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Cyclic mononucleotides modulate potassium and calcium flux responses to H_2O_2 in Arabidopsis roots

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ABSTRACT

Cyclic mononucleotides are messengers in plant stress responses. Here we show that hydrogen peroxide (H_2O_2) induces rapid net K^+-efflux and Ca^{2+}-influx in Arabidopsis roots. Pre-treatment with either 10 μM cAMP or cGMP for 1 or 24 h does significantly reduce net K^+-leakage and Ca^{2+}-influx, and in the case of the K^+-fluxes, the cell permeant cyclic mononucleotides are more effective. We also examined the effect of 10 μM of the cell permeant 8-Br-cGMP on the Arabidopsis microsomal proteome and noted a specific increase in proteins with a role in stress responses and ion transport, suggesting that cGMP is sufficient to directly and/or indirectly induce complex adaptive changes to cellular stresses induced by H_2O_2.

1. Introduction

Environmental stresses may cause plants to generate reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (HO) [1]. ROS, which are highly reactive to membrane lipids, proteins, and DNA, are believed to be the major contributing factors to stress injuries and to cause rapid cellular damage [2]. However, when ROS are generated at controlled levels, cells recognise these reactive molecules as signals to activate defence genes (for review, see [3]). The control of ROS level requires cells to rapidly produce and scavenge different forms of ROS in a coordinated manner. Enzymes, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR), and non-enzymatic antioxidants such as tocopherols, ascorbic acid (AsA), and glutathione (GSH) work in cooperation to the detoxification of ROS. Most ROS seem to exert signalling functions that lead to specific biological processes. At the transcription level, H_2O_2-responsive genes were initially identified by microarray and AFLP-based technologies [4]. Later signalling properties and distinct transcriptional responses were confirmed also for other ROS, including O_2^- [5]. However, H_2O_2 seems best suited to play a pivotal role as a signalling molecule due to a comparatively high stability and long half-life. H_2O_2 generation occurs in plants following exposure to a wide variety of abiotic and biotic stresses. These include temperature, UV irradiation, excess excitation energy, ozone exposure, some phytohormones (e.g. ABA), drought, salt, flooding, heavy metals, wounding and pathogen challenge e.g. [6,7]. In shoots, generation of ROS during salt stress is mainly attributed to declining CO_2 fixation, leading to higher leakage of electrons to O_2. In salt-stressed roots, ROS production is attributed to the disruption of mitochondrial function and salt-induced activation of the plasma membrane NADPH oxidases [8,9]. Furthermore, a number of studies show direct evidence of H_2O_2 accumulation *in planta* under salt stress [10] and it was reported that maize (*Zea mays*) plants pre-treated with H_2O_2 are capable of acquiring additional salt stress tolerance, suggesting that salt-induced ROS can induce antioxidant responses at the cell level [11]. However, salt stress triggers not just an

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oxidative burst but also an osmotic stress and an ion imbalance [12]. Na^+-influx into the cell results in a dramatic membrane depolarization, resulting in substantial loss of cytosolic K^+ [13]. Because K^+ is critical for the activity of many enzymes as well as cellular osmotic adjustment, a lack of K^+ can cause severe metabolic disorders [12] as well as trigger programmed cell death in salt-stressed tissues [14,15]. It has been shown that in plants K^+ is transported across the plasma membrane in and out of the roots through potassium channels or transporters [13]. The inward-rectifying K^+-channels (KIRs) mediate K^+-uptake, while the outward-rectifying K^+-channels (KORs) mediate K^+-release [16]. In addition, non-selective cation channels (NSCCs), the major system for Na^+ entry, are also involved in K^+-efflux and these channels have an important role in salt resistance in plants [13]. Constitutive NSCCs can be subdivided according to their voltage dependence [17]. In addition it has been demonstrated that ROS activated NSCCs are involved in HR response and in elongation/expansion of plant cells [18], and cyclic nucleotide-gated channels (CNGCs), which play a role in plant pathogen interaction in shoots [19] and in roots for non-selective cation uptake [20]. Given that H_2O_2 [10] and cGMP [21,22] are components of stress responses in general and the response to NaCl and drought stress in particular, we have undertaken, firstly, to study H_2O_2-dependent ion fluxes in *Arabidopsis thaliana* roots and determine whether cyclic nucleotides, and especially cGMP, may affect net K^+- and Ca^{2+}-fluxes. Secondly, we investigated if cGMP induces changes at the proteome level with a view to gain further insight into the possible physiological implications for cyclic nucleotide-dependent changes in ion fluxes.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana, ecotype Col-0, seeds were sterilized and distributed over Petri dishes containing sterile Murashige and Skoog (MS) medium, pH 6.2. The plates were place at 4 °C for 2 days for seed stratification. Afterwards, they were placed into a growth room and positioned vertically to allow roots to grow outside of the medium. Six day old seedlings were transferred into 55 × 15 mm Petri dishes so their roots were immersed in a shallow layer of the Basic Salt Medium (BSM) solution (0.1 mM KCl; 0.2 mM $CaCl_2$; pH 6.0 adjusted with Tris base and 2-(N-morpholino) ethanesulfonic acid (MES) buffer). For pre-treatment with cyclic nucleotides, appropriate chemical (adenosine 3′5′-cyclic monophosphate sodium salt monohydrate (cAMP; 10 μM); guanosine 3′5′-cyclic monophosphate sodium salt (cGMP; 10 μM); or the cell permeant analogues 8-Br-cAMP (10 μM) and 8-Br-cGMP (10 μM)) was added to the Petri dish for either 1 or 24 h.

