Physiological and Molecular Effects of the Cyclic Nucleotides cAMP and cGMP on Arabidopsis thaliana

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Master of Science

King Abdullah University of Science and Technology

Thuwal, Kingdom of Saudi Arabia

December 2012
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ABSTRACT

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*Natalia Maria Ordoñez Herrera*

The cyclic nucleotide monophosphates (CNs), cAMP and cGMP, are second messengers that participate in the regulation of development, metabolism and adaptive responses. In plants, CNs are associated with the control of pathogen responses, pollen tube orientation, abiotic stress response, membrane transport regulation, stomatal movement and light perception. In this study, we hypothesize that cAMP and cGMP promote changes in the transcription level of genes related to photosynthesis, high light and membrane transport in *Arabidopsis thaliana* leaves and, that these changes at the molecular level can have functional biological consequences. For this reason we tested if CNs modulate the photosynthetic rate, responses to high light and root ion transport. Real time quantitative PCR was used to assess transcription levels of selected genes and infrared gas analyzers coupled to fluorescence sensors were used to measure the photosynthetic parameters. We present evidence that both cAMP and cGMP modulate foliar mRNA levels early after stimulation. The two CNs trigger different responses indicating that the signals have specificity. A comparison of proteomic and transcriptional changes suggest that both transcriptional and post-transcriptional mechanisms are modulated by CNs. cGMP up-regulates the mRNA levels of components of the photosynthesis and carbon metabolism. However, neither cAMP nor cGMP
trigger differences in the rate of carbon assimilation, maximum efficiency of the photosystem II (PSII), or PSII operating efficiency. It was also demonstrated that CN regulate the expression of its own targets, the cyclic nucleotide gated channels - CNGC. Further studies are needed to identify the components of the signaling transduction pathway that mediate cellular changes and their respective regulatory and/or signaling roles.
I have the fortune to come to a place that opened its doors to scientists from all over the world and is keen to change the world destiny. Thanks to the King Abdullah University of Science and Technology for giving me the opportunity of seeing the world with new eyes.

I thank Prof. Chris Gehring for his encouragement to formulate creative questions and for supporting my work.

I would also like to acknowledge professors of the Bioscience department: Professor Ray Bressan and Liming Xiong for their teachings, suggestions and professionalism. I am grateful to the professors and researchers that opened the doors of their laboratories: Professors Sergey Shabala, Andrew Leaky, John Cheesman and Dr. Lana Shabala.

I thank the members of the Molecular Plant Biology: Drs Claudius Marondedze, Lara Donaldson, Stuart Meier, and Hisham Mansour. My appreciation also goes to Dr. Michael Van Oosten for his friendship, teachings and encouragement.

I thank to Danilo for his love, permanent support, dedication and for making me happy.

Thanks to my friends and colleagues for their generosity and for the memorable moments.

Finally, my heartfelt gratitude is extended to my family for their love.
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CHAPTER 1 GENERAL INTRODUCTION

1.1 The cyclic nucleotides as second messengers

Signaling mechanisms enable living organisms to adjust the cell behavior to the environment and development. These mechanisms sense external information, then transduce the messages into internal (chemical or electrical) signals and trigger highly specific responses. At the cellular level the signal transduction machinery involve many components, which include receptors, enzymes, adaptors, proteins and non-proteins molecules, transcription factors and effectors such as membrane transporters (Sanders et al., 2002). In plants several non-protein molecules have been identified as second messengers that mediate the primary signals. These molecules include, for example, cyclic nucleotides (CNs) (Newton and Smith, 2004), Ca\(^{2+}\) (Sanders et al., 2002), pH (Felle, 2001), ROS (Apel and Hirt, 2004) and lipids (Meijer and Munnik, 2003).

Some of the best described second messengers are the cyclic nucleotides adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP), which occur in diverse types of organisms: bacteria, protozoa, fungi, mammals and plants (McDonough and Rodriguez, 2012) where they mediate critical biological processes e.g. in mammals cAMP mediate Insulin effects and glucose metabolism in bacteria. Changes in the concentration of second messengers led to the activation or deactivation of downstream effectors generating variation in the phenotype. The concentration of cyclic nucleotides depends on the rate of synthesis by the nucleotidyl
or nucleotide cyclases (NCs) and the rate of degradation by phosphodiesterases (PDEs). The synthesis machinery include the adenyl cyclase (AC) that catalyzes the formation of cyclic AMP from ATP, with concomitant production of inorganic pyrophosphate and the guanylyl cyclase (GC) that by a similar esterification reaction produces cyclic GMP from GTP (Newton and Smith, 2004). The hydrolysis of cyclic nucleotides by PDEs produces nucleotide monophosphates, AMP and GMP. The activity of NCs is strictly regulated by activating events occurring at the plasma membrane such as the binding of specific ligands to protein receptors or the cytosol. Changes in the concentration of cyclic nucleotides are coupled to downstream effectors, namely protein kinases, G proteins, transcription factors and ion channels (McDonough and Rodriguez, 2012).

1.2 Cyclic nucleotides in plants

1.2.1 Synthesis and degradation

The occurrence and functional importance of cyclic nucleotides in plants was subject of controversy, however in the last years new findings indicate that cyclic nucleotides are part of plant signaling machinery (Newton and Smith, 2004).

Mass spectrometry and fluorescence-based assays have allowed the detection of cyclic nucleotides in plant cell extracts (Donaldson et al., 2004; Moutinho et al., 2001; Pacini et al., 1993). The concentration of cAMP and cGMP in plant tissue ranges between 0 and 20 pmol/fresh weight. These values are low compared to mammal concentrations, which are higher than 250 pmol/fresh weight (Gehring, 2010). Importantly, in plants...
changes in the cytosolic concentration of CN occur in response to specific stimuli e.g. cGMP concentration increases in response to salt and osmotic stress (Donaldson et al., 2004) and cytosolic cAMP increases in response to avirulent pathogens (Gehring, 2010).

Plant proteins with nucleotydyl cyclase and phosphodiesterase activity have been identified and they exhibit modular complexity and low degree of similarity with the animal counterparts suggesting that other members of the family remain unidentified (Gehring, 2010). The PsiP maize gene (AJ307886) is annotated as a plant AC. The PsiP gene was isolated from a Zea mays cDNA library and it encodes a protein that when heterologously expressed in E. coli significantly augments cytosolic cAMP concentrations and when silenced in pollen tubes by antisense oligodeoxynucleotides led to aberrant tube growth and orientation (Moutinho et al., 2001).

