The cyclic nucleotide cGMP is involved in plant hormone signalling and alters phosphorylation of Arabidopsis thaliana root proteins

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Abstract

The cyclic nucleotide cGMP has been shown to play important roles in plant development and responses to abiotic and biotic stress. Yet much controversy remains regarding the exact role of this second messenger. Progress in unravelling cGMP function in plants was hampered by laborious and time-consuming methodology to measure changes in cellular [cGMP] but the development of fluorescence-based reporters has removed this disadvantage. This study used the FlincG cGMP reporter to investigate potential interactions between phytohormone and cGMP signalling and found a rapid and significant effect of the hormones abscisic acid (ABA), auxin (IAA), and jasmonic acid (JA) on cytoplasmic cGMP levels. In contrast, brassinosteroids and cytokinin did not evoke a cGMP signal. The effects of ABA, IAA, and JA were apparent at external concentrations in the nanomolar range with EC50 values of around 1000, 300, and 0.03 nmoles for ABA, IAA, and JA respectively. To examine potential mechanisms for how hormone-induced cGMP signals are propagated, the role of protein phosphorylation was tested. A phosphoproteomics analysis on Arabidopsis thaliana root microsomal proteins in the absence and presence of membrane-permeable cGMP showed 15 proteins that rapidly (within minutes) changed in phosphorylation status. Out of these, nine were previously shown to also alter phosphorylation status in response to plant hormones, pointing to protein phosphorylation as a target for hormone-induced cGMP signalling.

Key words: abscisic acid, Arabidopsis, auxin, cGMP, jasmonic acid, phosphoproteomics, phosphorylation, protein, root, stress.
two membrane proteins were reported to have guanylate cyclase activity in vitro (Kwezi et al., 2007; Meier et al., 2010; Qi et al., 2010; Kwezi et al., 2011). The exact function of these putative cyclases in vivo has yet to be established but these studies provide good evidence that plants have the basic machinery to synthesize cNMPs. In addition, the recently developed reporter system to monitor the changes in cytoplasmic cGMP in real time (Isner and Maathuis, 2011), in a non-invasive manner at the single-cell level, should allow higher resolution in both time and space to study plant cNMP signalling and its integration in other signalling pathways.

Another issue that remains unclear is the generic mechanism of how cNMP signals are relayed to downstream components in plants. In mammalian cells, cNMPs accumulation can directly activate cyclic nucleotide-gated cation channels but more often leads to activation of cNMP-dependent protein kinases (e.g. PKG and PKA) or activation of phosphodiesterases that hydrolyse cGMP or cAMP. cAMP can also exert its action through the recently discovered class of exchange proteins directly activated by cAMP and also through cAMP-regulated guanine nucleotide exchange factors (Beavo and Brunton, 2002; Bos, 2003). Hence, cNMP signalling in mammals typically involves phosphorylation as an early event.

With the expanded molecular basis and the availability of improved tools, this study addressed whether there are hitherto-unknown areas where cGMP signalling may be physiologically relevant. A role for cGMP signalling in the action of giberrellic acid has previously been shown (Penson et al., 1996; Bastion et al., 2010; Isner and Maathuis, 2011) but is unclear for other major plant hormones. This study reports that abscisic acid (ABA), auxin (IAA), and jasmonic acid (JA) evoke rapid concentration-dependent changes in cytoplasmic cGMP concentration, whereas cytokinins and brassinosteroids (BL) do not. In addition, an isoformic tags for relative and absolute quantitation (iTRAQ)-based phosphoproteomics analysis was performed to examine whether protein phosphorylation is likely to be an integral part of cGMP signalling in plants and as such could provide a link between hormone, second messenger, and protein target.

**Materials and methods**

**Plant growth**

*Arabidopsis thaliana* (L) ecotype Columbia (Col-0) was grown hydroponically as described previously (Gobert et al., 2006) in a growth cabinet under short day conditions (10/14 light/dark, 23/17 °C, 200 μmol m−2 s−1 intensity, 70% relative humidity). The growth medium contained 1.25 mM KNO3, 0.5 mM Ca(NO3)2, 0.5 mM MgSO4 and 0.625 mM KH2PO4 as macronutrients and was renewed weekly.

