking a hypo-osmotic stimulus.

A stronger hypo-osmotic shock (100% ASW to 50% ASW) resulted in a rapid \([Ca^{2+}]_\text{cyt}\) elevation which initiated directly after the stimulus was applied (Figure 7D). In comparison, application of a cold shock from 34°C to 8°C triggered \([Ca^{2+}]_\text{cyt}\) elevations that rose less rapidly and exhibited a longer delay to their initiation (Figure 7D). Combining both shocks using perfusion with 50% ASW at 10°C led to biphasic \([Ca^{2+}]_\text{cyt}\) elevations in 71% of cells (42 cells, three separate experiments) (Figure 7D). These consisted of a very rapid initial peak in \([Ca^{2+}]_\text{cyt}\), followed by a second peak around 3 s later, which was of greater amplitude than the first peak in the majority of cells (24 out of 30). The mean maximal amplitude of the \([Ca^{2+}]_\text{cyt}\) elevations caused by the three different treatments were all significantly different from each other, with the cold shock alone causing the lowest and the combined cold- and hypo-osmotic shock causing the highest \([Ca^{2+}]_\text{cyt}\) elevations (Figure 7E).

Taken together, \([Ca^{2+}]_\text{cyt}\) elevations induced by hypo-osmotic shock exhibit significant differences in amplitude and timing in the presence of a simultaneous cold shock. This indicates that the cold shock stimulus is additive and of sufficient magnitude to influence cellular \([Ca^{2+}]_\text{cyt}\) signaling during hypo-osmotic stress. We therefore investigated whether \([Ca^{2+}]_\text{cyt}\) signaling during cold shock may influence the short-term survival of *P. tricornutum* under hypo-osmotic stress.
Simultaneous cold shock enhances survival during hypo-osmotic shock

Cells were treated with 25% ASW to administer a strong hypo-osmotic shock at control and low temperatures in the presence or absence of external Ca\(^{2+}\). Cell viability was determined after 3 min by staining with Sytox-Green. Administration of a cold shock alone, either in the presence or absence of external Ca\(^{2+}\), did not reduce cell viability (Figure 8). Application of a strong hypo-osmotic shock (25% ASW) significantly reduced cell viability, and this effect was greater following the removal of external Ca\(^{2+}\), supporting our previous observations that Ca\(^{2+}\) signaling is required for osmoregulation in P. tricornutum (Helliwell et al., 2021b). Surprisingly, application of 25% ASW in combination with a cold shock (4°C) led to a substantial reduction in cell mortality caused by hypo-osmotic shock (compared to the control temperature, 21°C). This effect was reduced by inhibiting Ca\(^{2+}\) signaling, although cell viability remained higher than at control temperature. Our data therefore indicate that rapid cooling has an important beneficial influence on the survival of P. tricornutum cells during a hypo-osmotic shock.

Cold shock is associated with Ca\(^{2+}\)-dependent K\(^{+}\) efflux

Ca\(^{2+}\)-dependent K\(^{+}\) efflux plays an essential role in cellular volume control in P. tricornutum during hypo-osmotic shock (Helliwell et al., 2021b). We therefore tested whether the [Ca\(^{2+}\)]\(_{cyt}\) elevations induced by cold shock also resulted in a K\(^{+}\) efflux that could influence cellular osmolarity. We settled a monolayer of P. tricornutum cells onto a microscopy dish and used a K\(^{+}\)-selective microelectrode to measure changes in extracellular K\(^{+}\) in the immediate vicinity of these cells. The cells were perfused with ASW at 25°C, before rapidly switching to 12°C. In each case, the cold shock induced a clear increase in extracellular K\(^{+}\) around the P. tricornutum cells (Figure 9, A–C). Application of a cold shock in the absence of external Ca\(^{2+}\) greatly reduced K\(^{+}\) efflux from the cells, indicating that the K\(^{+}\) efflux is Ca\(^{2+}\) dependent. Very little change in extracellular K\(^{+}\) was observed during a cold
Cold signalling in diatoms

