Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels

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Abstract A guanylyl cyclase has been recently identified in *Arabidopsis* but, despite the use of pharmacological inhibitors to infer roles of the second messenger 3′,5′-cyclic guanosine monophosphate (cGMP), very few measurements of actual cGMP levels in plants are available. Here, we demonstrate that cGMP levels in *Arabidopsis* seedlings increase rapidly ($\leqslant$5 s) and to different degrees after salt and osmotic stress, and that the increases are prevented by treatment with LY, an inhibitor of soluble guanylyl cyclases. In addition, we provide evidence to suggest that salt stress activates two cGMP signalling pathways – an osmotic, calcium-independent pathway and an ionic, calcium-dependent pathway.

Keywords: NaCl stress; Osmotic stress; cGMP; Cytosolic calcium; Second messenger; *Arabidopsis thaliana*

1. Introduction

Cyclic nucleotide monophosphates (cNMPs) are established signalling molecules in both micro-organisms and animals, but it is only over the last ten years that their presence has become accepted in higher plants. 3′,5′-cyclic guanosine monophosphate (cGMP) and 3′,5′-cyclic adenosine monophosphate (cAMP) have been detected in several plant species [1–5] but the number of reports about the presence and role of cyclic nucleotides in plants is still very small [6]. Guanylyl cyclase (EC 4.6.1.2) catalyses the formation of cGMP from guanosine 5′-triphosphate and has recently been identified in *Arabidopsis thaliana*, intensifying the search for cGMP regulated processes and downstream targets [7]. In animal systems, downstream targets of cNMPs include cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively), cyclic nucleotide gated ion channels (CNGCs) and phosphodiesterases [8–10]. *Arabidopsis* contains a family of 20 CNGCs [6], several of which have been shown to be activated by cNMPs [11–13]. These CNGCs have also been proposed as points of interaction between cNMP and calcium signalling [6].

Salt and osmotic stress result in transient increases in cytoplasmic free calcium concentration ($[Ca^{2+}]_c$), and disruption of this calcium transient affects downstream gene expression [14]. In *Arabidopsis*, exogenous application of cNMPs increases salt tolerance [15] and cNMPs down-regulate sodium ion influx, probably through a direct interaction with non-selective cation channels [15,16]. We measured cGMP levels in response to salt and osmotic stress and report the first demonstration of an increase in cGMP levels after abiotic stress in plants.

2. Materials and methods

2.1. Plant growth

All seedlings were transgenic *A. thaliana* Columbia-0 ecotype stably expressing apoaequorin under the control of the constitutive cauliflower mosaic virus 35S promoter [17]. Seedlings were grown on petri dishes containing plant nutrient agar [18] under constant fluorescent light (100 μM photons m^{-2} s^{-1}) at 24 °C for 14 days.

2.2. cGMP extraction and measurement

For each time point, four replicate pools of seedlings (each pool weighing 300 mg) were placed into a 6-well microtitre plate and aequorin was reconstituted overnight according to the method of [19]. The following morning, after addition of water, NaCl or sorbitol; samples were quickly blotted dry and immediately frozen in liquid nitrogen. A entire pool of seedlings could be removed from the well simultaneously enabling blotting and freezing to take place within 5 s. Frozen tissue was ground to a fine powder, added to 3 ml of ice-cold 6% trichloroacetic acid and the homogenate centrifuged at 4000 rpm for 15 min. The aqueous extract was extracted four times in 5 volumes of diethyl ether, dried under vacuum at 25 °C overnight and stored at −70 °C. cGMP content was determined according to the manufacturer's instructions for the cGMP (^{125}I) Assay System (Amersham Biosciences). The cGMP content of each pool of seedlings was determined separately.

2.3. $[Ca^{2+}]_c$ measurements

Seedlings were reconstituted in 6-well microtitre plates according to the method outlined in [17]. After reconstitution, individual seedlings were placed in cuvettes in 250 μl (or 200 μl when treating with inhibitors) water for 30 min. 50 μl of inhibitor was added 10 min (LY, final concentration 10 μM) or 1 h (neomycin, final concentration 0.1 mM) prior to treatment. The cuvette was placed inside a digital luminometer (Labsystems, Finland) and light units per second were recorded over 5-s intervals. Baseline counts were measured for 15 s after which 250 μl of treatment (water, NaCl or sorbitol) was delivered through a syringe attached to a light tight port. After luminescence counts returned to basal levels, the remaining aequorin was discharged by injecting 1 ml 2 M Ca_2Cl in 20% ethanol [14]. $[Ca^{2+}]_c$ was calibrated according to the

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Abbreviations: cGMP, 3′,5′-cyclic guanosine monophosphate; cAMP, 3′,5′-cyclic adenosine monophosphate; CNGCs, cyclic nucleotide gated ion channels

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method of [19] using the equation $p\mathrm{Ca} = 0.332588(-\log k) + 5.5593$, where k is a constant determined by the luminescence counts per second divided by the total remaining counts.