2.2. Non-invasive microelectrode ion flux measurements

Net fluxes of K^+ and Ca^{2+} were measured using non-invasive microelectrode ion flux (the MIFE) measuring system (ROCU, University of Tasmania) and are detailed elsewhere [23] as well as in the Supplementary file 1.

2.3. A. thaliana cell suspension culture and microsomal protein extraction

Cells derived from roots of *A. thaliana* (ecotype Columbia 0) and grown in 100 mL of Gamborg's B5 basal salt mixture (Sigma–Aldrich) with 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg mL^{-1}) and kinetin (0.05 μg mL^{-1}) in 250 mL sterile flask. Cells were grown in a growth chamber (Innova® 43, New Brunswick Scientific Co., NJ) with shaking at 120 rpm, and subcultured every 10 days. Light condition and cyclic nucleotide treatments and cell harvesting are further detailed in Supplementary file 1.

2.4. One-dimensional gel electrophoresis (1-DE) and in-gel trypsin digestion

Approximately 15 μg of the microsomal protein extract was reduced in 2 × SDS reducing buffer and electrophoresed in 12% one-dimensional (1D) gel electrophoresis at 150 V for 30 min. Gel staining, protein band section and *in gel* trypsin digestion are detailed in the Supplementary file 1.

2.5. Protein identification by LTQ-Orbitrap and functional analysis

Digested peptides were resuspended in 5% (v/v) ACN and 0.1% (v/v) formic acid (FA), and analysed with an LTQ Orbitrap mass spectrometer (Thermo-Scientific, Bremen, Germany) coupled to a nanoLC (Thermo-Scientific). Spectra were submitted to a local MASCOT (Matrix Science, London, UK) server and searched against *A. thaliana* in the TAIR database (release 10), with a precursor mass tolerance of 10 ppm, a fragment ion mass tolerance of ±0.5 Da, strict trypsin specificity allowing up to one missed cleavage, carbamidomethyl modification on cysteine as a fixed modification and methionine oxidation as a variable modification. Protein identification and data analysis methods are detailed in the Supplementary file 1.

2.6. Statistical analysis

All results are presented as mean ± S.E. (n = sample size). Data were analysed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Significant differences (at $P < 0.05$) between treatments were determined by one-way ANOVA based on Duncan's multiple range test and denoted by different lower case letters.

3. Results and discussion

Application of H_2O_2 to Arabidopsis roots induces a rapid and massive K^+-efflux. Just 1 h of treatment in either 10 μM of cell the permeant cyclic nucleotide (CN) analogue 8-Br-cAMP or 8-Br-cGMP was effective in significantly ($P < 0.05$) reducing the extent of this H_2O_2-induced K^+-leak (Fig. 1A). CN-treated roots lose >2-fold less K^+ compared to non-treated controls (Fig. 1B). No statistically significant ($P < 0.05$) differences between the Br-cAMP and Br-cGMP nucleotides were noted (Fig. 1). In the 1 h pre-treatment, membrane-permeable analogues were more efficient in preventing the K^+-efflux (significant at $P < 0.05$; Fig. 2A). Increasing the root pre-treatment with cyclic nucleotides from 1 to 24 h has further decreased the magnitude of H_2O_2-induced K^+-efflux (Fig. 2B); effect of timing was significant according to Duncan's multiple range test (data not shown). No significant ($P < 0.05$) difference between permeant and non-permeant analogues was found in this case (Fig. 2B); however, each CN has significantly ($P < 0.05$) reduced the magnitude of H_2O_2-induced K^+-loss compared with non-treated controls (Fig. 2B). Consistent with the notion that salinity stress results in a rapid elevation of H_2O_2 level in root tissues [8,9], root pre-treatment with CN reduced NaCl-dependent K^+-efflux (Supplementary file 2).