Figure 7 Interactions between the cold shock and hypo-osmotic shock Ca\(^{2+}\) signaling pathways. (A) R-GECO1 fluorescence ratio (F/F\(_0\)) of PtR1 cells in response to a mild hypo-osmotic shock (95% ASW, left) or a simultaneous hypo-osmotic and cold shock (10°C decrease, right). Twelve representative traces are shown. B, Percentage of cells exhibiting [Ca\(^{2+}\)]\(_{cyt}\) elevations for the experiment described in (A). Data are compiled from a minimum of two independent treatments. Number of replicates is shown in parentheses. C, Mean amplitude (±SE) of [Ca\(^{2+}\)]\(_{cyt}\) elevations from responding cells in (B). The two treatments are significantly different (Student’s t test P < 0.001). Number of replicates is shown in parentheses. D, R-GECO1 fluorescence ratio of PtR1 cells in response to stronger simultaneous cold- and hypo-osmotic shocks. Cells were treated with a single hypo-osmotic shock (50% ASW), a single cold shock (10°C) or a simultaneous cold- and hypo-osmotic shock (50% ASW, 10°C). Thirteen representative traces are shown. E, Mean maximal amplitude (±SE) of cells exhibiting [Ca\(^{2+}\)]\(_{cyt}\) elevations in (D). For biphasic peaks the higher amplitude was chosen. The data represent the combination of at least three independent experiments per treatment. Letters represent significant differences between treatments (one-way Kruskal–Wallis ANOVA Ranks P = 0.001, with Dunn post hoc). Number of replicates is shown in parentheses.

shock in the absence of cells, indicating that the performance of the K\(^+-\)selective microelectrode was not affected by the change in temperature. We conclude that cold shock induces Ca\(^{2+}\)-dependent K\(^+\) efflux in P. tricornutum cells, which may contribute to volume regulation during a simultaneous hypo-osmotic shock.

Discussion

This study shows that transient [Ca\(^{2+}\)]\(_{cyt}\) elevations are a consistent response to the rapid cold shocks likely to be experienced by intertidal diatoms. By using a continuous perfusion system, our study was able to avoid a shear-related [Ca\(^{2+}\)]\(_{cyt}\) response, which may have masked a cold [Ca\(^{2+}\)]\(_{cyt}\) response in earlier investigations using P. tricornutum expressing aequorin (Falciatore et al., 2000). The cold-induced [Ca\(^{2+}\)]\(_{cyt}\) elevations are shown to be specifically involved in sensing the rate of cooling rather than the absolute temperature. A similar dependence of the amplitude of [Ca\(^{2+}\)]\(_{cyt}\) elevations on the rate of cooling has been observed in Arabidopsis, which showed [Ca\(^{2+}\)]\(_{cyt}\) elevations at cooling rates down to 0.05°C s\(^{-1}\) (Plieth et al., 1999), indicating greater sensitivity of Arabidopsis to slower cooling rates. Phaeodactylum tricornutum and T. pseudonana did not show a Ca\(^{2+}\) signaling response to rapid warming, suggesting that the Ca\(^{2+}\) signaling pathways of animals and plants in response to elevated temperatures are not conserved in diatoms. Diatoms therefore likely use alternative cellular mechanisms for thermosensation in response to rapid heat shock, although as only short-term temperature increases were evaluated in our study, we cannot rule out a potential role for Ca\(^{2+}\) signaling in response to longer-term temperature increases.

Our environmental data indicate that rapid cooling is likely to occur following tidal immersion on days when the air temperature is substantially warmer than the sea temperature. The rate of cooling will depend primarily on the volume of the water in each pool, the temperature difference between the pool and the incoming tide and the rate of immersion (e.g. wave action). Diatoms only exhibited [Ca\(^{2+}\)]\(_{cyt}\) elevations in response to very rapid cooling (> 1°C s\(^{-1}\)) and so natural populations may not experience a sufficient rate of cooling if they are present in large volumes of water (e.g. deeper pools). However, diatoms are also abundant in many shallow environments in the intertidal zone, most notably in biofilms on the surface of tidal flats, rocks, or microalgae (Thompson et al., 2004). These low volume environments are likely to experience near instant changes in temperature following rapid tidal immersion.