Plant GCs comprise of two categories, the soluble and transmembrane proteins. From the first category, the ATGC1 from Arabidopsis thaliana has been shown to catalyze the conversion of GTP into cGMP when the recombinant protein is expressed in E. coli (Ludidi and Gehring, 2003). In plants, the second category includes the phytosulfokine receptors (Kwezi et al., 2011), brassinosteroid receptor (AtBRI1) (Kwezi et al., 2007) and the peptide signaling molecules receptors (AtPepR) (Qi et al., 2010). The existence of NCs may make it likely that plants have the machinery to synthesize CN although not all of them have been linked to functional processes (Isner et al., 2012). The AtPepR1 has been linked to the immune system and root development. The AtPepR1 has GC activity in vitro, AtPepR1 and cyclic nucleotide gated-channel 2 (DND1) null mutants do not
activate Ca\textsuperscript{++} currents in response to AtPeps leading to impaired immune transcriptional reprogramming and compromised immunity and root growth (Qi et al., 2010).

1.2.2 The cyclic nucleotide-gated channels (CNGC)

Genomic searches for CN targets in plants identified a family of ion channels, the CNGC that encode transporters containing the CN-binding region inside the carboxy-terminal domain (Kaplan et al., 2007), indicating that CN help to control ionic transport through a ligand-binding mechanism. The CNGC family contains at least 20 genes in Arabidopsis and there are orthologues in different plant species (Yuen and Christopher, 2010). These transporters mediate the passive transport of monovalent and divalent cations exhibiting differential selectivity to Na\textsuperscript{+}, Ca\textsuperscript{++} and K\textsuperscript{+}. The open probability of the channels is regulated weakly by voltage and by ligands CN and calmodulin (Ma et al., 2010). Experiments on the effects of cAMP and cGMP on specific CNGC showed that the CNs do not alter permeability of the channels in the same manner (Yoshioka et al., 2006) supporting the idea that sense they have different roles. Based on its physiological properties the CNGC are classified as voltage-independent nonselective cation channels (NSCC), although some of its members respond to voltage (Demidchik and Maathuis, 2007). Heterologous expression of the cDNA, in planta electrophysiology assays and knock-out mutants have been used to characterize some members of the family (Kaplan et al., 2007). Loss of function studies show that CNGC 2 and 4 have a role in plant defense to
avirulent virus regulating the hypersensitive response and that CNGC 11 and 12 are needed to trigger R-gene resistance (Talke et al., 2003). GUS-promoter expression analysis has shown that CNGC are expressed at different organs of the plants, for example CNGCs (1,2,3,6 and 10) are expressed in roots (Yuen and Christopher, 2010) and in young leaves transcripts of CNGCs (1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 17 and 20) have been detected (Genevestigator database). Mineral uptake and salt stress resistance are impaired in CNGC 1, 3 and 10 knock-out plants showing that CNGC might be essential for ion acquisition in the roots (Yuen and Christopher, 2010). CNGC 10 is an inward channel localized at the plasma membrane of root and leaf cells. It is essential for plant growth and development as the cngc10 null-mutant could not be recovered (Borsics et al., 2007). Heterologous expression of CNGC10 in yeast evidenced that the channel is responsive to cGMP and calmodulin but not to cAMP. In plants, antisense mutants have reduced K⁺ contents causes early flowering, reduced leaf area and accumulation of starch in the chloroplasts and impaired responses light (Borsics et al., 2007). It is likely that CNGCs mediate transport regulated functions such as stomatal opening, pollen tube development (Talke et al., 2003), senescence and metabolism. Recently, the CNCG 6 has been characterized as a transporter that is ubiquitously expressed and might participate in heat acclimation responses. CNGC 6 mediates Ca²⁺ fluxes through the plasma membrane that are activated by cAMP and heat. In addition, it is necessary for the induction of heat shock proteins expression under stress (Gao et al., 2012). Gene expression of some CNGC in roots is regulated by environmental soil conditions such as
nutrient deprivation (Yuen and Christopher, 2010) other regulatory mechanisms by which gene expression is regulated remain to be studied.

1.2.3 The physiological roles of the cyclic nucleotides

Cyclic nucleotides are involved in several plant physiological and developmental processes, although further research on potential regulated process is pending. Root ion transport has been shown to be modulated by CN presumably through the regulatory interactions of CN on plasma membrane transport (Pharmawati et al., 1999; Maathuis and Sanders, 2001). Cross-talk between cyclic nucleotide and calcium signaling is implicated in the effects of CNGC and CNs on the plant immune signaling (Dangl et al., 1996; Ma et al., 2009). Hormonal interactions are supported by phosphoproteomics and expression studies: the phosphoproteome of cGMP treated roots overlaps with the hormones induced phosphoproteome (ABA, gibelleric acid, auxin (IAA), jasmonic acid (JA) and cytokinins (Isner et al., 2012).

The cytosolic levels of cGMP are increased in response to salt and osmotic stress (Donaldson et al., 2004) as well as other abiotic stresses such as high ozone (Pasqualini et al., 2009). In guard cells, cGMP is part of the signaling cascade that is triggered by abscisic acid (ABA) and leads to stomatal closure (Meier et al., 2007). The evidence positions cGMP downstream of nitric oxide (NO) and upstream of the release of Ca\(^{+}\) storages, as the cGMP inhibitors impair the NO-dependent Ca\(^{+}\) spikes (Garcia-Mata et al., 2003).
Interestingly, studies in the macroalgae *Dictyota dichotoma* and *Gelidium sesquipedale* suggest that cAMP levels respond to light quality and that cAMP levels raise when light is switched on from the dark condition (Gordillo et al., 2004). Therefore, cAMP levels are sensitive to light and presumably are downstream of light sensors, suggesting that inhibition of adenylate cyclase activity would lead to impaired responses to light (e.g. photomorphogenesis developmental and metabolism). Pollen tube development is regulated by cAMP and Ca\(^{+}\) gradients (Mountinho et al., 2011). Recently, analysis of Arabidopsis leaf’s proteome in response to cAMP revealed that this CN alters the expression of proteins involved in a number of functional categories in one of these functional categories including photosynthesis, transport, defense, regulation of carbon metabolism and response to high light stress (Gehring, C., Unpublished data). In this study, we hypothesize that CN modulates photosynthesis, high light stress response and root transport, which might be linked to novel molecular mechanism important to further elucidate the role of CN in plants. Furthermore, we examine if the CN alter the gene expression of a selected set of genes involved in the biological functions under study.
CHAPTER 2 GENE EXPRESSION ANALYSIS

2.1 Introduction

Gene transcription is the cellular process by which the information in the DNA is used to synthesize mRNAs that in turn carry the message of what proteins are to be synthesized, thus contributing to define the composition of cells at specific biological conditions. Gene expression is a dynamic and highly regulated process by a complex network of genetic and epigenetic factors that include availability and activity of transcription factors, DNA and histone modifications. These factors are subject to regulation by high order mechanisms such as signaling pathways that ensure that specific subsets of genes are synthesized according to spatio-temporal and environmental conditions. Transcriptional studies have shown that drastic transcriptional reprogramming occurs in plants in response to developmental and hormonal cues as well as part of the acclimation to biotic and abiotic stresses (Kreps et al., 2002; Rossel et al., 2002; Delessert et al., 2004; Murchie et al., 2005; Chinnusamy et al., 2007;). Indeed, the expression of stress-responsive genes is necessary to achieve tolerance to the stresses.