3. Results

3.1. cGMP accumulates in response to salt and osmotic stress

The cGMP content of *Arabidopsis* seedlings increased rapidly after salt or osmotic stress (Fig. 1). Phosphodiesterase inhibitors were not added to the seedlings so the data represent actual cGMP levels at that time. The sorbitol concentrations are osmotically equivalent to the salt treatments (100 mM sorbitol corresponding to 50 mM salt, and 300 mM sorbitol to 150 mM salt) but there is an additional ionic component to salt stress.

Significant increases in cGMP were detectable within 5 s (the shortest time point technically possible), and 15 min after the stress treatment cGMP levels were dramatically higher than in control seedlings. Although after 15 min, there was not a large difference in cGMP content between salt and the corresponding osmotic stress, the initial rate of increase of cGMP levels was significantly higher after osmotic stress than salt stress. Similarly, the rate of increase also appears to be dose dependent.

3.2. Interaction of cGMP and calcium during abiotic stress

Membrane-permeable cGMP analogues have been shown to cause increases in $[Ca^{2+}]_c$ in tobacco protoplasts [20]. As salt and osmotic stress cause rapid increases in $[Ca^{2+}]_c$ [14] as well as cGMP (Fig. 1), we used inhibitors of cGMP accumulation and $[Ca^{2+}]_c$ increase to investigate interaction between these two second messengers. Treatment of *Arabidopsis* seedlings with salt or osmotic stress induced changes in $[Ca^{2+}]_c$ as expected [14] and were dose-dependent (Fig. 2). Interestingly, 50 mM (0.095 Os/kg) salt treatment caused a large increase in $[Ca^{2+}]_c$ but the iso-osmotic concentration of sorbitol (100 mM; 0.095 Os/kg) did not. Changes in $[Ca^{2+}]_c$ were transient and no additional responses were seen over longer time periods (data not shown).

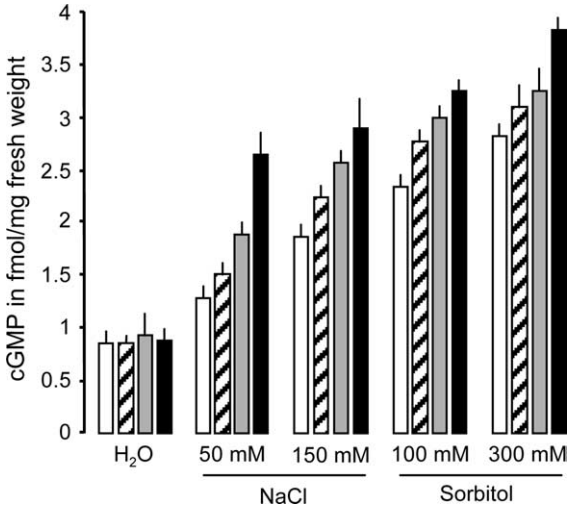

Fig. 1. cGMP content of *Arabidopsis* seedlings. cGMP levels in seedlings 5 s (white bars), 1 min (striped bars), 5 min (grey bars) and 15 min (black bars) after treatment indicated on *X*-axis. Data are the average of four replicates, standard errors are indicated and the data are representative of two independent experiments.

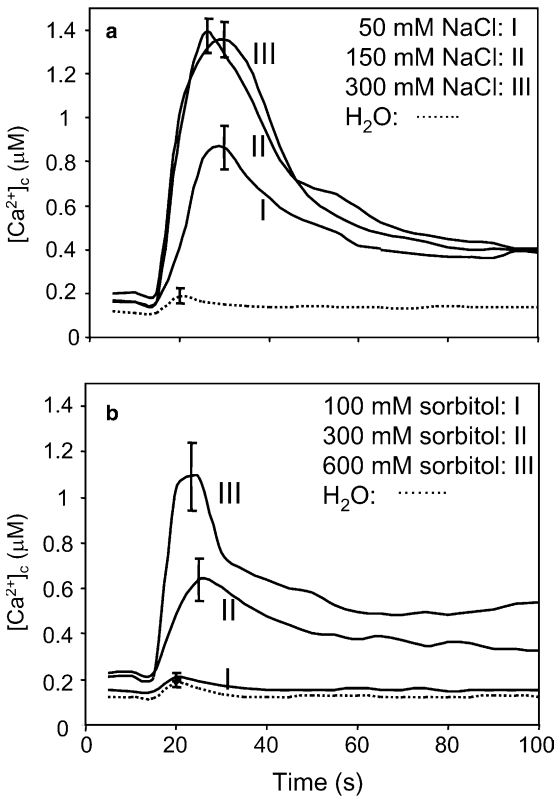

Fig. 2. $[Ca^{2+}]_c$ in *Arabidopsis* seedlings after abiotic stress. $[Ca^{2+}]_c$ in whole *Arabidopsis* seedlings after treatment with different concentrations of (a) NaCl and (b) sorbitol. Salt or sorbitol was added 15 s after the measurement began. Each trace is the average of at least 10 seedlings and standard error of the highest average reading is indicated. Each experiment was repeated at least twice with similar results.

