Research Paper

Cyclic GMP is involved in auxin signalling during Arabidopsis root growth and development

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Abstract

The second messenger cyclic guanosine 3′,5′-monophosphate (cGMP) plays an important role in plant development and responses to stress. Recent studies indicated that cGMP is a secondary signal generated in response to auxin stimulation. cGMP also mediates auxin-induced adventitious root formation in mung bean and gravitropic bending in soybean. Nonetheless, the mechanism of the participation of cGMP in auxin signalling to affect these growth and developmental processes is largely unknown. In this report we provide evidence that indole-3-acetic acid (IAA) induces cGMP accumulation in Arabidopsis roots through modulation of the guanylate cyclase activity. Application of 8-bromo-cGMP (a cell-permeable cGMP derivative) increases auxin-dependent lateral root formation, root hair development, primary root growth, and gene expression. In contrast, inhibitors of endogenous cGMP synthesis block these processes induced by auxin. Data also showed that 8-bromo-cGMP enhances auxin-induced degradation of Aux/IAA protein modulated by the SCF TIR1 ubiquitin-proteasome pathway. Furthermore, it was found that 8-bromo-cGMP is unable to directly influence the auxin-dependent TIR1-Aux/IAA interaction as evidenced by pull-down and yeast two-hybrid assays. In addition, we provide evidence for cGMP-mediated modulation of auxin signalling through cGMP-dependent protein kinase (PKG). Our results suggest that cGMP acts as a mediator to participate in auxin signalling and may govern this process by PKG activity via its influence on auxin-regulated gene expression and auxin/IAA degradation.

Key words: Arabidopsis thaliana, auxin signalling, cGMP, guanylate cyclase, PKG, TIR1-Aux/IAA interaction.

Introduction

The plant hormone auxin plays a central role in plant responses to physiological and environmental changes. It regulates cell division and differentiation, embryogenesis, organogenesis, phototropic and gravitropic responses, and root and shoot architecture formation (Woodward and Bartel, 2005; Vanneste and Friml, 2009). Optimal post-embryonic root growth requires tight control of indole-3-acetic acid (IAA) activity, which can be regulated by diverse mechanisms including IAA biosynthesis, its transport, and signal transduction (Hayashi, 2012).

It has been demonstrated that the auxin signal transduction system operates via the SCF TIR1/AFB proteasome machinery (TIR1/AFB is TRANSPORT INHIBITOR RESPONSE1/AUXIN RECEPTOR-BOX), which plays a key role in the regulatory process of transcription and leads to auxin-related developmental responses (Chapman and
In this process, auxin promotes the degradation of auxin/indole-3-acetic acid (Aux/IAA) transcriptional repressors through the proteasome pathway by enhancing the ubiquitination of Aux/IAA proteins (Gray et al., 2001). Aux/IAAs are recognized as substrates and the F-box protein TIR1 is a component of the SCF<sup>TIR1/AFB</sup> type E3 ubiquitin ligase complex (Kepinski, 2007). Furthermore, TIR1 and five TIR1 homologue proteins (AFB1–AFB5) redundantly function as nuclear auxin receptors in Arabidopsis (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Parry et al., 2009; Greenham et al., 2011). Recent studies demonstrated that auxin acts as a ‘molecular glue’ in binding to TIR1 and stabilizing the interaction between TIR1 and Aux/IAA proteins (Santner and Estelle, 2009; Greenham et al., 2011). This interaction results in Aux/IAA ubiquitination and subsequent degradation by the 26S proteasome and therefore releases the AUXIN RESPONSE FACTOR (ARF) proteins to regulate the expression of target genes (Tan et al., 2007). Auxin rapidly alters the expression of hundreds of genes within minutes and early auxin-inducible genes are classified into three major families: SAUR (Small auxin up RNA), GH3 (Gretchen Hagen3), and AUX/IAA genes (Goda et al., 2008; Chapman and Estelle, 2009). The Arabidopsis Aux/IAA family comprises 29 members, which encode short-lived nuclear proteins that function as unstable repressors regulating auxin-inducible gene expression (Worley et al., 2000; Remington et al., 2004). When auxin levels are low, Aux/IAAs are relatively stable and able to exert repression on target genes. As auxin levels rise, TIR1-mediated proteolysis of Aux/IAAs relieves the repression, and target genes are expressed. The SCF regulatory proteins AXR1, ECR1, and RCE1 are involved in the RUB/NEDD8 conjugation of CUL1. Mutations in these components confer auxin-resistant phenotypes and result in defects in auxin-related developmental processes (del Pozo et al., 2002; Dharmasiri et al., 2003). Auxin signalling components have been conserved throughout the evolution of land plants and have proliferated and specialized to control specific developmental processes (Chapman and Estelle, 2009).