The characterization of the molecular changes is a necessary step to understand the mechanisms by which the CNs trigger physiological changes. Recently, a transcriptome study in Arabidopsis thaliana roots has shown that cGMP regulate the relative transcription levels of several genes, many of those related to membrane transport were significantly up-regulated including the CN targets CNGC 2, 6, 12, 15, 16, 17 and 20
(Maathuis, 2006). However, it is unclear if cAMP trigger changes at the transcriptional levels and to what extent the responses to the different CN overlap. Information in CN could be encoded as changes in Ca\(^{++}\) signatures. Rapid changes (1h) in the transcription levels of 230 Arabidopsis genes are induced by Ca\(^{2+}\) spikes, which were obtained by using chemical antagonists of calmodulins (Kaplan et al., 2006). In this study they found Ca\(^{2+}\)-dependent genes to include aquaporins, stress-responsive, signaling related genes and only two photosynthetic genes (these two are also Ca2+-dependent? Otherwise, you need a separate sentence) that were up-regulated (Kaplan et al., 2006).

We have collected proteomics evidence that shows that cAMP induces changes in the protein composition of Arabidopsis leaves (Gehring C., Unpublished) and it was proposed that cAMP-dependent regulatory mechanisms could be initiated at the transcriptional level or could be triggered by post-transcriptional mechanisms. Based on these new findings, we selected a group of candidate genes that are expressed in Arabidopsis leaves to analyze if cAMP triggers changes at the transcription level (Table 1). These genes belong to functional categories that might be subject to modulation by the cyclic nucleotides: Carbon metabolism, transport and responses to high light. Furthermore, our goal is to explore the dynamics of the changes that occur over a time course of three hours (after cAMP or cGMP treatment). In addition we aim to determine if there is a dose-dependent behavior by exposing plants to CN at two concentrations (1 and 10 µM).
Table 1. List of candidate genes that were subject of transcription analysis. Descriptions and functions were taken from Geninvestigator Database and (Nutricati et al., 2006; Borsics et al., 2007; Cai et al., 2008; Johnson and Ruban, 2010; Bahaji et al., 2011; Gao et al., 2012). Significant changes at the proteomic level were taken from Gehring C.

Unpublished data.

<table>
<thead>
<tr>
<th># Accession</th>
<th>Name</th>
<th>Function</th>
<th>Description</th>
<th>cAMP-dependent proteome</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCG00490</td>
<td>Large subunit of RUBISCO (RBLC)</td>
<td>Photosynthesis</td>
<td>The Ribulose-bisphosphate carboxylase It catalyses the first step of the Carbon fixation pathway.</td>
<td>Up-regulated</td>
</tr>
<tr>
<td>AT1G53580</td>
<td>Glyoxylase II 3</td>
<td>Response to high light and oxidative stress.</td>
<td>Mononuclear Fe(II)-containing member of the b-lactamase fold superfamily.</td>
<td>Up-regulated</td>
</tr>
<tr>
<td>AT5G48300</td>
<td>ADP glucose pyrophosphorylase 1</td>
<td>It is the main regulator of starch synthesis.</td>
<td>Small subunit of ADP-glucose pyrophosphorylase, it is required for large subunit stability.</td>
<td>Up-regulated</td>
</tr>
<tr>
<td>AT1G06680</td>
<td>Photosystem II subunit P-1</td>
<td>The photosynstem II harvest the light, taking the energy to catalyze photolysis of water. Involved in absorption and dissipation of energy.</td>
<td>Encodes a 23 kD protein, is part of photosystem II and participates in the regulation of oxygen evolution.</td>
<td>Down-regulated</td>
</tr>
</tbody>
</table>
### 2.2 Materials and Methods

#### 2.2.1 Plant growth

Wild type *Arabidopsis thaliana*, ecotype Columbia-0 (*Col-0*) seeds were sterilized by washing with 70% ethanol, 7% Sodium hypochlorite and 0.2% (w/v) Triton X-100 for 8 minutes. Then, seeds were rinsed three times with distilled water, re-suspended in distilled water and stratified at 4°C for two days. The planting procedure was performed under a laminar flow chamber using aseptic conditions. Murashige and Skoog (MS) media supplemented with 3% sucrose and 0.7 % agar was autoclaved and poured into 10 cm Petri dishes and allowed to cool. Afterwards, a canvas mesh was put on the top of
the medium and approx. 50 seeds were spread separately using a Pasteur pipette. The Petri dishes were sealed with Parafilm and transferred to the growing chamber for 14 days, under long day conditions (16 h light/8 dark), at 24°C and a light intensity of 80–120 μmol m⁻² s⁻¹ photons.

2.2.2. Plant treatment
Concentrated (100 μM) stock solutions of the treatments were prepared by dissolving 8-Bromoadenosine 3',5'-cyclic monophosphate sodium salt (Br-cAMP) and 8-Bromo-guanosine 3',5'-cyclic monophosphate sodium salt (Br-cGMP) (Sigma-Aldrich, USA) in distilled water. The 14-days old seedlings were transferred to 6 cm Petri dishes containing MS liquid media and allowed to equilibrate for 30 minutes. Later, the treatment solutions were added to the medium reaching final concentrations of 10 μM and 1 μM, the control plants were treated with MS medium only. The dishes were transferred to the growth room and allowed to incubate for specific time points (10, 30, 60 and 180 minutes). Two independent biological replicates were performed for each treatment.

2.2.3. Quantitative real time PCR

2.2.3.1. RNA extraction
Roots were excised and immediately the rosette tissue was frozen in liquid Nitrogen. Total RNA was extracted from 100 mg of rosette tissue using the RNeasy kit (Qiagen,
USA). DNase I digestion was performed according to the manufacturer instructions.

RNA was quantified using a Nanodrop and the absorbance ratio of 260 nm and 280 nm checked to be approx. 2. The RNA quality was also verified using a 1.2% agarose gel electrophoresis made in 1X MOPS buffer containing 6.2% v/v formaldehyde. The 10X MOPS stock buffer [0.2 M MOPS (pH 7.0), 20 mM sodium acetate and 10 mM EDTA (pH 8.0)] was prepared in DEPC-treated water. In parallel 2.5 µg of RNA was mixed with 10 µl of loading dye [60% Formamide, 9% Formaldehyde, 67 µg/mL Ethidium Bromide and 1X MOPS] and and heated at 65° C for 5 minutes. The samples were loaded into the gel and afterwards electrophoresed in 1X MOPS running buffer at 100 volts for one hour. Finally, the bands were visualized with a Gel Doc system (BioRad Inc., USA).