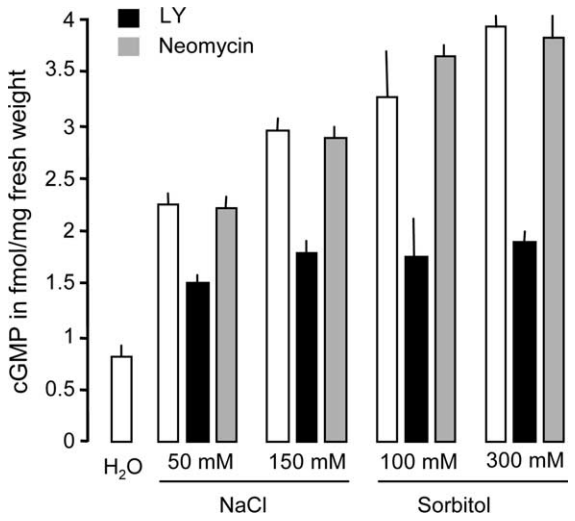

Fig. 3. Effect of inhibitors on *Arabidopsis* seedling cGMP content. cGMP levels in seedlings 15 min after treatment indicated on *X*-axis. Seedlings received no pre-treatment (white bars), pre-treatment with LY (black bars) or pre-treatment with neomycin (grey bars). Data are the average of four replicates, standard errors are indicated and the data are representative of two independent experiments.

LY83583 is an inhibitor of soluble guanylyl cyclases [21] and pre-treatment with 10 μM LY significantly reduced cGMP accumulation in seedlings after salt or osmotic stress (Fig. 3). Pre-treatment with 0.1 mM neomycin, an inhibitor of IP_3-gated calcium channels [22], had no effect on endogenous cGMP levels after abiotic stress (Fig. 3), despite dramatic reduction in the corresponding $[Ca^{2+}]_c$ (Fig. 4). This indicates that lack of a proper calcium response does not affect cGMP accumulation suggesting that cGMP production is not activated downstream of the increase in $[Ca^{2+}]_c$. Interestingly, pre-treatment of seedlings with LY decreased the amplitude of the $[Ca^{2+}]_c$ increase after 50 mM salt treatment (Fig. 4a), suggesting that synthesis of cGMP is required for the full calcium transient. However, LY had no effect on the $[Ca^{2+}]_c$ increase after 150 mM salt or 300 mM sorbitol treatment (Fig. 4c and d).