H_2O_2 application caused a rapid onset of transient Ca^{2+}-influx (Fig. 3A–C). One hour pre-treatment with the cell permeant cyclic nucleotides, 8-Br-cAMP and 8-Br-cGMP, were similarly efficient in reducing both the magnitude of the peak H_2O_2-induced Ca^{2+}-influx (~3-fold difference compared with non-treated controls; Fig. 3D; significant at $P < 0.05$). As a result, CN-treated roots accumulated 2 to 3-fold less Ca^{2+} over the first 15 min after stress onset

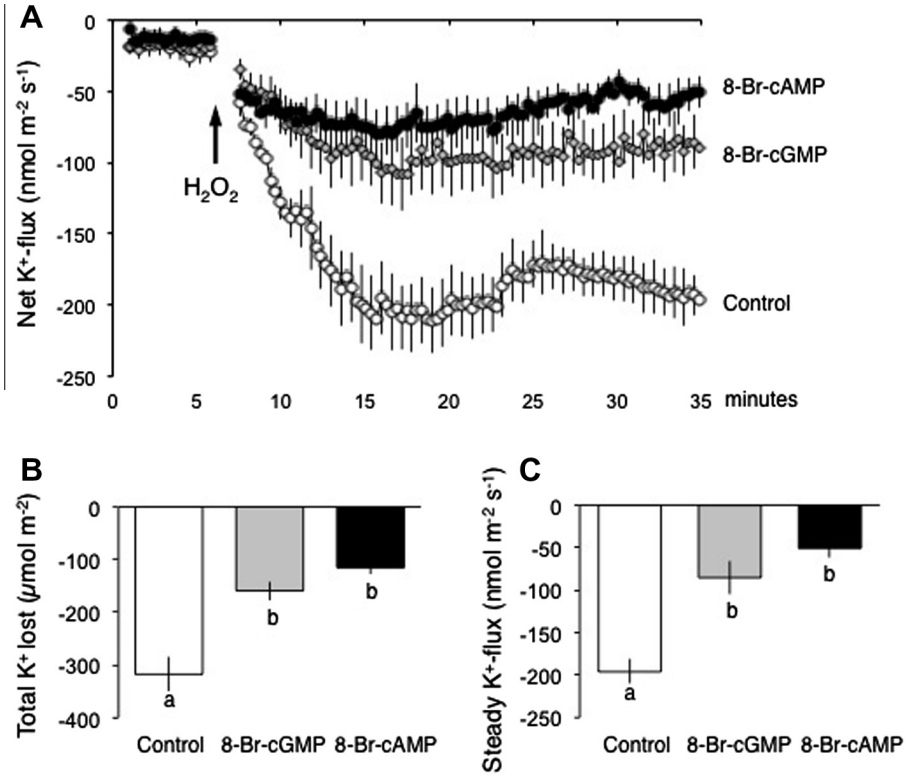

Fig. 1. Net K^+-fluxes in Arabidopsis roots in response to 10 mM H_2O_2. (A) Transient K^+-flux responses for control (untreated roots) and roots pretreated for 1 h with 10 μM of either 8-Br-cGMP or 8-Br-cAMP. (B) Total amount of K^+ (in μmol m^{-2}) lost from roots within 30 min of H_2O_2 stress onset. (C) Steady-state K^+-fluxes at the end of H_2O_2-induced transient in control and 8-Br-cGMP or 8-Br-cAMP- pretreated roots. Data are mean ± S.E. (n = 5, 6 individual roots). For all MIFE measurements the sign convention is "efflux negative". Different lower case letters indicate a significant difference between treatments at $P < 0.05$ (Duncan's multiple range test).