2.2.3.2. cDNA synthesis

The cDNA was synthesized using the High capacity cDNA reverse transcription Kit (Applied Biosystems, USA). The master mix without RNA inhibitor (10X RT buffer, 10X random primers, 25X dNTPs and MultiScribe Reverse transcriptase) was prepared according to manufacturer instructions and maintained on ice. Each reaction mix was made up of 10 µl of master mix, 2µg of RNA and the final volume was adjusted by adding RNAs-free water. The conditions for reaction program was: 25°C (10 min), 37°C (120 min), 85°C (5 min) and 4°C until collection. The cDNA concentration was measured with a Nanodrop, and 1:20 aliquotes prepared and stored at 4°C for immediate use or -20°C for longer storage.
2.2.3.2. Primers

We selected eight target genes and one housekeeping gene and designed pairs of primers using the Primer express software. For each set of primers, we performed amplification on a serial dilution of a cDNA pool and analyze the fluorescence as a function of the number of cycles plot and the melting curve. This method was used to verify that the primer sets were annealing to the target gene by producing a single PCR product, generating a minimum amount of primer dimers and had efficiency greater than 1.7. The sequences of the primers used to amplify the fragments of the selected genes are shown in Table 2.

2.2.3.3. Relative Quantification

Quantitative real-time PCR (qRT-PCR) based on SYBR-green fluorescence was used to determine changes in gene expression. Amplification reactions contained 2 µL of cDNA (50ng/µL), water and master mix (Roche) in a final volume of 20 µL. The reaction mix were placed in separated wells of a 96-well reaction plate and loaded into a thermal cycler (Roche). The amplification protocol comprised 45 cycles (95 °C, 60°C and 72 °C) and a cooling step (40°C). Each sample was amplified at least twice in the same plates to guarantee reproducibility. Relative transcription levels were calculated comparing threshold cycle values (Ct) of control and treatment samples normalized with respect to the fluorescence of the Ubiquitin conjugating enzyme (At5g25760) reaction by the ΔΔCT method (Kubista et al., 2006).
Table 2. Primer Sequences used for the Quantitative Real Time PCR. Sequences are given from 5’ to 3’.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin Conjugating Enzyme</td>
<td>At5g25760</td>
<td>GGACCCTCTTT</td>
<td>CTTGAGGAGGTTGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATCAAGGA</td>
<td>AAAGGA</td>
</tr>
<tr>
<td>RBCL</td>
<td>ATCG00490</td>
<td>CGTTGGAAGACC</td>
<td>TCACCTGTTCAGCC</td>
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<tr>
<td></td>
<td></td>
<td>GTTTCTATT</td>
<td>TGTGATT</td>
</tr>
<tr>
<td>Rotamase CYP4</td>
<td>AT3G62030</td>
<td>CGGTGTAAAGTCG</td>
<td>AGGAACGAGCTCA</td>
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<tr>
<td></td>
<td></td>
<td>CAATTAGTTTC</td>
<td>AAGCAA</td>
</tr>
<tr>
<td>Glyoxylase II 3</td>
<td>AT1G53580</td>
<td>TTATCTTCTCGCG</td>
<td>CAGTCTTTGTCACCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACTTACTTC</td>
<td>GATCAAT</td>
</tr>
<tr>
<td>CNGC 6</td>
<td>At2g23980</td>
<td>AAGCCCTTACCG</td>
<td>TTGCCACGAATTTCA</td>
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<td></td>
<td></td>
<td>AAGTGAA</td>
<td>GCTCAT</td>
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<td>CNGC 10</td>
<td>AT1G01340</td>
<td>TGTGTGTACGCCG</td>
<td>CAGATGTAAGCTG</td>
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<td></td>
<td></td>
<td>ATCGAT</td>
<td>TCCTGAAC</td>
</tr>
<tr>
<td>PSII PsbI</td>
<td>AT5G66570</td>
<td>CTTCAGACGGAAG</td>
<td>GTTGGACTGTGACT</td>
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<td>CGTCAATT</td>
<td>GCAGCATAAGTG</td>
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<tr>
<td>PS II subunit P-1</td>
<td>AT1G06680</td>
<td>ATCAGACGAAACG</td>
<td>AACCACCTTTGTC</td>
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<td></td>
<td></td>
<td>TGAATGG</td>
<td>CCAGCTT</td>
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<td>ADP- Glucose Pyrophosphorylase 1</td>
<td>AT5G48300</td>
<td>AGGGCCATCATCG</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>ACAAAAA</td>
<td>TGTCTTG</td>
</tr>
</tbody>
</table>

2.3 Results

We performed 60 independent experiments in which the seedlings were incubated with Control, Br-cGMP and Br-cAMP dissolved in water, these analogues of the CN could penetrate the plasma membrane and reach the cytoplasm [reference?]. Our study was performed using relatively high concentrations of the CN (1 and 10 µM) attempting to allow the treatment to overcome the physical barriers, such as root epidermis, and reach the targets. The total RNA was extracted from the aerial tissue and subsequent quantification was performed, and degradation and purity were assessed by measuring
the ratio of the absorbance at A260/A280, which were approximately 2. RNA quality check was performed on the samples and clear bands were visualized following electrophoresis (Figure 1).

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>cAMP</th>
<th>cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. µM</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Time</td>
<td>10</td>
<td>180</td>
<td>30</td>
</tr>
</tbody>
</table>

**Figure 1.** RNA quality check on 1.2% agarose gel electrophoresis. Arabidopsis seedlings were sown on MS medium (Control, C) or MS medium supplemented with Br-cAMP (1 or 10µM) or Br-cGMP (1 or 10µM). The plants were incubated with treatments for 15, 30, 60 and 180 minutes of followed and the aerial tissue used for RNA extraction. Total RNA of 18 plant samples were loaded into each lane and separation was carried using agarose gel (1.2%) electrophoresis. For all the lanes, there are distinguishable bands corresponding to ribosomal RNA.

In order to detect induced changes in the messenger RNA levels of the candidate genes specific primers were designed and used to perform cybergreen-based qRT-PCR. Both
cGMP and cAMP treatments induced changes in gene transcription levels of the selected specific genes. In most of the cases, we observed transient transcriptional changes within the first 30 minutes. The analysis of the effects of CN in a group of three genes that are involved in the photosynthesis revealed that cGMP (10 µM) up-regulate Rubisco (ATCG00490) and components of PSII (AT1G06680 and AT5G66570), whereas cAMP (1 µM) do not alter mRNA levels of Rubisco but induced the PSII Oxygen-evolving complex (Figure 2a, 2b, 2c). In addition, a starch synthesis regulator, ADP phyrophosphorylase (AT5G48300), was up-regulated 2.6 fold by cGMP (10 µM) and not by cAMP (Figure 2d).

We analyzed the relative expression of genes related to photoprotection, the *Arabidopsis* CYP4 (AT3G62030) and Glyoxylase II (AT1G53580). Expression of the former was modulated by cAMP in an oscillatory pattern. mRNA levels were elevated in the first 10 minutes and repressed later on 180 minutes after exposure to the stimuli (Figure 3a). Moreover, after 30 minutes treatment with 1 µM and 10 µM cGMP of the mRNA level of CYP4 was down-regulated by 0.6-fold and 0.74-fold, respectively (Figure 3a). In contrast, there is no significant change in the Glyoxylase II mRNA levels between the controls and the CN treated plants (Figure 3b).