Cold shock-induced [Ca\(^{2+}\)]\(_{cyt}\) elevations in P. tricornutum do not play an obvious role in acclimation to low
temperatures. We found no longer-term growth effects of experimentally blocking the cold shock Ca$^{2+}$ signal. Cold signaling in *P. tricornutum* therefore differs from plants and insects (Knight and Knight, 2012; Teets et al., 2013), in which the [Ca$^{2+}$]$_{cyt}$ elevations play a direct role in acclimation to lower temperatures. The [Ca$^{2+}$]$_{cyt}$ response in *P. tricornutum* is specifically induced by rapid cooling, which points to a potential role in short-term regulation of cellular processes rather than longer term acclimation to a change in temperature. Of particular interest is the interaction between cold shock and osmotic shock, since intertidal organisms are often likely to experience these stresses simultaneously, during an incoming tide or rain precipitation (Lewin and Guillard, 1963; Kirst, 1990). Given the nature of the osmotic and cold shock Ca$^{2+}$ signals identified in *P. tricornutum*, it is most likely that they involve distinct sensory pathways, as evidenced by their additive nature and the appearance of a biphasic [Ca$^{2+}$]$_{cyt}$ elevation when cells were treated with simultaneous cold and osmotic shocks. Whether these distinct responses represent Ca$^{2+}$ entry through different Ca$^{2+}$ channels or are due to sequential activation of the same Ca$^{2+}$ channel by different stimuli with little or no refractory period remains to be determined, although it is worthy of note that both the osmotic (Helliwell et al., 2021b) and the cold-induced Ca$^{2+}$ signals initiate at the cell apices (Figure 2B). Cold and osmotic Ca$^{2+}$ signals also both require the presence of external Ca$^{2+}$, indicating a shared requirement for plasma membrane Ca$^{2+}$ channels, at least in the initiation of the [Ca$^{2+}$]$_{cyt}$ elevation.

The protective effect of cold shock on survival of *P. tricornutum* in response to severe hypo-osmotic shock may arise directly from cooperative Ca$^{2+}$ signaling (Supplemental Figure S4). The hypo-osmotic shock-induced [Ca$^{2+}$]$_{cyt}$ elevations lead to rapid efflux of K$^+$ in *P. tricornutum*, which restricts cell volume increase and prevents bursting (Helliwell et al., 2021b). The results here strongly suggest that cold-induced [Ca$^{2+}$]$_{cyt}$ elevations may also act directly to trigger K$^+$ efflux from the cytosol, for example through the activation of Ca$^{2+}$-dependent K$^+$ channels. Whether the rapid loss of K$^+$ plays a physiological role in acclimation to low temperature is unclear, but it would clearly serve to lower the osmolarity of the cell. Given the frequent co-occurrence of cold and hypo-osmotic shocks, the cold-induced [Ca$^{2+}$]$_{cyt}$ elevations may therefore function primarily to support osmoregulation. Rapid cooling does not appear to adversely harm the cell when Ca$^{2+}$ signaling is inhibited, whereas a severe hypo-osmotic shock will lead to cell bursting within seconds if cell volume is not controlled (Helliwell et al., 2021b).