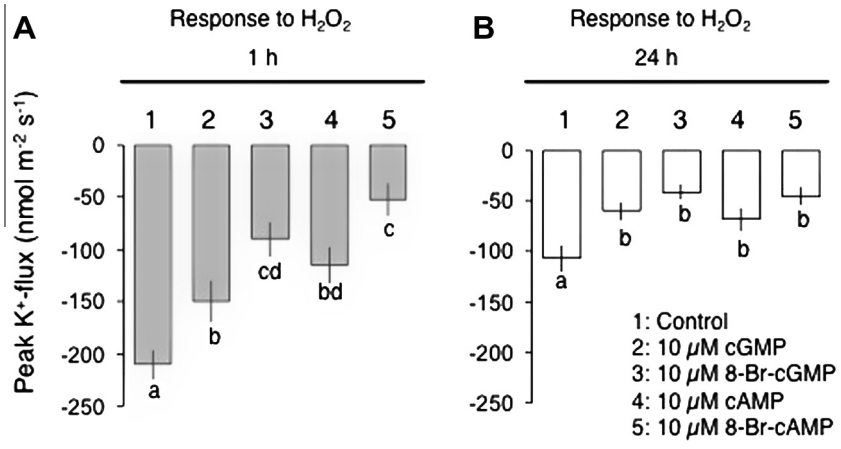

Fig. 2. Summary of effects of 1 h (A) and 24 h (B) cyclic nucleotide pre-treatment on H_2O_2-dependent net K^+-leak from Arabidopsis roots. Data are mean ± S.E. (n = 5–7 individual roots). Different lower case letters indicate a significant difference between treatments within the same time of exposure range at $P < 0.05$ (Duncan's multiple range test).

(Fig. 3E; significant at $P < 0.05$). The effect on Ca^{2+}-influx was also observed after treatment with cAMP and cGMP (Supplementary file 2).

To shed light on the cellular responses that may be involved in reducing the H_2O_2-induced K^+- and Ca^{2+}-net fluxes, a proteomics analysis was undertaken. Microsomal proteins were extracted from Arabidopsis cell suspension cultures pre-treated with 10 μM of 8-Br-cGMP for 1 h, fractionated by 1D gel electrophoresis, trypsin digested and analysed by tandem mass spectrometry. Data was processed with Mascot and Scaffold for identification and label free quantitation, respectively. A total of 939 proteins from microsomal enriched fractions were identified and 93 of these showed significant differential expression between the control and treatment. Of the latter, 46 proteins were up-regulated (Table 1) and 47 down-regulated (Table 2).

The most up-regulated proteins included insulinase (AT1G51980; fold change (FC) 2.8), aconitase (AT2G05710; FC 2.7), aldolase (AT3G52930; FC 2.6) and voltage dependent anion channel (VDAC) 3 (AT5G15090; FC 2.5). Insulinase is a membrane protein involved in ATP and metal ion binding, glycolysis and proteolysis induced in response to salt stress and cadmium ion. It is also localized in the mitochondrial respiratory chain complex III,