The qPCR experiments showed that either CNs repress or induce different CNGC transcript levels. CNGC 10 (AT1G01340) expression was diminished by cAMP treatments
(1 and 10 µM) and by cGMP (10 µM) (Figure 4a), whereas CNGC 6 (At2g23980) mRNA concentration was enhanced by cGMP (10 µM) and unchanged by cAMP (Figure 4b).

a)

![Graph showing relative cDNA level over time for different treatments](image1)

b)

![Graph showing relative cDNA level over time for different treatments](image2)
Figure 2. Modulation of mRNA expression of genes involved in photosynthesis and starch metabolism by cAMP and cGMP. Relative mRNA expression of treated as compare to the control plants measured with qPCR. The gene amplification of a) Rubisco RBLC, b) PSII Oxygen evolving complex, c) Photosystem II subunit P-1 and d)
ADP Phyrophosphorylase were normalized against the *Ubiquitin conjugating enzyme*. Bars represent the arithmetic mean of the relative expression ±SE (n= 2, 3 biological; ≥ 2 technical replicates). Changes were considered significant when the relative levels were at least 1.5-fold compared to the control levels*.

a).

![Graph a)](image)

b).

![Graph b)](image)
Figure 3. Response of high light response genes to cAMP and cGMP treatment. Relative mRNA expression of treated as compare to the control plants measured with qPCR. The gene amplification of a) Glyoxylase II and b) Rotamase were normalized against the *Ubiquitin conjugating enzyme*. Bars represent the arithmetic mean of relative expression ±SE (n= 2, 3 biological; ≥ 2 technical replicates). Changed were considered significant when the relative levels were at least 1.5-fold compared to the control levels*.

a).
b).

Figure 4. Effect of cAMP and cGMP on the expression of CNGC 10 and CNGC 6. Relative mRNA expression of treated as compare to the control plants measured with qPCR. The gene amplification of a) CNCG 10 and b) CNCG 6 were normalized against the Ubiquitin conjugating enzyme. Bars represent the arithmetic mean of relative expression ±SE (n= 2, 3 biological; ≥ 2 technical replicates). Changes were considered significant when the relative levels were at least 1.5-fold compared to the control levels*.

2.4 Discussion

It has been previously shown that cGMP modulates gene expression in Arabidopsis roots (Maathuis, 2006). In the current study we show that cAMP is also involved in the regulation of gene transcription. To further characterize the specificity of the cAMP signal, we measured mRNA levels of seedlings treated with either cAMP or cGMP under
the same experimental conditions. Treatment for 30 minutes with cGMP resulted in CNGC 6 mRNA up-regulation (1.73 fold), whereas no significant change in expression change was observed by cAMP treatment. In other cases the responses of the two cyclic monophosphates overlapped. For example the relative expression of CNGC 10 was down-regulated by both cGMP and cAMP after 30 minutes, although these changes were not reflected at the protein levels. Our data reveals that cyclic monophosphates cAMP and cGMP have their own specificity and differentially regulate signal transduction and possibly downstream process.

In addition the evidence presented here shows that cGMP up-regulates the expression of genes that are related in the light and dark reactions of photosynthesis, Rubisco (ATCG00490) and Photosystem II – P subunit (AT1G06680), whereas cAMP does not alter the mRNA levels of Rubisco but up-regulates components of the light harvesting machinery (AT5G66570). These molecular data indicate that cGMP could be involved in the regulation of the rate of carbon assimilation. As suggested by the transcriptional analysis of other aspects of carbon metabolism could also be regulated by cAMP, for example the synthesis of starch which is the main product of photosynthesis and the main storage of sucrose (Zeeman et al., 2007). The down-regulation (0.58-fold) of the ADP glucose pyrophosphorylase (AT5G48300) would diminish the capacity of the cells to convert glucose into ADP-glucose that is the limiting reaction in the synthesis of starch occurring in the chloroplast stroma (Bahaji et al., 2011). In addition, cAMP reduces the expression of the membrane transporter CNGC10 which in turn might decrease the
capacity to export starch from the chloroplast to the cytosol (Borsics et al., 2007) as the ion gradients play a vital role in the co-transport of metabolites such as glucose and maltose that are the final products of degradation of starch (Zeeman et al., 2007). Taken together the data suggests that cAMP would mediate the regulation of starch metabolism, leading to decreased leaf starch contents. Further quantification assays, such as mass spectrometry analysis and electron microscopy visualization of iodine-stained starch granules, are needed to validate this claim.

Further, cAMP has been shown to modulate the gene expression of CYP4 which is a chaperone proteins involved in the repair of PSII after high light stress. CYP4 is one of the most abundant proteins in the stroma and the CYP4 loss of function mutant is sensitive to oxidative stress and high light (Cai et al., 2008). Therefore, cAMP may contribute to sensing and triggering responses to high light. In the future, it would be important to clarify if the redox state of the stroma and the expression of other antioxidant proteins (e.g. ascorbate peroxidase) are also regulated by cAMP. Our work supports the fact that CNs regulate cation transport in leaves by affecting the expression of some NSCC (CNCG6 and CNGC10). CNs modulate the expression of their own targets, the CNGCs, suggesting that the magnitude of the signals can be finely tuned by feedback mechanisms to generate highly specific responses.
Most of the tested transcriptional changes occurred as early transcriptional responses, suggesting that the signal is driven rapidly from the roots to the leaves and that electrical or Ca$^{2+}$ signatures could be used downstream to decode the information into transcriptional changes. However, we did not find conclusive matches between the CN- and Ca$^{2+}$ targets at the transcription level (Kaplan et al., 2006). This disagreement could be explained by the complexity of Ca$^{2+}$ signals, in which different types of spatio-temporal oscillations encode different messages including changes at the transcription level.

To discern if the effects of cAMP on protein levels are directly reflected at the level of transcriptional regulation we performed qPCR on a subset of cAMP regulated proteins and on additional genes that might be involved in cAMP signaling pathway. Transcript levels of the selected genes were measured at 10, 30, 60 and 180 minutes and the cAMP concentrations were 1 and 10 µM. We found that some differentially regulated proteins were also transcriptionally modulated by cAMP, whereas in other cases protein and mRNA levels did not correlate. Seedlings treated with 1 µM cAMP showed a rapid ($\leq$ 10 minutes) and transient induction of Rotamase CYP4 (AT3G62030) mRNA transcription (up-regulated of 2.39 fold) with no significant changes observed at the protein levels during the first 60 minutes but were significantly up-regulated at 180 minutes after the treatment (Figure 3b). It is noteworthy that a higher concentration of cAMP (10 µM) did
neither affect the transcript nor protein levels and that cGMP had no significant influence on the transcript levels.