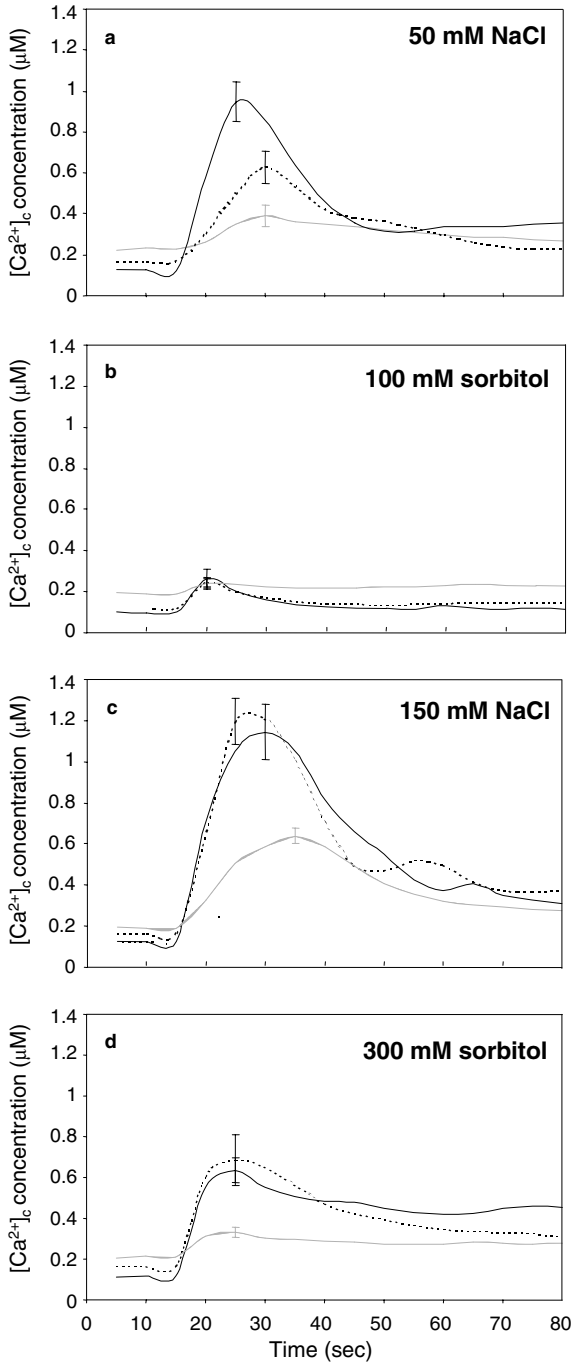

Fig. 4. Effect of inhibitors on $[Ca^{2+}]_c$ in *Arabidopsis* seedlings after abiotic stress. $[Ca^{2+}]_c$ in whole *Arabidopsis* seedlings after treatment with (a) 50 mM NaCl, (b) 100 mM sorbitol, (c) 150 mM NaCl and (d) 300 mM sorbitol. Seedlings were pre-treated in water (black lines), LY (dashed lines) or neomycin (grey lines) before stress treatment. Salt or sorbitol were added 15 s after the measurement began. Each trace is the average of at least 10 seedlings and standard error of the highest average reading is indicated. Each experiment was repeated at least twice with similar results.

4. Discussion

The key result reported here is that cGMP is produced in response to salt and osmotic stress. This is the first direct measurement of cGMP levels in response to abiotic stress in plants. The accumulation of cGMP after abiotic stress is extremely rapid with increases detectable within 5 s. In whole-cell and patch clamp experiments, cGMP has been shown to down-regulate Na^+ influx [15,16]. This down-regulation of Na^+ influx via cGMP-sensitive voltage-independent channels can only occur if cGMP is synthesised in response to salt. Our demonstration that cGMP levels increase after salt treatment therefore links exogenous cGMP experiments to the situation in vivo. As yet, the role of cGMP accumulation in response to osmotic stress is not clear. In contrast to the increase in salt tolerance seen in response to exogenous cGMP, no enhancement of plant growth in the presence of sorbitol has been observed [15].

cGMP levels in other reports vary considerably from basal levels of 0.05 pmol/g FW and 0.3 pmol/g FW after gibberellin treatment of barley aleurone tissue [1], to basal levels of 2–10 pmol/g FW and approx. 90 pmol/g FW after nitric oxide treatment in tobacco leaves [2]. Our results, showing basal levels of less than 1 pmol/g FW and almost 4 pmol/g FW after stress treatment, lie between these extremes.

As both cGMP and calcium increases occur within a similar time frame, we looked at possible interaction between these signalling molecules. It appears that the increase in cGMP after salt and osmotic stress does not depend on the increase in $[Ca^{2+}]_c$. Similarly at 150 mM salt stress and corresponding osmotic stress, the increase in $[Ca^{2+}]_c$ appears to be independent of any preceding cGMP production (Fig. 4c and d). However, at lower salt concentrations (50 mM) a reduction in cGMP production significantly reduced the amplitude of the $[Ca^{2+}]_c$ increase (Figs. 3 and 4a) suggesting that cGMP was acting upstream of the calcium transient. cGMP-sensitive voltage-independent channels include the CNGCs, which could function as a link between cGMP synthesis and $[Ca^{2+}]_c$. Interestingly, there is no $[Ca^{2+}]_c$ increase at the corresponding osmotic stress (100 mM sorbitol) which raises the tantalizing possibility that this cGMP-dependent calcium increase is activated only in response to the ionic component of salt stress and not the osmotic component. At higher salt concentrations, the larger cGMP-independent osmoticum-activated increase in $[Ca^{2+}]_c$ may effectively swamp the contribution of the increase due to the ionic stimulus. We found that LY still significantly reduced the calcium transient in response to 75 mM salt but not 100 mM salt (results not shown). Recent experiments have highlighted the fact that calcium responses to salt are always faster and higher than those to the corresponding osmotic

stress (Scrase-Field and Knight, unpublished data), possibly due to the additional activation pathway.

The existence of two calcium-signalling pathways in response to salt stress (one ionic-activated and cGMP-dependent, the other osmoticum-activated and cGMP independent) would provide a means for plant cells to discriminate between salt and osmotic stress. Such discrimination must occur as salt and osmotic stress result in different downstream gene expression patterns and the SOS3–SOS2–SOS1 system is only activated in response to salt and not osmotic stress [23]. SOS3 is thought to sense a salt-specific calcium signal and together with SOS2, regulates activity of SOS1, a plasma membrane Na^+/H^+ antiporter [23]. It is thus conceivable that the ionic-activated cGMP-dependent calcium increase may function via the SOS system, linking cGMP to increases in Na^+ efflux, as well as decreases in Na^+ influx.

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