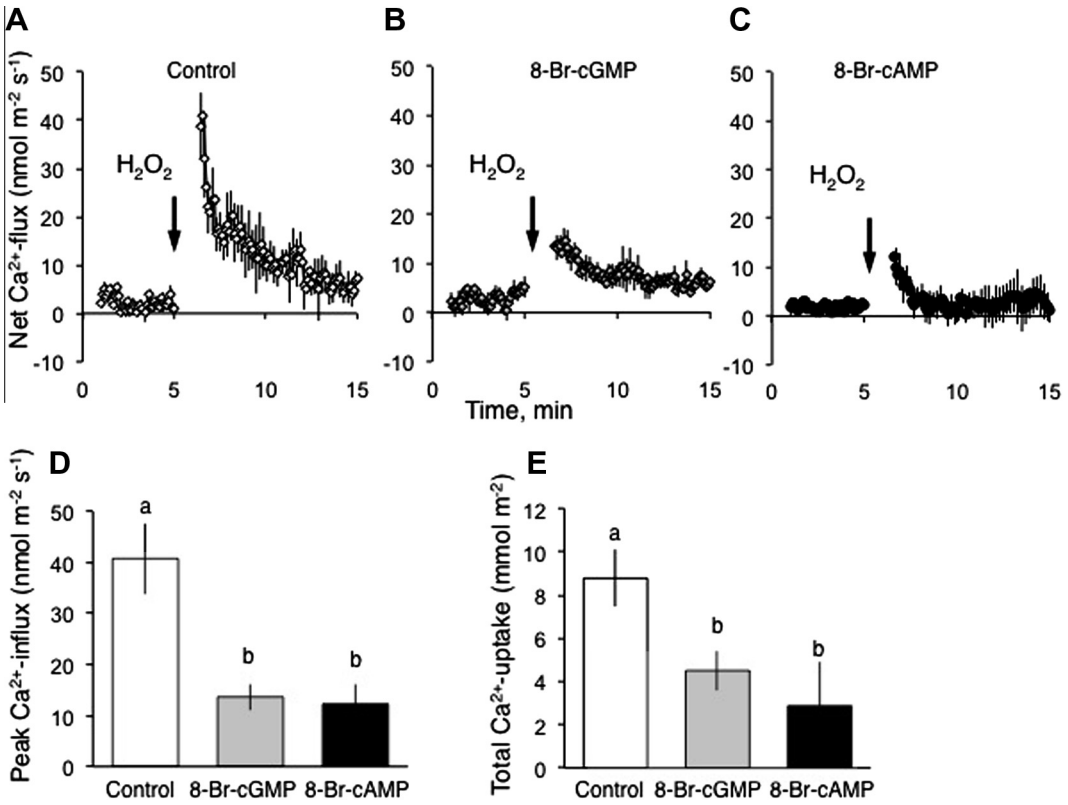

Fig. 3. Net Ca^{2+}-fluxes in Arabidopsis roots in response to 10 mM H_2O_2. (A–C) Transient Ca^{2+}-uptake in control (untreated roots), and roots pretreated for 1 h with 10 μM of either 8-Br-cGMP or cAMP, respectively. (D) Peak Ca^{2+}-influx (nmol m^{-2} s^{-1}) in CN-treated roots as compared with untreated controls. (E) Total amount of Ca^{2+} (in μmol m^{-2}) taken by Arabidopsis roots within the first 15 min of H_2O_2 stress onset. Data are mean ± S.E. (n = 5, 6 individual roots). Different lower case letters indicate a significant difference between treatments at $P < 0.05$ (Duncan's multiple range test).

mitochondrion and vacuolar membranes. Aconitase, an enzyme that catalyses the conversion of citrate to isocitrate, is known to affect superoxide dismutase CSD2 transcription and has been implicated in response to salt stress and regulating resistance to oxidative stress and cell death in Arabidopsis and *Nicotiana benthamiana* [24]. Aldolase superfamily protein has been shown to be involved in glycolysis and response to cadmium ion [24].

Three VDACs 1, 2 and 3 (AT3G01280, AT5G67500 and AT5G15090, respectively) were detected as up-regulated. VDACs are localized in the outer mitochondrial membrane and involved in metabolite exchange between the cytosol and organelle i.e. regulation of anion transport [25], whereas VDAC 3 may also play a role in the defense against bacterial pathogens. Also up-regulated, albeit to a smaller extent, were proteins associated to H^+-ATPase activity (AT3G28710 – FC 1.9; AT4G30190 – FC 1.8) suggesting possible control over cell energy balance and membrane potential maintenance.

The most highly down-regulated proteins included coatomer subunit (AT2G21390; FC 0.3), ribosomal protein L6 (AT1G33120; FC 0.4), N-terminal nucleophile aminohydrolase (AT4G31300; FC 0.4), phosphate transporter 3.1 (AT5G14040; FC 0.4) and peroxidase (AT5G17820; FC 0.4). Coatomer is involved in intracellular protein transport and ER to Golgi vesicle-mediated transport [26], while the N-terminal nucleophile aminohydrolase is involved in proteolysis and response to stress. It encodes 20S proteasome subunit PBA1, which acts as a plant caspase-3-like enzyme. Phosphate transporter 3.1 encodes a mitochondrial phosphate transporter, and it modulates plant responses to salt stress [25]. Peroxidase superfamily protein is involved in oxidation–reduction process as well as response to oxidative stress.

A gene ontology (GO) analysis on the 46 up-regulated proteins revealed significant enrichments in "molecular functions", "biological processes" and "metabolic pathways" categories. In the "molecular function" classification, the most enriched categories included molecules that are structural constituent of the ribosome, molecules with ion trans-membrane transporter activity, GTP binding proteins, molecules with guanyl nucleotide-binding and ATPase activity (Supplementary file 3). Proteins that respond to cadmium ions, osmotic/salt stress and temperature stimulus, and ion transport were the most enriched "biological processes" (Supplementary file 3). The 47 down-regulated proteins were also classified into three "molecular functions" and 10 "biological processes". The structural constituent of the ribosome was the most enriched "molecular function", while responses to cadmium ion, osmotic/salt stress, modification-dependent protein catabolism and proteolysis formed the top five "biological processes" (Supplementary file 3). The cGMP-dependent microsomal proteome revealed that cGMP might play an important role in the regulation of ribosomal structural activity and ion transmembrane transporter activity, in addition to stress responses.

A "metabolic pathway" analysis was performed using the Kyoto encyclopedia of genes and genomes (KEGG), a reference base for functional annotation linking interaction networks of biological systems [27,28]. In the "metabolic pathway" analysis, the most enriched KEGG pathways in the up-regulated proteins included the ribosome pathway involved in translation (19 proteins; ath03010), followed by oxidative phosphorylation (nine proteins; ath00190), making them the most extensive cGMP-dependent interaction networks. With regards to the down-regulated proteins, the most enriched pathways included ribosome pathway

Table 1
cGMP-responsive up-regulated microsomal proteins.