We identified Glyoxylase II 3 (AT1G53580) and the large subunit of Rubisco (ATCG00490) as genes that are modulated by cAMP at protein level but not at mRNA level. The 10 µM cAMP treatment that led to up-regulated protein concentration of the large subunit of Rubisco (ATCG00490) showed no significant up-regulation at the transcript level neither at 10, 30, 60, or 180 minutes Figure 2a). Likewise, Glyoxylase II 3 was up-regulated after 60 minutes of cAMP (1µM) treatment but no significant up-regulation was observed at the mRNA. Therefore, the data suggest that processes like transcriptional and post-transcriptional factors such as pre-mRNA processing, RNA export from nucleus, RNA and protein turn-over plays an important part in cAMP dependent changes of the proteome.

Another group of genes exhibit a complex behavior in which the mRNA and protein concentrations are not correlated and depend on time and concentration factor of the CN treatment. In response to cAMP the mRNA levels of the ADP glucose pyrophosphorylase 1 (AT5G48300) decreased 180 minutes after the treatment (down-regulated 0.58 fold), and contrastingly the protein levels are increased at the same time point. The protein concentration of PSII oxygen-evolving complex I (AT5G66570) are down-regulated by 10 µm cAMP after 1 and 3 hours of treatment, however mRNA levels are unaltered and up-regulated in response to the lower concentration of 1 µm cAMP.
Extensive examination on cAMP downstream components and how they sense cAMP concentrations are therefore crucial.

In conclusion, we showed that the cyclic nucleotides cAMP and cGMP modulate foliar gene transcription. Since there is a low degree of correlation between gene transcription and protein expression effects, both transcriptional and post-transcriptional processes are downstream of the cyclic nucleotides. In some cases, the two cyclic monophosphates triggered different responses, indicating that the signals are specific and may be recognized by specific effectors proteins. The molecular results support the role of CN in the transduction of light signals and membrane transport and the new findings also open the probability to novel roles for the CN in the control of metabolism.
CHAPTER 3 THE PHOTOSYNTHESIS AND HIGH LIGHT STRESS IN RESPONSE TO CYCLIC NUCLEOTIDES

3.1 Introduction

3.1.1. Photosynthesis

The photosynthesis is a central metabolic pathway in which light is harvested by plants, cyanobacteria and algae to generate chemical energy. The energy is collected and can be used to drive the carbon dioxide (CO₂) fixation into three carbon carbohydrates that are furthermore transformed into more complex organic molecules. The plants have developed regulation systems that make photosynthesis flexible to cope with the energy demands of sessile organisms that live in a changing environment.

The photosynthesis encompasses a series of reactions divided into two phases: Electron transport chain and carbon reduction cycle. In eukaryotes, both phases occur at the chloroplasts. In the first phase, the light is harvested by the Photosystem II (PSII) and the excitation energy used to drive the split of water molecules into Oxygen (O₂), protons and electrons. The protons are accumulated in the thylakoid lumen and the electrons are transferred through the electron transport chain until they reach the final acceptor NADP (Linear electron flow). This transfer employs additional energy provided by the light harvesting reaction at the Photosystem I (PSI). The photosystems are molecular complexes that contain the chlorophylls, accessory pigments (e.g.
carotenoids) and proteins (e.g. Light harvesting complexes). The electron transfer reactions are coupled to the pumping of protons from the stroma to the thylakoid lumen, producing a proton gradient across the thylakoid membrane called the proton motive force (pmf) (Kramer et al., 2003; Mitchell, 1966). Dissipation of the pmf is coupled to the synthesis of ATP catalyzed by the ATPase. In addition the pmf acts as a regulator of the energy dissipation by nonphotochemical quenching (NPQ) (Niyogi et al., 1998).

In the carbon reduction cycle, the ATP and NADPH are spent to convert the low energy molecule CO$_2$ into organic molecules. The assimilation involves the carboxylation reaction by the ribulose-1, 5 bisphosphate (RuBp) carboxylase (Rubisco), the reduction steps and RuBp regeneration. Rubisco can accept either O$_2$ or CO$_2$ as substrates, starting the photorespiration or carbon fixation pathways. In the later, one molecule of phosphoglycerate (PGA) and one of glyceraldehyde 3-phosphate are generated using 1 ATP and 1 NADPH. In photorespiration one molecule of CO$_2$ is released and energy is spent in regenerating RuBP. Assimilation can be stated in terms of carboxylation rate that accounts to fixed carbon minus released carbon by dark respiration and is a parameter that can be measured by gas exchange analysis (Long and Bernacchi, 2003). Other parameters like the efficiency of PSII and the linear electron transport rate can be evaluated by combining chlorophyll fluorescence and gas exchange measurements (Baker, 2008).
3.1.2. Regulation of photosynthesis

The photosynthetic rate of the plants is a dynamic process influenced by several extrinsic and intrinsic factors which confer high flexibility to the reactions. For the C3 plants, the Farquhar model studied the limitations of the photosynthetic flux at light saturating conditions considering the contribution of the enzymatic activity, rate of parallel reactions (e.g. photorespiration), substrate concentrations (O₂, CO₂), temperature and rate of light reactions (Farquhar et al., 1980). The model shows that light saturated photosynthesis is limited by one of the following factors: rate of Rubisco carboxylation (Vc, max), rate regeneration of ribulose bisphosphate (J max) and triphosphate consumption (VTPU). Most of the CO₂ uptake into the plant takes place at the leaf’s stomata; therefore the assimilation rate is affected by the stomatal conductance (Yoo et al., 2009). Biological parameters such as the plant developmental stage and the sink demands would affect VTPU. The Vc,max depends on the amount and activation state of Rubisco as well as the concentration of CO₂ at the chloroplasts (Long and Bernacchi, 2003). Plants change the cell concentration of Rubisco or other components of the photosynthesis as a general mechanism of control, changes are attained by differential gene expression. For instance, the mRNA levels of photosynthesis-related components have been shown to be altered in response to light, pathogen attack (Kangasjarvi et al., 2012), abiotic stresses (Saibo et al., 2009) and the concentration of the final products of the pathway (Paul and Pellny, 2003).
The photosynthetic efficiency is also tuned by post-translational control of the protein components, the covalent modifications contribute to switch the enzymes to the active configurations or to target them for degradation. Activation of some of the Calvin-Benson enzymes is achieved by protonation; the redox regulation mechanism is mediated by thioredoxin and ferredoxin and requires alkanization of the stroma by the light reactions. The accumulation of protons in the stroma is correlated to the production of Oxygen reactive species (ROS), the onset of NPQ (Niyogi et al., 1998) and activation of protein regulators such as the kinases (Ferris et al., 2006). Recently, it has been demonstrated that the concentration of small metabolites can also be part of the redox signaling in the chloroplast, the 3’-phosphoadenosine 5’-phosphate (PAP) is accumulated in high light and it translocates to the nuclei where it changes gene expression (Estavillo et al., 2011).