Osmoregulation in response to hypo-osmotic stress in diatoms (and most other eukaryotes) is most likely initiated by activation of mechanosensitive channels due to the increase in cell volume (Helliwell et al., 2021b). Mechanosensitive channels only activate when the membrane is under tension, that is, when swelling has already occurred, and cell viability is therefore under immediate threat if rapid osmoregulation cannot be achieved. The K$^+$ efflux in response to a cold shock would allow the cell to reduce its osmolarity even if this critical increase in membrane tension is not perceived. By associating K$^+$ efflux with an additional stimulus that commonly co-occurs with hypo-osmotic shock, diatoms can augment the osmoregulatory response and help prevent cell swelling to critical levels. Consistent with this hypothesis, cold-induced [Ca$^{2+}$]$_{cyt}$ elevations were only associated with very rapid cooling. A more gradual exposure to hypo-osmotic stress conveys a much lower risk of cell bursting, reducing the need to augment the osmoregulatory response.

We should also consider that low temperature may have a direct effect on reducing mortality during hypo-osmotic stress that is independent of the signaling component, for example by increasing cell wall rigidity. However, the protective effect of cold shock in the absence of Ca$^{2+}$ was small compared to the much greater reduction in mortality in the presence of external Ca$^{2+}$. We were unable to identify pharmacological inhibitors to selectively inhibit either osmotic or cold associated Ca$^{2+}$ signaling and the removal of external Ca$^{2+}$ completely inhibited both signaling pathways. Dissecting the individual contributions of these signaling pathways to cell survival during simultaneous shocks is therefore not currently easily achieved. Selective inactivation of the underlying molecular mechanisms through genetic approaches will most likely be required to fully understand the nature of the cross-talk between the signaling pathways.

Cellular responses to stressors are commonly examined in isolation in the laboratory in order to simplify the
elucidation of the signaling pathways responsible. However, organisms often have to respond to inputs from multiple stimuli simultaneously in their natural environment, leading to cross-talk between signaling pathways. Cross-talk in cell signaling can occur when two distinct stimuli trigger a shared cellular response that confers tolerance to both stressors. This may involve activation of a common receptor or activation of independent receptors that converge on a specific node in the signaling pathway (Knight and Knight, 2001). Cross-talk with temperature sensing is likely to have evolved when another stress occurs simultaneously with temperature or with a predictable temporal link (i.e., one stimulus consistently precedes the other) (Sinclair et al., 2013). In the case of intertidal zone, many environmental parameters will exhibit a degree of covariance associated with tidal immersion and emersion. It seems likely that organisms inhabiting these environments have developed mechanisms of cross-talk in their pathways of environmental perception that enable them to optimize their physiological responses.

There are multiple examples of cross-talk between temperature and osmotic stress signaling pathways in other eukaryotes. In plants, freezing temperatures can lead to cellular water loss due to external ice formation and many of the genes within the cold-responsive (COR) regulon are also inducible by drought (Boyce et al., 2003). The cold-responsive C-repeat binding factors/dehydration-responsive element-binding (CBF/DREB1) and drought-responsive DREB2 transcription factors both bind to a common promoter element (DRE), leading to convergence of the cold and drought signaling pathways (Boyce et al., 2003). Overexpression of the cold-responsive DREB1A transcription factor in Arabidopsis resulted in enhanced tolerance to both freezing and drought stress (Liu et al., 1998). In addition, Arabidopsis plants treated with the phytohormone abscisic acid, which plays a primary role in drought tolerance, also show enhanced freezing tolerance (Mantyla et al., 1995). Cross-talk between temperature and osmotic stress signaling pathways have also been documented in yeast. Saccharomyces cerevisiae exhibits a high osmolarity (HOG) response to hyper-osmotic stress that results in increased production of the compatible solute, glycerol. The HOG response is mediated by a mitogen-activated protein kinase.