Accession No.	Protein description	Fold change	GO enrichment
AT1G51980	Insulinase (Peptidase family M16) protein	2.8	OiM, RtOS
AT2G05710	Aconitase 3	2.7	RCi, RtOS, CW
AT3G52930	Aldolase superfamily protein	2.6	RCi, RtOS, CW
AT5G15090	Voltage dependent anion channel 3	2.5	IT, OM, RBS
AT3G27240	Cytochrome C1 family	2.4	OP, OiM
AT4G02520	Glutathione S-transferase PHI 2	2.4	RCi, RtOS, RTS, RBS, M
AT1G04820	Tubulin alpha-4 chain	2.3	RCi, RtOS, CW
AT1G77120	Alcohol dehydrogenase 1	2.3	RCi, RtOS, PM
AT2G36530	Enolase	2.3	RCi, RtOS, RTS, M
AT1G64520	Regulatory particle non-ATPase 12A	2.2	RTS, RBS, M∗
AT2G33040	ATP3 γ subunit of Mt ATP synthase	2.2	IT, OP, OiM
AT4G13010	Oxidoreductase, zn-binding dehydrogenase	2.1	OiM
AT4G20890	Tubulin beta-9 chain	2.1	RCi, Vac M, PM
AT4G37910	Mitochondrial heat shock protein 70–1	2.1	RCi, RtOS, CW
AT5G40810	Cytochrome C1 family	2.1	OiM
AT3G14990	Glutamine amidotransferase-like protein	2	RCi, PM, Vac
AT1G53240	Lactate/malate dehydrogenase family protein	1.9	RCi, RtOS, RTS, RBS, CW
AT1G55490	Chaperonin 60 beta	1.9	RTS, RBS, M
AT2G42210	Mitochondrial import translocase	1.9	OiM
AT3G02360	6-Phosphogluconate dehydrogenase protein	1.9	RtOS, M
AT3G08580	ADP/ATP carrier 1	1.9	OiM
AT3G28710	ATPase, V0/A0 complex, subunit C/D	1.9	IT, OP, PM
AT3G46430	Unknown protein	1.9	OiM
AT4G32470	Cytochrome b ubiquinol oxidase	1.9	mATPet, OP, OiM
AT5G16050	General regulatory factor 5	1.9	RCi, CW
AT5G62690	Tubulin beta chain 2	1.9	RCi, RtOS, CW
AT5G66760	Succinate dehydrogenase 1–1	1.9	mATPet, OP, OiM
AT5G67500	Voltage dependent anion channel 2	1.9	IT, OM, RBS
AT1G20620	Catalase 3	1.8	RCi, RTS, CW
AT2G39460	Ribosomal protein L23AA	1.8	RTS, CW
AT4G24190	Chaperone protein htpG family protein	1.8	RCi, RtOS, RTS, M, ER
AT4G30190	H^+-ATPase 2	1.8	IT, OP, OiM
AT5G40770	Prohibitin 3	1.8	RtOS, M
AT1G07920	GTP-binding Elongation factor Tu protein	1.7	RCi, M, Vac
AT3G42050	Vacuolar ATP synthase subunit H protein	1.7	IT, OP, RBS, Vac M, PM
AT4G34200	D-3-phosphoglycerate dehydrogenase	1.7	IT, M
AT5G15650	Reversibly glycosylated polypeptide 2	1.7	RCi, RtOS, CW
AT3G16450	Mannose-binding lectin superfamily protein	1.6	RTS, OM
AT3G23990	Heat shock protein 60	1.6	RCi, RTS, Vac M
AT4G11150	Vacuolar ATP synthase subunit E1	1.6	IT, OP, OiM, RtOS, RTS, RBS
AT5G08670	ATP synthase alpha/beta family protein	1.6	IT, OiM
AT5G35530	Ribosomal protein S3 family protein	1.6	RtOS, M
AT3G01280	Voltage-dependent anion channel 1	1.5	IT, OM, RBS
AT3G15950	DNA topoisomerase-related	1.5	RtOS, M
AT3G52990	Pyruvate kinase family protein	1.5	RCi, M
AT5G13450	Delta subunit of Mt ATP synthase	1.5	IT, OiM

CW-cell wall, ER-endoplasmic reticulum, IT-ion transport, mATPet-mitochondrion ATP synthase coupled electron transport, M-membrane (based on TAIR annotation), M∗-proteosome and chloroplast envelope, OiM-organelle inner membrane, OM-outer membrane, OP-oxidative phosphorylation, PM- plasma membrane, RTS-response to temperature stimulus, RBS-response to biotic stimulus, RCi-response to cadmium ion, RtOS-response to osmotic/salt stress, Vac-vacuole.

