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Claudius Marondedze, Arnoud J. Groen, Ludivine Thomas, Kathryn S. Lilley, Chris Gehring

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A Quantitative Phosphoproteome Analysis of cGMP-dependent Cellular Responses in
Arabidopsis thaliana

Claudius Marondedze\textsuperscript{1,2*}, Arnoud J. Groen\textsuperscript{2*} Ludivine Thomas\textsuperscript{3}, Kathryn S. Lilley\textsuperscript{2} and Chris Gehring\textsuperscript{1}

\textsuperscript{1} Division of Biological and Environmental Sciences and Engineering, 4700 King Abdullah University of Science and Technology, 23955-6900 Thuwal, Kingdom of Saudi Arabia
\textsuperscript{2} Cambridge Centre for Proteomics, Cambridge Systems Biology Centre, and, Department of Biochemistry, University of Cambridge Tennis Court Road, Cambridge CB2 1QR, UK
\textsuperscript{3} Bioscience Core Laboratory, Division of Biological and Environmental Sciences and Engineering, 4700 King Abdullah University of Science and Technology, 23955-6900 Thuwal, Kingdom of Saudi Arabia

Running Title: The Arabidopsis cGMP-dependent phosphoproteome

* Both authors have contributed equally to the work

Correspondence: Chris Gehring, Division of Biological and Environmental Sciences and Engineering, 4700 King Abdullah University of Science and Technology, Thuwal, 23955-6900, Tel.: +966-544700029; E-mail: Christoph.Gehring@KAUST.edu.sa
Dear Editor,

The second messenger cyclic nucleotide 3′,5′-cyclic guanosine monophosphate (cGMP) is increasingly recognised as a key signalling molecule that mediates many physiological and developmental processes in plants (Supplemental Figure 1A). While cGMP-dependent phosphorylation of Arabidopsis proteins is a known phenomenon (Isner et al., 2012), a quantification of the cGMP-dependent protein phosphorylation at the system level has not been reported previously. Here we applied Ti⁺⁴⁺-IMAC phosphopeptide enrichment technique combined with tandem mass spectrometry to analyse the cGMP-dependent phosphoproteome of *Arabidopsis thaliana* cell suspension culture cells metabolically labelled with ¹⁵N (Supplemental Figure 1B) and show highly specific response signatures.

Labelled cells were treated with 100 nM 8-Br-cGMP for 5 min or with dH₂O as a mock treatment (Supplemental Materials and Methods). A total of 5406 phosphopeptides, corresponding to 2006 unique proteins, were identified from the analysis of four biological replicates. Of the latter, 633 phosphopeptides detected in at least two biological replicates and in both the control and the treatment were differentially phosphorylated, corresponding to 417 unique proteins, and these were subjected to quantitative analyses (Supplemental Table 1). Of the 633 differentially phosphorylated peptides, 142 phosphopeptides from 127 unique proteins showed statistically significant changes (Supplemental Table 1). A total of 90 phosphopeptides increased in their phosphorylation state at 5 min, while in the case of 52 peptides, the phosphorylation state decreased. The confirmed position of each of the detected phosphosites is shown in Supplemental Table 1 and an example of the spectrum showing the identification of a phosphosite on a peptide of the arginine/serine-rich splicing factor 41 (At5g52040) is shown in Supplemental Figure 1C and D. Fifty-five of the differentially phosphorylated peptides could be tracked in at least two biological replicates at the protein expression level and of those only two showed a significant (p≤0.05) change in protein expression levels (Supplemental Table 1).

The phosphopeptides were tested for enriched motifs of phosphorylation target sites (Supplemental Figure 1E-H). Two motifs occur in all differentially phosphorylated peptides (Supplemental Figure 1E and G), while two motifs are specific for up- (Supplemental Figure 1F) or down-regulated phosphopeptides (Supplemental Figure 1H). The first (….sP…) is diagnostic for proline-directed kinases, the second (.R..s....) for protein kinase A (PKA) and protein kinase C (PKC) (Supplemental Table 1). The proline directed kinase motif occurs in 39 cGMP-dependently phosphorylated peptides and 25 specifically dephosphorylated peptides. The PKA and PKC kinase motifs occur in 16 phosphorylated and six dephosphorylated peptides. In addition, eight of the phosphopeptides identified belong to six proteins annotated as kinases (Supplemental Table 1), and four of these proteins, a protein kinase family protein (AT1G67580), a pyruvate orthophosphate dikinase
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(AT4G15530), a S-locus lectin protein kinase (AT1G11330) and the brassinosteroid (BR)-signalling kinase 1 (AT4G35230) showed an increase in their phosphorylation states. The cGMP-dependent phosphorylation of the BR-signalling kinase is particularly noteworthy since the BR receptor (At4g39400), a plasma membrane localized leucine-rich repeat receptor kinase, has been shown to contain a functional cytosolic guanylyl cyclase domain (Kwezi et al., 2007) thus further supporting a role for cGMP in BR signalling.