Figure 9 Cold shock induces a Ca\(^{2+}\)-dependent K\(^+\) efflux. A, K\(^+\) efflux from P. tricornutum cells during a cold shock. A K\(^+\) microelectrode was placed adjacent to densely packed P. tricornutum cells to measure K\(^+\) in the immediate vicinity of the cell. A cold shock was applied by perfusion. The increase in extracellular K\(^+\) is the result of K\(^+\) efflux from the cells. The temperature in the dish is also shown (upper trace). B, Extracellular K\(^+\) during a cold shock in the absence of external Ca\(^{2+}\) (perfusion with ASW-Ca\(^{2+}\) + 200 \(\mu\)M EGTA). C, Mean change in extracellular K\(^+\) around P. tricornutum cells during a cold shock. "No cells" indicates control experiments where the experimental setup was identical, but no P. tricornutum cells were present in order to assess whether the performance of the K\(^+\) microelectrode was influenced by temperature. The total number of replicates for each treatment are shown in parentheses, error bars = se.
pathway that can also be activated by other stimuli including both cold and heat shocks. Heat shock activates the HOG pathway indirectly by stimulating loss of glycerol, leading to hyper-osmotic stress (Winkler et al., 2002; Dunayevich et al., 2018).

Our results indicate that cross-talk between Ca\(^{2+}\)-mediated cellular signaling mechanisms is an important consideration in the response of marine organisms to multiple stressors. While our results are discussed primarily in the context of the intertidal zone where rapid substantial changes in temperature are a regular occurrence, the conserved nature of cold-induced Ca\(^{2+}\) signaling in *T. pseudonana* suggests that this pathway may be important more widely in diatom ecology. The cold-induced [Ca\(^{2+}\)]\(_{cyt}\) elevations in *T. pseudonana* exhibit different characteristics from *P. tricornutum* that may reflect differences in their physiological response. Planktonic diatoms will undoubtedly encounter substantial fluctuations in temperature and salinity in near-shore and estuarine environments or when they are mixed through the thermocline, although the magnitude and rate of the temperature changes are likely to be lower. Diatoms inhabiting sea ice environments may also experience rapid changes in temperature and salinity, for example, during flushing of hyper-saline brine channels with melt water (Mock and Junge, 2007). Future elucidation of the mechanisms of cross talk in these signaling pathways will allow us to understand how diatoms successfully integrate inputs from multiple environmental stimuli, which has likely played a major role in their success in diverse and highly dynamic environmental regimes.

**Materials and methods**

**Recording of rockpool temperature**

Temperature data were recorded using a 27-mm Envlogger v2.4 (ElectricBlue, Porto, Portugal) encased in acrylic resin, recording in 30-min intervals with a resolution of 0.1°C. The Envlogger was secured to the substrate using Z-Spar A-788 epoxy resin roughly 3 cm below the surface waters of a shallow mid-shore rockpool measuring ~8-cm deep at Looe Hannfore, Cornwall, UK (50.3411, −4.4598) from July 1, 2019 to July 7, 2019.

**Strains and culturing conditions**

The wild-type *P. tricornutum* strain used in this study was CCAP 1055/1 (Culture Collection of Algae and Protozoa, SAMS, Scottish Marine Institute, Oban, UK). A *P. tricornutum* strain transformed with the R-GECO1 Ca\(^{2+}\) biosensor (*PtR1*) was generated as described previously (Helliwell et al., 2019). Three *eukcata1* knockout strains in the *PtR1* line (labeled A3, B3, and B6) were generated by CRISPR–Cas9-mediated gene editing, with two single-guide RNAs ~50-bp apart targeted to the pore region of *PtEUKCATA1* resulting in deletions of 38–124 bp (Helliwell et al., 2019). The *T. pseudonana* strain expressing the R-GECO1 biosensor (*TpR1*) was generated as described in Helliwell et al (2021a, 2021b). Cultures were maintained in natural seawater with f/2 nutrients (Lewin and Guillard, 1963; Guillard, 1975); modified by the addition of 106-μM Na\(^2\)SiO\(_3\)·5H\(_2\)O and the exclusion of vitamins (*P. tricornutum* only). For imaging experiments, cells were acclimated to an ASW medium for minimum 10 days prior to analysis. ASW contained 450-mM NaCl, 30-mM MgCl\(_2\), 16-mM MgSO\(_4\), 8-mM KCl, 10-mM CaCl\(_2\), 2-mM NaHCO\(_3\), 97-μM H\(_2\)BO\(_3\), f/2 supplements, and 20-mM HEPES (pH 8.0). Cultures were grown at 18°C with a 16:8 light/dark cycle under illumination of 50 μmol m\(^{-2}\) s\(^{-1}\).