**cGMP measurements in protoplasts**

In order to measure the effect of ABA on cGMP content, the δ-FlincG reporter sensor was used as described by Isner and Maathuis (2011). Briefly, protoplasts were extracted from *Arabidopsis* roots (2–4 weeks old) and transiently transformed with fluorescence cGMP reporter δ-FlincG. When fluorescence was at its maximum (20–30 h after transformation), protoplasts were used for experimentation by placing them in a chamber perfused with incubation medium without or with hormone (ABA, IAA, JA, kinetin, or BL; Sigma) at the indicated concentrations, previously dissolved with incubation medium. Fluorescence intensity was monitored using epifluorescence microscopy (Diaphot-TMD, Nikon) with a ×100 air objective. cGMP-dependent fluorescence was recorded every 30 s using 480/20-nm excitation and 520/40-nm emission. Fluorescence images were acquired with a Rolera-XR digital camera (Hamamatsu) and analysed with SIMPLE PCI 6.1.2 offline imaging software (Compix Imaging Systems).

**cGMP treatment and preparation of root microsomal fractions**

Cyclic GMP treatment consisted of exposing roots to the membrane-permeable, non-hydrolysable cGMP analogue Br-cGMP (20 μM final concentration) for 2, 5, or 10 minutes. *Arabidopsis* root material from control and cGMP-treated plants was harvested at fixed times during the light/dark cycle from 5-week-old plants. In total, protein was pooled from four independently grown batches of plants (for each treatment) across a 6-month period. After grinding in liquid nitrogen, root microsomes were prepared as described (Rea et al., 1992) in the presence of phosphatase inhibitor (Roche, Burgess Hill, UK). Briefly, material was homogenized in 10 ml of medium (1.1 M glycerol, 5 mM EGTA, 1.5% (w/v) polyvinylpyrrolidone, 1% (w/v) ascorbic acid, 1 mM phenylmethanesulphonylfluoride, 1 mM benzamide, 50 mM TRIS-MES; pH 7.6). Homogenate was passed through Miracloth and centrifuged at 3600 g for 10 minutes. Supernatant was centrifuged at 100,000 g for 30 minutes. The microsomal membrane pellet was resuspended in overlay medium (1.1 M glycerol, 5 mM TRIS-MES pH 7.6, 1 mM EGTA, 2 mM dithiothreitol).

**Phosphopeptide preparation**

Prior to phosphoproteomic analysis, for each sample 400–700 μg microsomal protein was reduced with tris(2-carboxyethyl)phosphine in the presence of 0.1% SDS, and alkylated with methylmethanethiosulphonate as in the iTRAQ procedure (Ross et al., 2004). Samples were digested with trypsin in 50 mM triethylammonium bicarbonate (TEAB) overnight at a protein/trypsin ratio of 1:30. Samples were acidified to a final concentration of 1% trifluoroacetic acid (TFA) and separated from the membranes by ultracentrifugation and the supernatant was desalted over an OligoR3 microcolumn and eluted with 0.25 M acetic acid and 40% acetonitrile (ACN). Phosphopeptides were isolated via immobilized metal ion affinity chromatography (IMAC) (Andersson and Porath, 1986; Ficarro et al., 2002) with PhosSelect beads (Sigma; 40 µl beads/mg protein digest), according to the manufacturer’s instructions, and eluted with 200 mM sodium phosphate (pH 8.5). The original flowthrough from IMAC was adjusted to 65% CAN and 2% TFA and saturated with glutamic acid for a second round of phosphopeptide isolation with titania (TopTip titania beads, 30% relative humidity). The original flowthrough from IMAC was recombined and separated by strong cation exchange HPLC. Cation exchange fractions containing peptides were reduced in volume by speed-vac and analysed by LC-MS/MS.

**Phosphoproteomics analyses**

Phosphopeptides were redissolved in 0.5 M TEAB and labelled with the appropriate iTRAQ label. iTRAQ-labelled peptides were combined and separated by strong cation exchange HPLC. Cation exchange fractions containing peptides were reduced in volume by speed-vac and analysed by LC-MS/MS.

LC-MS/MS analysis was performed using an integrated Famos autosampler, SwitchosII switching pump, and UltiMate micro pump.
system (LC Packings, Amsterdam, Netherlands) with an Hybrid Quadrupole-TOF LC/MS/MS mass spectrometer (QStar Pulsar i, Applied Biosystems) equipped with a nano-electrospray ionization source (Proxeon, Odense, Denmark) and fitted with a 10 µm fused-silica emitter tip (New Objective, Woburn, USA). Chromatographic separation was achieved on a 75 µm × 15 cm C18 PepMap Nano LC column (3 µm, 100 Å, LC Packings) and a 300 µm × 5 mm C18 PepMap guard column (5 µm, 100 Å, LC Packings) was in place before switching in-line with the analytical column and the MS. The mobile phase consisted of water/ACN (98:2, v/v) with 0.05% formic acid and a flow rate of 200 nl/min. A linear gradient was created upon switching the trapping column inline by mixing with ACN/water (98:2, v/v) with 0.05% formic acid and a flow rate of 200 nl/min.