The redox state of the chloroplast, activity of electron fluxes and redox plastoquinone state would act as primary sensors of light reception (Galvez-Valdivieso et al., 2009). It is not clear which other intermediates participate in the signal transduction pathway that ends with transcriptional changes. For example, if Ca$^{2+}$, changes in membrane potential and the concentration of other small metabolites might play a role. In this study we hypothesize that the CNs modulate the net CO$_2$ assimilation and chlorophyll fluorescence under different light intensities and after exposure to high light.
3.1.3 The high light response

Plants are exposed to fluctuating light conditions, in order to be able to survive plants have the ability to sense light and adjust its metabolism and development according to the conditions, as well as defense mechanism against the exposure to an excess of energy. The light that is absorbed by the leaves can be reflected as fluorescence, dissipated as heat or used for chemical quenching. Therefore, changes in the light fluorescence (specifically PSII) reflect changes of the plant’s capacity to assimilate and dissipate the energy (Baker, 2008).

In the latter, light is primarily used in the photosystems as source of energy for the electron transport chain that has a limited capacity to transfer the electrons and receive photons. When the systems receives additional excitation energy it produces ROS, oxidative damage, aberrations in the integrity of lipids and proteins, reversible or irreversible damage of the photosynthetic components (photoinhibition), cell death and eventually plant death (Galvez-Valdivieso et al., 2009). This condition can occur in plants exposed to excessive light with an intensity that is greater than the required growing light by more than 10 times, whereas leaves exposed to high light stress (less than 10-fold of growing light) do not experience photoinhibition and oxidative damage (Fryer et al., 2003). To overcome the oxidative stress, leaves undergo transcriptional reprogramming with induction of antioxidant proteins (e.g. ascorbate peroxidase2) that is partially dependent on ABA (Karpinski et al., 1999; Fryer et al., 2003). After
Arabidopsis leaves are exposed to high light stress, the maximum quantum yield of PSII photochemistry decrease. This response is also triggered by ABA and reflecting the deactivation of PSII reaction centers by PSII component (D1) degradation (Gao et al., 2012). Lack of ABA receptors is not sufficient to explain all the HL-triggered molecular and physiological changes (Galvez-Valdivieso et al., 2009), therefore other hormones or small molecules such as cAMP or cGMP might be mediating and or modulating signaling events.

3.2 Materials and Methods

3.2.1. Plant growth and treatment

Arabidopsis thaliana seeds, ecotype Col-0, were planted in a 1:1 mixture of soil and vermiculite into 1L containers. The plants were maintained in growth chamber for 6 to 7 weeks under controlled conditions: temperature: 22°C, light was provided by fluorescent lights with an intensity of approximately 100 mmol quanta m⁻² s⁻¹, short-day photoperiod (8 hours of light/ 16 hours of dark). Two sets of experiments with different type of treatments were performed. In the first experiment, the leaves were sprayed with treatment solutions and in the second, the solutions were inoculated by syringe infiltration. The leaves were treated with one of the following solutions: 10 μM Br-cAMP, 10 μM Br-cGMP, 10 μM ABA or distilled water.
3.2.2. Photosynthesis and chlorophyll fluorescence parameters

Leaf gas exchange and fluorescence parameters were measured from fully expanded leaves with infrared gas analyzers coupled with fluorescence attachments (LI-COR Biosciences) (Figure 5). Net CO₂ assimilation was measured under constant CO₂ concentration of 400 ppm (atmospheric), the relative humidity of the air ranged between 60 and 70% and the leaf to air vapor pressure deficit (VpdL) was controlled to be lower than 1.3. In the light response experiment plants where light acclimated for 30 minutes after which they were sprayed with appropriate treatments and the net CO₂ assimilation was measured under the following light intensities PAR: 1500, 1200, 1000, 800, 500, 200, 150, 100, 80, 50, 25, 20 and 0 µmol m⁻² s⁻¹ changing every 5 minutes. In the constant light experiment (Figure 6), dark-adapted leaves were clamped into the IRGA and exposed to a far-red pulse of light for a period of 30 minutes. The minimal florescence F₀, fluorescence F and maximal fluorescence after a saturating pulse Fm of dark-adapted leaves were recorded followed by a saturating pulse of light. The actinic light was switched on to an intensity of the growing light (150 µmol m⁻² s⁻¹) after 30 minutes a saturating light was given and the fluorescence parameters were monitored until stable before taken the light-adapted parameters: minimal fluorescence (Fo’), fluorescence at actinic light (F’) and maximal fluorescence (Fm’). These set of measurements were done before the treatments and were taken as basal controls. The same plants were then inoculated with the solution treatments using a syringe and the plants maintained for one hour at either growing light or high light (1000 µmol m⁻² s⁻¹). After the exposure to light and chemical treatments a new set of F’, F₀’ and Fm’ were
measured and the light was switched off. The plants were kept in the dark for additional 30 minutes at the end of which dark-adapted parameters were measured as described elsewhere (Galvez-Valdivieso et al., 2009). Chlorophyll fluorescence parameters were used to estimate the variable fluorescence Fv and Fv’ that is equal to the difference among Fm and F in dark and light adapted leaves, the maximum quantum yield of photosynthesis Fv/Fm, the Photosystem II (PSII) maximum efficiency Fv'/Fm'.

Figure 5. Assembly for the photosynthesis experiment. 60-days old plants were clamped into the IRGA.
Figure 6. The light regime applied to leaves that were treated with CNs and subject of photosynthetic analysis. The figure shows the intensity of the light at which the plants were exposed in the course of the experiment. The experimental units were subject to treatments and exposed to high (Black line) or low light (Red line). The numbers indicate the moments at which the chlorophyll fluorescence and carbon assimilation was measured. The arrow indicates the time point at which the treatment was inoculated.

3.3 Results

First we examined whether cGMP or cAMP modulate the net CO$_2$ assimilation rate of individual leaves at different light intensities. Treatment solutions, 10 µM cAMP, cGMP, ABA and water as a control, were spread on leaves and photosynthesis was measured
from the same leaves. The measurements were taken 5 minutes after the onset of stimulation and lasted for 2 hours in which the light intensities where switched every 2 minutes approximately. Plants were first exposed to high light (1500 μmol photons m\(^{-2}\) s\(^{-1}\)) and gradually the light intensity was decreased until darkness. The light saturation curves show that the saturation point of the photosynthesis ranges from 380 - 400 μmol photons m\(^{-2}\) s\(^{-1}\). At this intensity plants exposed to the different treatments assimilate between 4.4 and 6.6 μmol CO\(_2\) m\(^{-2}\) s\(^{-1}\) (Table 2). No statistically significant difference was found in the photosynthesis of leaves treated with control and the cAMP, cGMP or ABA by the t-student test. However, it was observed that ABA and cAMP treated plants have slightly lower levels of photosynthesis than the control at high light intensities, whereas cGMP has the opposite effect (Figure 7).

**Table 3.** Photosynthetic rate of single leaves exposed to variable light intensity and treated with CNs. The numbers show the average (n=4) of CO\(_2\) assimilation measured with IRGAs after exposure to water-solved treatments.