**Epifluorescence imaging of R-GECO1 fluorescence**

Cell culture measuring 500 μL of was added to a 35-mm microscope dish with glass coverslip base (In Vitro Scientific, Sunnyvale, CA, USA) coated with 0.1% poly-L-lysine (Merck Life Science UK, Gillingham, Dorset) to promote cell adhesion to the glass surface. Cells were allowed to settle for 5–20 min at RT under light. R-GECO1 was imaged using a Leica DMI8 inverted microscope (Leica Microsystems, Milton Keynes, UK) with a 63 × 1.4NA oil immersion objective, using a Lumencor SpectraX LED light source (4% intensity) with a 541–551-nm excitation filter and 565–605-nm emission filter. Images were captured with a Photometrics Prime 95B sCMOS camera (Teledyne Photometrics, Birmingham, UK) with a 300-ms exposure. Images were captured at 3.33 frames per second using Leica application suite X-software v.3.3.0.

**Administration of temperature shocks to cells in the imaging setup**

The dish was perfused with ASW without f/2 nutrients at a standard flow rate of 16 mL min\(^{-1}\). To achieve rapid changes in temperature in the dish, the perfusion was switched between solutions of different temperature to achieve target temperatures of approximately 10°C, 22°C, or 30°C respectively. Actual dish temperature was recorded using a Firesting micro optical temperature sensor (Pyroscience GmbH, Aachen, Germany). For the majority of experiments, cells were perfused with warmer media (dish temperature 30°C) for 1 min prior to application of the cooling shock (these conditions reflect those observed in the rockpool observations). The perfusion flow rate was altered to achieve different temperature change rates. As cooling rate was not linear, the maximum cooling rate was defined as the largest decrease temperature within a one second period.

**Application of inhibitors and elicitors**

External Ca\(^{2+}\) was removed by perfusion with ASW without CaCl\(_2\) containing 200-μM EGTA. RR was added to cells at a final concentration of 10 μM 5 min prior to cold shock treatment. Menthol was prepared as a 1-M stock solution in DMSO and used at concentration of 1 mM, resulting in a final DMSO concentration of 0.1% v/v.

**Processing of imaging data**

Images were processed using LasX software (Leica). The mean fluorescence intensity within a region of interest (ROI) over time was measured for each cell by drawing an ROI
cultures were grown at 18°C for 10 min, which acts to inhibit Ca²⁺ signaling during rapid cooling but avoids prolonged exposure to very low external Ca²⁺.

Statistical analysis
Graphs and statistical analyses were performed using Sigmaplot v14.0 (Systat Software, Slough, UK). Error bars represent standard error of the mean. Unless indicated otherwise, imaging experiments were repeated three times with independent cultures on different days to ensure reproducibility of the response.

Normal distribution of respective datasets was tested using Shapiro–Wilk’s normality test. When passed, statistical analysis of datasets with two groups were done with Student’s t test, and when not passed with Mann–Whitney’s rank sum test. Statistical analysis of datasets with more than two groups were performed using an analysis of variance (ANOVA) followed by a Holm–Sidak post hoc test when the normality test was positive. When the normality test was negative, Kruskal–Wallis’ one-way ANOVA on Ranks was used instead. All statistical tests were performed with Sigmaplot v14.0.3.192 (Systat software Inc).