Data analysis
The MS results were searched for protein identification and relative abundance using Protein Pilot version 3.0. Data were searched against the IPI Arabidopsis database. From all identified phosphopeptides (Supplementary Table S1, available at JXB online), those with a sum of iTRAQ intensities peak areas of >300 and a peptide confidence score of >50 were included for further analysis (Supplementary Table S1 sheet 2). iTRAQ reporter ion intensities less than twice the background were highlighted in red but retained for analysis. The iTRAQ ratios for each time point versus control were divided by the median ratio to account for systematic errors of peptide load (Supplementary Table S1 sheet 3). Peptide IDs with missing iTRAQ ratios were discarded. After log2 transformation of the 2, 5, and 10 min datasets, standard deviations were calculated for each dataset. Only peptides whose ratios changed by values outside the 90% interval around the median (±1.64 SD) were assumed to show significant changes in phosphorylation and included for further analysis (annotated with blue or red background in Supplementary Table S1 sheet 4). Peptides with predicted non-canonic cleavage were then discarded. In cases where multiple spectra were recorded for a peptide, ratio values were averaged across spectra (Supplementary Table S1 sheet 5). Averaged ratios for all significantly changing peptides are summarized in Supplementary Table S1 sheet 6.

Results and discussion
The plant hormones ABA, IAA, and JA evoke concentration-dependent changes in cellular cGMP

Fig. 1 shows example traces of continuously recorded fluorescence signals derived from the cGMP FlincG reporter (Nausch et al., 2008; Isner and Maathuis, 2011). Addition of 100 nM (final concentration) ABA to the external medium leads to a large increase in fluorescence within 1 minute, indicating a rise in cellular [cGMP]. Increasing concentrations of ABA led to saturation of the cGMP signal with an approximate EC50 of 1000 nM (inset), a value that is similar to that determined for endogenous ABA levels in cells (Harris et al., 1988). Similarly, external IAA changed cellular cGMP with slightly faster kinetics than ABA and also with a higher apparent affinity of approximately 350 nM. The most sensitive response was recorded in the presence of JA: concentrations as low as 1 nM evoked a discernible change in cellular cGMP. For JA an EC50 of 25 pM was derived. In stark contrast, addition of kinetin or BL did not cause any reproducible change in cellular cGMP, even at concentrations as high as 40 µM, which is well beyond those deemed physiologically relevant (Werner et al., 2003).

The data depicted in Fig. 1 strongly suggest a connection between ABA signalling and cGMP. Currently, the evidence for a direct connection between ABA and cGMP is unclear. In barley aleuore cells the application of ABA did not change cGMP levels (Penson et al., 1996), nor was any effect found of ABA on cGMP levels in Arabidopsis mesophyll cells by Wang et al. (2007). In contrast, the ABA-induced stomatal closure in pea guard cells appears to require the synthesis of cGMP (Neill et al., 2002). In Arabidopsis too, there are clear indications that guard cell ABA signalling occurs via cGMP (Neill et al., 2002; Dubovskaya et al., 2011). In all the above studies, cGMP levels were measured with immunoassays (e.g. Lomovatskaya et al., 2011), a method that has intrinsic disadvantages (Isner and Maathuis, 2011). The current data suggest that, when measured in real time at the single-cell level, ABA perception increases cGMP levels. In most cells, the response of plants to salt and drought critically relies on the potency of ABA to regulate water homeostasis, for example by modulating aquaporin conductance (Parent et al., 2009) or stomatal apertures (Harris et al., 1988; Zhang et al., 2001). The well-documented function of cGMP in the same phenomena (salinity, stomatal function; Maathuis and Sanders, 2001; Essah et al., 2003; Donaldson et al., 2004; Maathuis, 2006; Li et al., 2011) gives further credence to the notion that cGMP is an important component of the downstream signal cascade induced by ABA.

A link between IAA and cGMP via nitric oxide (NO) has been inferred: Pharmacological studies (Lanteri et al., 2006) provided indirect evidence for IAA-controlled adventitious root formation in cucumber to occur via NO while NO itself is generally believed to function through activation of the cGMP-producing guanyl cyclase. Application of GC antagonists reduced root formation whereas addition of membrane-permeable cGMP counteracted the effect of the GC agonist. Gravitropism is another IAA-controlled process where cGMP may play a role (Hu et al., 2005). These authors showed that the gravitropic response included an NO-dependent rise in root cGMP levels during a 15–20 h period. Thus, the current results confirm and expand on these findings by showing a rapid and direct effect of IAA on cellular cGMP.