A pathway analysis identified cGMP-dependent phosphorylation of seven proteins that are part of the spliceosome (Figure 1A and B) (Matera and Wang, 2014) and belong to four different spliceosome complexes (Figure 1B). Two of these proteins, the pre-mRNA processing protein (AT1G44910) and splicing factor PW1 (AT1G60200), belong to the U1 complex; the WD40 repeat protein (AT2G32700) is part of the U4/U6 complex; the periodic tryptophan protein 2 (AT1G15440, AtPWP2) belongs to the U5 complex; UBP1-associated protein 2A (AT3G56860), RNA-binding protein 47C' (AT1G47500) and the arginine/serine-rich (RS) splicing factor are common components. The analysis of sequence patterns flanking the phosphorylated residues (see Supplemental Materials and Methods) indicates that different kinases are involved in the phosphorylation of spliceosome components (Figure 1B) and that the proline-directed protein kinase motif (….sP…) is the most prevalent, observed in two of the three proteins in the common components, all U1 complex proteins and one U4/U6 complex protein. Incidentally, phosphorylation of the spliceosome peptides identified here has also been reported in large-scale phosphorylation studies albeit not in response to cGMP (Supplemental Table 1).

The spliceosome is a multi-megadalton ribonucleoprotein (RNP) complex that catalyses pre-mRNA splicing. Its assembly occurs following the orderly and dynamic interaction of five spliceosomal small nuclear (sn)RNP s and several other splicing factors and, much like in yeast and metazoa, the major five spliceosomal snRNPs, U1, U2, U4, U5 and U6, are U-rich snRNAs. Conformation and composition of the spliceosome is highly dynamic, thus allowing accuracy and flexibility and interaction of the RNA-RNP requires repeated rearrangements of the spliceosome components (Figure 1A). The complex RNA-RNA and RNP interaction serves to align the intricate reactive moieties of the pre-mRNA and RNP for catalysis. Of the five spliceosome complexes, four contain at least one protein that is cGMP-dependently phosphorylated or dephosphorylated thus pointing to a role of cGMP in the modulation of spliceosome activity through post-translational modification (Figure 1A and B).

It is becoming increasingly clear that alternative splicing, as performed by the spliceosome, influences many plant processes and notably responses to biotic and abiotic stresses. Not only are mutations in splicing factors and spliceosomal proteins predicted to modulate the circadian clock and the highly complex plant defense responses, for example, (Staiger and Brown, 2013), it has also been shown that
the phosphorylation status of spliceosome components can affect splicing (Feng et al., 2008; Lipp et al., 2015) resulting in these modifications being a key regulatory element of the response to stimuli. Given that cGMP-transients are a part of plant responses to stress including hormone-mediated stress responses (Meier et al., 2009; Muleya et al., 2014; Qi et al., 2010), we predict that specific cGMP-dependent phosphorylation of spliceosome components is a mechanism that modulates the transcriptional response to plant stress.

Differentially phosphorylated proteins are also enriched in the gene ontology (GO) categories ‘biological processes’, ‘molecular functions’ and ‘cellular localization’ (Supplemental Materials and Methods). The most enriched ‘biological processes’ include ‘regulation of cellular component size’ and ‘regulation of cell size’ (Supplemental Table 2). Given this enrichment in the process ‘regulation of cell size’, we experimentally tested the effect of a 5 min treatment with 100 nM of 8-Br-cGMP on protoplasts and noted a doubling of their volume (Figure 1C and D). Phosphatase inhibitors alone increase the volume, while the combination of cGMP and phosphatase inhibitors decrease the volume (Figure 1C). In contrast, the kinase inhibitors on their own do not significantly alter protoplast volumes (Figure 1D). Cyclic GMP in the presence of kinase inhibitors causes a significant increase in cell volume. Treatment with 100 nM of 8-Br-cGMP also resulted in significant cell volume increases in guard cell protoplasts (Figure 1E) as well as the opening of stomatal pores in vivo (Figure 1F). This data is therefore consistent with the fact that cGMP-dependent volume changes depend critically on both the activation of kinases and phosphatases.

Changes in phosphorylation status occur in five proteins with a role in cell size regulation. In two, it was previously reported and in two it is predicted (Supplemental Tables 1). Phosphorylation increases in Tortifolia 1, a plant-specific microtubule-associated protein controlling the anisotropic orientation of cortical microtubules during cell elongation and hence the direction of organ growth (Peña et al., 2014). The dephosphorylated proteins, include one of two orthologous of E3 ubiquitin, histone mono-ubiquitination 1, involved in mono-ubiquitination of histone H2B that in turn regulates diverse processes including cell trafficking, signalling and auxin transport (Del Pozo and Manzano, 2014).