It has been shown that the second messenger cyclic guanosine 3',5'-monophosphate (cGMP) is an important signalling component with multiple biological functions in plants (Newton and Smith, 2004), similar to the situation in animals. cGMP has been detected in several plant species, including barley, mung bean, tobacco, soybean, and Arabidopsis (Newton and Smith, 2004), and the genome contains sequences suggesting the existence of plant cGMP-responsive kinases (Newton and Smith, 2004), and the Arabidopsis genome contains sequences that encode gene products with both a cyclic nucleotide-binding domain and a protein kinase (Meier and Gehring, 2006). However, specific cGMP targets in plants are largely unknown and in particular there is little molecular evidence available of bona fide cGMP-dependent kinases.

In plants, cGMP is involved in stress responses, seed germination (Teng et al., 2010), α-amylase production (Penson et al., 1996; Wu et al., 2013), stomatal movement (Dubovskaya et al., 2011; Joudoi et al., 2013), reorientation of pollen tube and cell polarity (Prado et al., 2004; Salmi et al., 2007), and anthocyanin and flavonoid biosynthesis (Bowler et al., 1994; Suita et al., 2009). Moreover, cGMP plays a role in plant responses to various phytohormones, including gibberellic acid, auxin, and abscisic acid (Penson et al., 1996; Pagnussat et al., 2003; Dubovskaya et al., 2011). In some of these responses, cGMP is the downstream molecule of phytohormone action and mediates phytohormone signalling, suggesting possible crosstalk between phytohormones and cGMP. In particular, cGMP is an important molecule in auxin-regulated signalling in determining root morphology during growth and development (Bai et al., 2012). cGMP accumulation was reported in response to auxin treatment during adventitious root formation and asymmetric cGMP accumulation in root tips during the gravitropic response (Pagnussat et al., 2003; Hu et al., 2005; Bai et al., 2012). Auxin-induced adventitious roots and root gravitropic responses were blocked by GC inhibitors 6-anilino-5,8-quinolinedione (LY83583) or 1H-[1,2,4]-oxadiazole-[4,3-a]-quinazolin-1-one (ODQ), suggesting a key role for endogenous cGMP in these processes (Pagnussat et al., 2003; Hu et al., 2005; Bai et al., 2012). However, the molecular mechanism of cGMP and auxin interaction in the root development of plant is still poorly understood.

In the study we used auxin-related mutants and transgenic plants to analyse how cGMP is involved in the auxin signalling and thus affects root growth and development in Arabidopsis. Our results demonstrated that cGMP modulated auxin-dependent gene expression and Aux/IAA protein degradation through the stimulation of PKG activity. The results presented here provide evidence for a link between the auxin signalling pathway and the cGMP signalling pathway.

### Materials and methods

**Plant materials, growth conditions, and chemicals**

The Arabidopsis mutants tir1-1 (Ruegger et al., 1998), axr1-3, and axr1-12 (Lincoln et al., 1990) and the transgenic lines DRS::GUS (Ulmosov et al., 1997), HS::AXR3NT-GUS, HS::axr3-1NT-GUS, and HS::GUS, myc-TIR1 (Gray et al., 2001) have been previously described, and all of them are in the Col-0 background. Seeds were sterilized with 1.5% NaClO for 15 min, washed with sterile water three times, placed in 4 °C for 3 days and then planted on