There is no report of a direct link between JA and cGMP. However, both JA and cGMP cause accumulation of stress related proteins like CHS, PAL, and PR1 (Bowler et al., 1994; Durner et al., 1998; Sharan et al., 1998; Suita et al., 2009). Moreover, cGMP levels have been shown to be raised in response to stimuli that also increase JA such as pathogen attack and oxidative stress (Meier et al., 2009; Lin et al., 2011). The current data show that the effects of picomolar concentrations of JA on cellular cGMP and the derived EC50 value (Fig. 1) is lower than the JA concentrations found in plant tissue, which vary from around 1 to 5 nM (Nojiri et al., 1992; Méndez-Bravo et al., 2011). Though the relevance of this remains to be explained, it may suggest that JA has targets other than cGMP that require higher hormone concentrations.

No cGMP response was recorded after cell exposure to cytokinin (Fig. 1). This suggests that the action of this
cGMP exposure leads to rapid phosphorylation of root proteins

In animal cells, cGMP signalling is primarily relayed via cGMP-dependent kinases, PKGs, which in turn modulate protein activity of downstream targets such as transcription factors (Martinez-Atienza et al., 2007). cGMP signalling in plants has been shown to impact on a multitude of functions (Bowler et al., 1994; Penson et al., 1996; Durner et al., 1998; Maathuis and Sanders, 2001; Essah et al., 2003; Newton and Smith, 2004; Maathuis, 2006; Rubio et al., 2007; Ma et al., 2009), yet no cGMP-dependent kinases have been identified in plants to date. The apparent absence of cNMP-dependent kinases (Bridges et al., 2005; Martinez-Atienza et al., 2007) suggests that cyclic nucleotide signalling in plants may be fundamentally different. Indeed, it is unclear whether phosphorylation per se is relevant in transducing plant cNMP signals. To assess whether protein phosphorylation is an integral part of cGMP signalling in plants, the current study used phosphoproteomics approaches to determine the phosphorylation status of root proteins in the absence and presence of membrane-permeable Br-cGMP. Root samples from independently grown batches of plants were pooled and iTRAQ labelled to quantify changes in phosphopeptide abundance amongst microsomal proteins (see Supplementary Fig. S1 for peptide spectra). The data show that cGMP caused a rapid and significant change in the phosphorylation status of 19 peptides (Table 1). Most of these were previously shown to be phosphopeptides (PhosPhAt database, http:// phosphat.mpimp-golm.mpg.de/db.html) but four novel phosphopeptides were found: one in the syntaxin SYP122, one in the inorganic phosphate transporter APT1 and two in an LRR-type protein kinase (At5g49770). In nine cases, phosphorylation status increased whereas in the remaining ten cases, protein domains were dephosphorylated. In many

concentration. Sigmoidal correlations were fitted to the average values for changes in fluorescence for each concentration across three or four independent measurements. EC50 values for ABA, IAA, and JA were 970 nM, 350 nM, and 25 pM respectively.
cases, significant changes were apparent within 2 min exposure to Br-cGMP and this rapid effect suggests that cGMP could directly impact on kinases and phosphatases to relay cGMP signals.

Several studies have investigated the phosphoproteome in response to plant hormones (Chen et al. 2010; Kline et al., 2011) which allows cross referencing of phosphoproteins with those identified in this study. A total of nine proteins (At1g02520; At1g04780; At1g08090; At1g22280; At1g59870; At1g80930; At2g18960; At3g25070; At3g52400) listed in Table 1 have been shown earlier to be altered in phosphorylation in response to ABA (Kline et al., 2011) or a cocktail of five hormones (ABA, gibberrellic acid, JA, IAA, and kinetin; Chen et al., 2010), and in two cases the same protein was identified in all three studies: the 2C type protein phosphatase At1g22280 and the proton pump interactor At4g27500. Although Kline et al. used 5–30 min incubation times and Chen et al. much longer periods of 1–6 h, the data suggest that there may be considerable overlap between cGMP- and hormone-induced changes in phosphorylation.

In conclusion, these findings provide further evidence of important roles for cGMP in relaying the action of the plant hormones ABA, IAA, and JA but not BL and cytokinins. ABA, IAA, and JA exposure in the nanomolar range evoked a cGMP elevation within minutes of administering. In addition, artificially induced elevated levels of cGMP rapidly lead to (de)phosphorylation of proteins whose phosphorylation status is also affected by plant hormones. In combination, these results suggest that cGMP is involved in ABA, IAA, and JA signal transduction and that protein (de)phosphorylation is one of the mechanisms by which this process is sustained.

Supplementary material
Supplementary data are available at JXB online.

Supplementary Table S1. iTRAQ phosphoproteomics data and their analysis.

Supplementary Fig. S1. MS-MS spectra for all identified phosphopeptides.

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References