(nine proteins) and biosynthesis of phenylpropanoids (seven proteins; ath01061). The KEGG analysis indicated that many of the differentially expressed microsomal proteins play a role in modulating the level of translation and oxidative phosphorylation networks. The cGMP-dependent increase in oxidative phosphorylation associated proteins as well as proteins with a role in abiotic stress responses is consistent with findings in animal systems that showed that cGMP-dependent stress responses are linked to increases in energy demand [29,30].

Cyclic nucleotides and notably, cGMP and cAMP are increasingly becoming recognized as important second messengers in higher plants. Cyclic nucleotide-dependent signalling is often linked to transient increases in cytosolic Ca^{2+}, and it is likely that the cooperation of both messengers is important in modulating abiotic stress [22] and biotic stress responses e.g. [31,32]. Cooperation between these second messengers is further supported by the fact that Cyclic Nucleotide Gated Channels (CNGCs) harbour a calmodulin-binding site in their C-terminal cytosolic domain [33]. Furthermore, cGMP has also been shown to cause a time-dependent increase in the content of oxidised methionine residues in a group of proteins functionally enriched for stress responses, suggesting that this cGMP-dependent post-translational modification may act as a cellular signal [34] that induce a cellular defence response.

Activation of Ca^{2+}-permeable channels in the plasma membrane have been reported in several plant systems including guard cells [35], and it was shown recently that AtCNGC5 and AtCNGC6 are unique cGMP-activated non-selective Ca^{2+}-permeable cation channels in the plasma membrane of Arabidopsis guard cells [36]. Furthermore, Ca^{2+}-currents mediated by CNGC6 channels in Arabidopsis root epidermal protoplasts were activated by cytosolic cAMP [37] under the heat treatment. Such a cAMP-dependent activation is not seen in our *in planta* studies (Fig. 3). There are two possible explanations for this. Firstly, it could be that in our experiments, channels other than CNGC5 or CNGC6 may mediate H_2O_2-induced Ca^{2+}-uptake. Indeed, it was shown recently that a ROS-regulated Ca^{2+}-transport protein, Annexin 1 (AtANN1), can mediate H_2O_2-induced Ca^{2+}-uptake in Arabidopsis roots and is

Table 2
cGMP-responsive down regulated microsomal proteins.

Accession No.	Protein description	Fold change	GO enrichment
AT2G21390	Coatomer subunit	0.3	VM
AT1G33120	Ribosomal protein L6 family	0.4	PM, Vac M
AT4G31300	N-terminal nucleophile aminohydrolases	0.4	RtOS, Vac M
AT5G14040	Phosphate transporter 3,1	0.4	CW
AT5G17820	Peroxidase superfamily protein	0.4	CW
AT5G19990	Regulatory particle triple-A ATPase 6A	0.4	M
AT1G47260	Gamma carbonic anhydrase 2	0.5	RtOS, M
AT1G53750	Regulatory particle triple-A 1A	0.5	PM
AT1G70600	Ribosomal protein L18e/L15 protein	0.5	M
AT2G21660	Cold, circadian rhythm & RNA binding 2	0.5	RCi, RtOS, M*
AT2G22420	Peroxidase superfamily protein	0.5	ExR
AT2G44350	Citrate synthase family protein	0.5	RCi, CW
AT3G02530	TCP-1/cpn60 chaperonin family protein	0.5	RCi, PM
AT3G09440	Heat shock protein 70	0.5	RCi, CW
AT3G09630	Ribosomal protein L4/L1 family	0.5	CW
AT3G09840	Cell division cycle 48	0.5	RCi, CW
AT3G13790	Glycosyl hydrolases family 32 protein	0.5	CW
AT4G13930	Serine hydroxymethyltransferase 4	0.5	RCi, PM
AT4G16720	Ribosomal protein L23/L15e protein	0.5	PM
AT5G02870	Ribosomal protein L4/L1 family	0.5	M, Vac M
AT5G20010	RAS-related nuclear protein-1	0.5	RCi, RtOS, CW
AT5G64100	Peroxidase superfamily protein	0.5	CW
AT1G08360	Ribosomal protein L1p/L10e family	0.6	PM
AT1G20200	PAM domain protein	0.6	PM
AT1G66280	Glycosyl hydrolase superfamily protein	0.6	RtOS, M
AT1G71695	Peroxidase superfamily protein	0.6	CW
AT1G78900	Vacuolar ATP synthase subunit A	0.6	RtOS, CW
AT1G79530	Glyceraldehyde-3-phosphate dehydr.	0.6	M
AT2G04390	Ribosomal S17 family protein	0.6	PM
AT2G41810	Protein of unknown function, DUF642	0.6	ExR
AT3G03960	TCP-1/cpn60 chaperonin family protein	0.6	M
AT3G04720	Pathogenesis-related 4	0.6	RtOS, ExR
AT3G07110	Ribosomal protein L13 family protein	0.6	M
AT3G07390	Auxin-responsive family protein	0.6	PM
AT3G08530	Clathrin, heavy chain	0.6	VM
AT3G11130	Clathrin, heavy chain	0.6	VM
AT3G11400	Eukaryotic translation IF factor 3G1	0.6	PM
AT3G14600	Ribosomal protein L18ae/LX protein	0.6	M
AT3G43190	Sucrose synthase 4	0.6	PM
AT3G51800	Metallopeptidase M24 family protein	0.6	PM, Nuc
AT3G54400	Eukaryotic aspartyl protease protein	0.6	CW
AT4G24820	26S proteasome, regulatory subunit Rpn7	0.6	M
AT4G39260	Cold, circadian rhythm & RNA binding 1	0.6	RtOS, CW
AT5G02500	Heat shock cognate protein 70–1	0.6	RCi,M, CW
AT5G20290	Ribosomal protein S8e family protein	0.6	CW
AT5G26260	TRAF-like family protein	0.6	ExR
AT5G52470	Fibrillarin 1	0.6	M, Nuc