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**References**

1. **Gray et al.** 2001
2. **Kepinski** 2007
3. **Dharmasiri et al.** 2005a
4. **Kepinski and Leyser** 2005
5. **Parry et al.** 2009
6. **Greenham et al.** 2011
7. **Tan et al.** 2007
8. **Worley et al.** 2000
9. **Remington et al.** 2004
10. **Hu et al.** 2005
11. **Bai et al.** 2012
12. **Penson et al.** 1996
13. **Newton and Smith** 2004
14. **Suita et al.** 2009
15. **Teng et al.** 2010
16. **Salmi et al.** 2007
17. **Bowler et al.** 1994
18. **Pagnussat et al.** 2003
19. **Dubovskaya et al.** 2011
20. **Joudoi et al.** 2013
the half-strength Murashige and Skoog (1/2MS) medium (pH 5.8) containing 1% sucrose and 0.8% agar in the growth room at 23 °C under 100–120 μmol photons m⁻² s⁻¹ with a 16 h/8 h light/dark photoperiod.

In the study, LY83583 and ODQ were used as the GC inhibitors and 8-bromoguanosine 3’,5’-cyclic guanosine monophosphate (8-Br-cGMP) was used as a cell-permeable cGMP derivative; Rp-8-Br-cGMP, MG132, and 1-naphthylphthalalamic acid (NPA) were used as the PKG, proteasome, and auxin transport inhibitors, respectively. The above chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) except NPA (Chem Service, West Chester, PA, USA). They were contained in the medium for different treatments.

Phenotypic analysis and statistics

The length of the primary roots and lateral roots (LRs) was measured with NIH Image software (Image J, version 1.43). Emerged LRs and β-glucuronidase (GUS)-staining sites were counted using an anatomical lens. Root hairs were photographed with a Leica stereo microscope and the density was counted in a 2.5 mm region from the primary root tip.

GUS staining and quantitative GUS activity assays

GUS staining was carried out according to the methods described by Pelagio-Flores et al. (2011) with some modifications. Briefly, seedlings were fixed in 90% acetone at −20 °C for 1 h, washed twice in 50 mM sodium phosphate buffer (pH 7.0) and then incubated in GUS-staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 100 mM sodium phosphate (pH 7.5), 0.5 mM K₂[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆], 10 mM EDTA, and 0.1% Triton X-100. The seedlings were incubated at 37 °C for 6–18 h and then cleared using HCG solution (chloroacetalddehyde/water/glycerol = 8:3:1) for 12 h. Individual representative seedlings were photographed using a Leica Microsystems DM500B microscope.

Quantitative GUS activity assay was performed as described by Hu et al. (2012). Root samples were homogenized in GUS extraction buffer (50 mM potassium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, and 0.1% SDS). The extract was centrifuged at 12000 g for 15 min at 4 °C. The fluorogenic reaction was carried out in a reaction mixture containing 2 mM 4-methylumbelliferone (4-MU; Sigma-Aldrich) as a substrate and 0.1% Triton X-100. The reaction was incubated for 50 min at 37 °C and stopped with 0.2 M Na₂CO₃. Fluorescence was measured with excitation at 365 nm and emission at 455 nm on a Thermo Scientific NanoDrop 2000c spectrophotometer. Enzyme activity was calibrated by standard curve of 4-methylumbelliferone (4-MU; Sigma-Aldrich). Protein content was normalized according to the method of Bradford (1976).

Quantitative real-time PCR analysis

Total RNA was extracted with Trizol (TaKaRa) from roots, and then was treated with RNA-free DNase (Promega, Madison, WI, USA). First-strand cDNA was synthesized with the PrimeScript II 1st Strand cDNA Synthesis Kit (TaKaRa, Mountain View, CA, USA). Quantitative real-time PCR was performed using the SYBR PrimeScript RT-PCR Kit (Perfect Real Time; TaKaRa). PCR was performed using a CFX 96 Real-Time system (Bio-Rad, Hercules, CA, USA) with the following standard cycling conditions: 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The cycle threshold (Cₚ) was calculated using the ΔΔCₚ method. The specific primers for each gene are listed in Table S1. Expression levels of genes were normalized to ACTIN2 levels.

cGMP content and GC activity assay

For cGMP content assay, 200 μg roots were ground in liquid N₂. Then 1.5 ml of ice-cold 6% (v/v) trichloroacetic acid was added, and the homogenate was centrifuged at 1000 g for 15 min at 4 °C. The supernatant was extracted four times in five volumes of water-saturated diethyl ether. The aqueous extract was dried under a stream of N₂ at 60 °C and stored at −70 °C. The cGMP content was measured according to the manufacturer’s protocol of cGMP enzyme immunoassay kit (Sigma-Aldrich). The standard curve is presented in Tables S2 and S3 and Fig. S1.