Growth at different temperatures after a cold shock
For the growth curves, cells were grown to mid exponential phase (2.73 × 10⁶ cells mL⁻¹). The culture was divided into 10-mL aliquots and cells were pelleted by centrifugation (1250 g at 18°C). Cells were washed in 40-mL ASW ± Ca²⁺ and pelleted again by centrifugation. Cells were then re-suspended in their respective treatments (20 mL of ASW ± Ca²⁺ at 18°C or 4°C to administer a rapid cold shock). The temperature of the ASW was monitored after mixing and found to have remained at 4°C. After 10 min, 2 mL of each culture was used to inoculate culture vessels containing 18 mL of standard ASW F/2 media containing 10-mM Ca²⁺ (approximately starting density of 6.8 × 10⁶ cells mL⁻¹) and cultures were grown at 18°C or 4°C for 5 days or 30 days, respectively. The cells were therefore only in media without Ca²⁺ for 10 min, which acts to inhibit Ca²⁺ signaling during rapid cooling but avoids prolonged exposure to very low external Ca²⁺.

Cell survival during hypo-osmotic shock
To examine the effect of temperature on cell viability during hypo-osmotic shock, 10 mL of a late log phase culture (6 × 10⁶ cells mL⁻¹) were pelleted by centrifugation (1250 g at 18°C). Cells were washed twice with 10-mL ASW ± Ca²⁺ and 250-µL aliquots were taken. To apply the hypo-osmotic and cold shock treatments, 750 µL of ASW (±Ca²⁺) or deionized water at two different temperatures (20°C or 4°C) were added to each tube. Addition of water results in a severe hypo-osmotic osmotic shock (final concentration 25% ASW) simultaneously with the temperature shock. The cells were then incubated at their respective temperatures for 3 min prior to addition of 5-µM Sytox Green (Thermo Fisher Scientific, Loughborough, UK). All treatments were then incubated at 20°C 15 min in darkness. Cell viability was measured with a LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems, Villeneuve d’Ascq, France) to count live (displaying red chlorophyll fluorescence) versus dead cells (Sytox Green stain) with following settings: Excitation intensity green = 11, red = 7, count threshold for both = 3.

Quantification of K⁺ efflux in P. tricornutum populations using K⁺-selective microelectrodes
K⁺ microelectrodes were fabricated as described previously (Helliwell et al., 2021b). Clark GC-1.5 borosilicate glass capillaries (Harvard Apparatus, Cambridge, UK) were pulled to a fine point using a P-97 pipette puller (Sutter, Novato, CA, USA). The pipette tips were then gently broken to produce a diameter of ca 10–20 µm. The capillaries were salanized by exposure to N,N-dimethyltrimethylsilylamine (TMSDMA) vapor at 200°C for 20 min within a closed glass Petri dish. The K⁺ microelectrodes were prepared by introducing K⁺ ionophore I (Sigma Aldrich, Gillingham, Dorset, UK) into the pipette tip by suction. Pipettes were then back-filled with the filling solution (100-mM NaCl, 20-mM HEPES pH 7.2, and 10-mM NaOH). The reference electrode was filled with 3-M KCl, and data were recorded using an AxoClamp 2B amplifier, with pClamp v10.6 software (Molecular Devices, CA, USA). Each K⁺ microelectrode was calibrated using a two-point calibration with standard KCl solutions. The mean slope of the calibrated electrodes was 53.0 ± 1.3 mV per decade (±se).

For the measurements, 10 mL of P. tricornutum cells from exponential culture containing 10⁶−10⁷ cells mL⁻¹ were centrifuged at 1250 g for 10 min and re-suspended in 1 mL of ASW. The cells were then allowed to settle on a poly-L-lysine coated microscope dish. Cells were perfused with ASW or ASW-Ca²⁺ (0-µM Ca²⁺ + 100-µM EGTA) and cold shocks were applied as described for the microscopy observations. Control experiments were performed in the absence of P. tricornutum cells to ensure that the change in temperature did not alter the performance of the K⁺ microelectrodes.

Supplemental data
The following materials are available in the online version of this article.

Supplemental Figure S1. [Ca²⁺]Cyto elevations in response to repeated cold shocks.

Supplemental Figure S2. Cold shocks from different starting temperatures.
Supplemental Figure S3. Cell volume during cold shock. 
Supplemental Figure S4. Proposed Ca\(^{2+}\) signaling pathways in response to osmotic and cold stress.

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