CW-cell wall, ExR-extracellular region, M-membrane (based on TAIR annotation), M*-peroxisome, Nuc-Nucleus, PM- plasma membrane RCi-response to cadmium ion, RtOS-response to osmotic/salt stress, Vac-vacuole, VM-vesicle membrane.

responsible for the stress-induced elevation of cytosolic free Ca^{2+} [38]. Secondly, it is conceivable that effects of CNs on CNGC activity *in planta* may be indirect and mediated by messengers absent in patch-clamp experiments.

Since the treatment of roots with H_2O_2 has induced massive K^+-efflux that is reduced by cyclic nucleotides, we propose that the CNGC are at least in parts responsible for the flux. This hypothesis is supported by several findings. Firstly, CNGCs are gated by cyclic nucleotides on the cytosolic side that harbours the cyclic nucleotide-binding site. This would also explain why the cell permeant analogue is more effective at preventing K^+-loss. Secondly, CNGCs belong to the group of non-selective cation channels (NSCC) and, as such, are permeable to both K^+ and Ca^{2+} therefore accounting for the modulation of the Ca^{2+}-flux by the cyclic nucleotide pre-treatment. Importantly, the role of CNGC in plant adaptive responses to salt stress was clearly demonstrated (e.g. CNGC10; [39,40]). Thirdly, patch-clamp experiments have suggested that some NSCC channels may indeed be activated by either H_2O_2 [41] or hydroxyl radicals [42]. However, the molecular identity of these channels remains to be discovered, and it remains to be answered of whether the observed H_2O_2-induced changes in K^+- and Ca^{2+}-fluxes are mediated by the same specific CNGC, or by different channels from this group. A further possibility is that observed effects of CN on net K^+-fluxes in CN-pre-treated roots may be indirect and mediated by the voltage-gated K^+-selective (e.g. GORK; [13]) channels. Indeed, the observed 1.8–1.9 increase in amount of H^+-ATPase or its subunits may be indicative of CN activation of this electrogenic transport system. This hypothesis should be tested in direct experiments including net H^+-flux and membrane potential measurements, as well as biochemical assays of H^+-ATPase transport activity.

Given that cyclic nucleotide pre-treatment does markedly change the proteome, we were interested to learn if the changes in proteomic signature can shed some light on the systems response that in turn can explain the protective effect of the treatment. The two most noteworthy characteristics of the proteomics response induced within 1 h post-cyclic nucleotide treatment were, firstly, the up-regulation of proteins involved in abiotic stress

responses and secondly proteins with a direct or indirect role in ion transport. These would suggest that cyclic nucleotides act as messengers that are sufficient to trigger an abiotic defense response that in turn has a marked effect on H_2O_2-dependent ion fluxes. The cyclic nucleotide-dependent accumulation of proteins involved in ion transport are consistent with changes in H_2O_2-dependent ion flux signatures and also with specific cGMP-dependent transcript accumulation of genes that encode monovalent cation transporters such as non-selective ion channels and cation:proton antiporters [43]. However, it also appears that cGMP is sufficient to directly and/or indirectly induce a highly selective change in the proteome showing that it does induce many proteins, some that are directly involved in ion transport and others annotated as having a role in metabolism and abiotic stress responses. It is conceivable that cGMP does so indirectly by activating via CNGCs that in turn increase cytosolic Ca^{2+} that then acts as a second messenger in downstream signaling. The changes in the cGMP-dependent proteome that result in changed H_2O_2-dependent ion fluxes are indication of the complex mechanisms that govern ion transport and ion homeostasis at the systems level.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.01.062.

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