In summary, our data reveal that the second messenger cGMP causes rapid phosphorylation changes of a specific set of proteins with roles in many different physiological processes including spliceosome function and cell volume regulation. Furthermore, these data are a valuable resource for the analyses of complex signalling pathways that are critically dependent on the second messenger cGMP.

SUPPLEMENTARY MATERIAL
Supplemental Figure and “Material and Methods”
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Supplemental Table 1 - Differentially phosphorylated peptides/proteins
Supplemental Table 2 - GO enrichments and pathway analysis

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AUTHOR CONTRIBUTIONS
CG and KSL have conceived of the project, CM, AJG and LT have performed the experiments and all authors have contributed to the analyses of the data and the writing of the manuscript. CM and AJG have contributed equally to the project.

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REFERENCES


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FIGURE 1. cGMP-dependent (de)phosphorylation of spliceosome components and cell volume changes.

(A) Dynamic reorganizations of the spliceosome assembly system. (B) cGMP-dependent changes in the phosphorylation status of spliceosome components. Arrows under phosphorylation indicate either increase (arrow facing up) or decrease (arrow facing down) in phosphorylation of the respective proteins. Also included is the sequence logo representation of all phosphorylated sites in spliceosome enzymes that are differentially changing presented as WebLogo sequences using WebLogo version 3.4 (http://weblogo.threeplusone.com/create.cgi). In the plot, the phosphorylated residue is at the centre and flanked between six amino acids on both the N- and C-terminal. (C) Protoplasts prepared from Arabidopsis cell suspension cultures were suspended in water (control) or treated with buffer containing either 100 nM 8-Br-cGMP or 100 nM phosphatase 1 inhibitor (Pase 1) cocktail for 5 min. Experimental details are described in the Supplementary “Materials and Methods”. The data were analysed by Student’s t-test and bars represent the mean +/- s.e. of three independent experiments (n ≥ 50 protoplasts per experiment). Left inset: Light microscopic image of control and cGMP-treated mesophyll protoplasts. Right inset: Western blot of protoplast proteins probed with anti-NCK1 or the (phosphorylation-dependent) anti-pNCK 1 as detailed in the Supplementary “Materials and Methods”. (D) Protoplasts suspended in 0.02% (v/v) DMSO or treated with buffer containing either 100 nM 8-Br-cGMP or 100 nM of the protein kinase inhibitor K-252a and treated for 5 min before cell volumes were calculated. Different letters over the bars indicate that the values are significantly different (p<0.05). (E) Guard cell protoplasts suspended in water (control) or treated with 100 nM 8-Br-cGMP for 5 min. Inset: Guard cell protoplasts exposed to either water (control) or 100 nM 8-Br-cGMP. The bar represents 5 µm. (F) Stomatal apertures changes in response to 100 nM 8-Br-cGMP treatment (≥ 10 min) in the dark. The data were analysed by Student’s t-test and bars represent the mean +/- s.e. of three independent experiments (n≥20 guard cells per experiment). Inset: Scanning electron microscope image of an open Arabidopsis stomatal pore.
**A**

- Pre-RNA → Intron → mRNA
- Pre-spliceosome: U1, U2, U6, U5
- Pre-catalytic spliceosome: U1, U4, U6, U5
- Catalytic spliceosome: U1, U5

**B**

**Common components:**
- SR - AT3G56860 (UBP1-associated protein 2A)
- hnRNPs - AT1G47500 (RNA-binding protein 47 C')
- SR - AT5G52040 (arginine/serine-rich (RS) splicing factor)

**U1 complex:**
- FBP11 - AT1G44910 (pre-mRNA processing protein)
- S164 - AT1G60200 (splicing factor PW1)

**U4/U6 complex:**
- Prp4 - AT2G32700 (WD40 repeat protein)

**U5 complex:**
- Prp8BP - AT1G15440 (periodic tryptophan protein 2)

**Phosphorylation:**
- \( \Delta \)
- \( \nabla \)

**C**

- Guard cell protoplast volume (\( \mu m^3 \))

Control: a, b, c, d
8-Br cGMP: e, f
8-Br cGMP + Pase I: g, h

pNCK 1(\( p=0.36 \))
pNCK 1(\( p=0.003 \))

**D**

Protoplast volume (\( \mu m^3 \))

Control: a, b, c, d
DMSO: e, f
8-Br cGMP: g, h
8-Br cGMP + K-252a: i, j

**E**

- Guard cell protoplast volume (\( \mu m^3 \))

Control: a, b

**F**

- Stomatal pore in \( \mu m \)

Control: a, b

- Stomatal pore in \( \mu m \)

Control: a, b

- Stomatal pore in \( \mu m \)

Control: a, b

- Stomatal pore in \( \mu m \)

Control: a, b