<table>
<thead>
<tr>
<th>Light PAR</th>
<th>ABA 10μM</th>
<th>cAMP 10μM</th>
<th>cGMP 10μM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.3613</td>
<td>-0.1379</td>
<td>-0.0531</td>
<td>-0.3046</td>
</tr>
<tr>
<td>20</td>
<td>0.6804</td>
<td>0.8148</td>
<td>0.8403</td>
<td>1.0635</td>
</tr>
<tr>
<td>25</td>
<td>0.8989</td>
<td>1.0339</td>
<td>0.9187</td>
<td>1.0500</td>
</tr>
<tr>
<td>50</td>
<td>1.7070</td>
<td>2.1656</td>
<td>2.1913</td>
<td>1.9056</td>
</tr>
<tr>
<td>80</td>
<td>2.8533</td>
<td>3.1211</td>
<td>2.9909</td>
<td>3.2046</td>
</tr>
<tr>
<td>100</td>
<td>3.7338</td>
<td>3.5828</td>
<td>3.8758</td>
<td>3.6996</td>
</tr>
<tr>
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<td>4.4239</td>
<td>4.5181</td>
<td>5.1178</td>
<td>4.7628</td>
</tr>
<tr>
<td>200</td>
<td>5.1771</td>
<td>4.7831</td>
<td>5.7945</td>
<td>5.1734</td>
</tr>
<tr>
<td>500</td>
<td>5.7787</td>
<td>5.3836</td>
<td>6.5862</td>
<td>6.1926</td>
</tr>
</tbody>
</table>
To corroborate that the results were due to differences in the variation of chemical concentration inside cells and not due to the penetration of the solutions into the plant, we performed a second experiment in which the treatments were inoculated by syringe infiltrated into the leaves. Photosynthesis was measured over increasing light levels and the experiment shows that ABA significantly reduces the net CO$_2$ assimilation whereas neither cAMP nor cGMP are different from the control (Figure 8). Leaves were exposed to a high or low light for one hour to test whether cAMP or cGMP improves the condition of the PSII after stress (Figure 6). The chlorophyll fluorescence emissions were used to estimate the maximum quantum efficiency of PSII ($F_v/F_m$) and the PSII operating efficiency ($F_v'/F_m'$). For both low and high light, the chlorophyll fluorescence parameters of the controls and treated leaves were indistinguishable (Table 4). Our experiment shows that the high light stress reduces the maximum quantum efficiency of PSII which is in agreement with previous reports (Figure 9) (Galvez-Valdivieso et al., 2009) and indicates that CNs do not interfere with the PSII efficiency after a HL episode. We also verified that the electron transport rate, NPQ and PSII efficiency factor of the control and CNs treated leaves are not different under these experimental conditions.
Figure 7. Light response curve of photosynthetic rate. Changes in CO₂ assimilation for leaves exposed to different light intensities and treated with cAMP, cGMP, ABA and water. The points represent the average of 4 independent samples and the bars the standard deviation.

Figure 8. Photosynthetic rate of infiltrated leaves. Leaves were inoculated and exposed to high light 1000 μmol photons m⁻² s⁻¹ (HL) or low light 150 μmol photons m⁻² s⁻¹ (LL).
Table 4. Chlorophyll fluorescence parameters at low and high light for control, cAMP (10 µM) and cGMP (10 µM) treated Arabidopsis leaves.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>cAMP (10µM)</th>
<th>cGMP (10µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low Light</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>0.78 ± 0.016</td>
<td>0.78 ± 0.009</td>
<td>0.803 ± 0.060</td>
</tr>
<tr>
<td>Fv'/Fm'</td>
<td>0.73 ± 0.004</td>
<td>0.70 ± 0.019</td>
<td>0.730 ± 0.011</td>
</tr>
<tr>
<td><strong>High Light</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>0.71 ± 0.006</td>
<td>0.72 ± 0.01</td>
<td>0.700 ± 0.057</td>
</tr>
<tr>
<td>Fv'/Fm'</td>
<td>0.47 ± 0.004</td>
<td>0.48 ± 0.006</td>
<td>0.472 ± 0.003</td>
</tr>
</tbody>
</table>

Figure 9. Maximum quantum efficiency of PSII photochemistry for control, cAMP and cGMP stimulated leaves. Single leaves were infiltrated with cAMP, cGMP, ABA or water and subsequently clamped into the IRGAs. After 30 minutes of light acclimation, the leaves were exposed to low or high light and finally to complete darkness. The chlorophyll fluorescence parameters Fo, Fm, Fo’, Fm’ were measured and used to calculate the maximum quantum efficiency of PSII.
3.4 Discussion and conclusion

The adjustment of the photosynthetic rate to the environmental and metabolic conditions is central for plant survival. The plants need specialized control mechanisms that carry information between the organelles at the cellular level and the organs at the whole plant level. At the whole plant level, hormonal signals such as ABA function to control stomatal opening, gene transcription and the architecture of photosystems. Other small metabolites (e.g. PAP) mediate the retrograde communication between chloroplasts and nuclei (Estavillo et al., 2011). On the other hand, the CNs have been shown to play a role in the light signaling transduction (Moutinho et al., 2001), stomatal opening (Garcia-Mata et al., 2003) and stress tolerance (Newton and Smith, 2004). Cyclic nucleotides trigger changes ion trans-membrane transport and importantly, CNs stimulation is correlated with the occurrence of Ca^{2+} spikes. In this study we have demonstrated that CNs modulate gene transcription in leaves and importantly that cGMP up-regulates central components of the photosynthesis reactions (RBCL, PSII subunit P-1). Therefore we rationalized that CNs would act as second messengers affecting the photosynthetic rate and response to high light by using one or many of the mentioned mechanisms.

We characterized the effects of CNs on the photosynthesis of adult plants under different light conditions. After treatment with water, the control plants saturated at 400 μmol photons m^{-2} s^{-1} showing assimilating 6 μmol CO_{2} m^{-2} s^{-1} (Figure 7), which is
lower than the expected 8 μmol CO$_2$ m$^{-2}$ s$^{-1}$ uptake by Arabidopsis adult plants treated under similar conditions of this age (Yoo et al., 2010). This might be due the treatment infiltration or touching of the leaves that can induce stomatal closure. However we performed all experiments under the same experimental conditions. We corroborated that the chlorophyll fluorescence parameters of control leaves are in agreement with previously reported data (Galvez-Valdivieso et al., 2009).

In any of the measured photosynthetic parameters the control and CNs are indistinguishable, in contrast with the ABA treated plants that exhibit significant reductions in the CO$_2$ assimilation rate. Therefore, we conclude that CNs do not modulate photosynthesis and the PSII efficiency after HL exposure. It is still possible that under different physiological conditions, such as pre-exposure to stress, CNs can work as regulators of carbon assimilation and metabolism. Further studies may shed light on whether the relative mRNA expression of the ascorbate peroxidase and ROS levels are modulated by CNS.

At the moment, there is not adequate genetic evidence demonstrating that CN are sufficient and necessary for mediating cell signaling in vivo. Nevertheless, recent CNs detection techniques, genetic tools and the identification of downstream targets are opening promising solution to some of these outstanding issues.
BIBLIOGRAPHY