For the GC activity assay, roots were homogenized in a medium containing 175 mM Tris/HCl (pH 7.9), 20 mM theophylline, and a protein inhibitor cocktail for plant cell and tissue extracts (SIGMA-Aldrich). The homogenate was centrifuged at 1300 g for 5 min at 4 °C. GC activity was measured by estimating the rate of cGMP formation from Mn²⁺-GTP in a reaction mixture containing 175 mM Tris/HCl (pH 7.9), 20 mM theophylline, 20 mM MnCl₂, 1 mM GTP, and 60 μg of total protein in final volume of 0.25 ml (Dubovskaya et al., 2011). The reaction mixture was incubated at 25 °C for 10 min. Then 0.25 ml of 0.2 M Na₂CO₃ was added and the solution was mixed, frozen at −70 °C, thawed, mixed again, and centrifuged at 6000 g for 10 min. cGMP content in the supernatant was measured using cGMP enzyme immunoassay kit.

Proteasome assay

The ATP-dependent 20S core unit activity of the 26S proteasome in 7-day-old wild-type (WT) seedlings was measured by peptide hydrolysis activity using succinyl-Leu-Leu-Val-Tyr-4-methyl-coumaryl-7-amide (Sigma-Aldrich) as the substrate with or without ATP and Mg²⁺ (Fujinami et al., 1994). Briefly, roots were homogenized with 50 mM Tris/HCl buffer (pH 8.0) containing 20 mM 2-mercaptoethanol. The extract was centrifuged at 12000 g for 15 min at 4 °C. The supernatant was added to the reaction solution (50 mM Tris/HCl buffer, 20 mM 2-mercaptoethanol, and 10 mM substrate with or without 4 mM ATP and 10 mM MgCl₂) and then the mixture was incubated for 50 min at 37 °C and stopped with 0.2 M Na₂CO₃. The fluorescence from the hydrolysed substrate was measured using a fluorometer (excitation 380 nm, emission 440 nm).

Yeast two-hybrid assay

The vectors and strains used for the yeast two-hybrid assay were provided in the Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Mountain View, CA, USA). The yeast two-hybrid assay was performed according to the Yeast Protocol Handbook (Clontech). The AD-IAA3, AD-IAA7, AD-IAA17, and BD-TIR1 plasmids were constructed by inserting PCR fragments of full-length cDNAs into the appropriate plasmids. The PCR fragments of IAA3, IAA7, IAA17, and TIR1 were amplified with specific primers containing an EcoRI or BamHI site. The resultant fragment was digested with EcoRI and BamHI, and cloned into pGAD7-p and pGBK7 to generate plasmid AD-IAA3, AD-IAA7, AD-IAA17, and BD-TIR1. The plasmids were co-transformed into yeast strain AH109. All primers used for yeast two-hybrid assays are listed in Table S1.

Pull-down assay

Pull-down assays with bacterially produced glutathione-S-transferase (GST)-IAA7/AXR2 were performed as described previously (Gray et al., 2001). Briefly, 100 μl of TIR1-Myc protein extracted from 7-day-old seedlings of the transgenic line tir1-1[TIR1-Myc] was incubated for 2.5 h at 4 °C with >50 μg of glutathione-agarose beads (Sigma-Aldrich) on which GST-IAA7/AXR2 protein was immobilized in 500 μl of reaction buffer (20 mM Tris pH 8.0, 200 mM NaCl, and 5 mM dithiothreitol) in the presence of the indicated compounds. After incubation at 4 °C the agarose beads were collected by brief centrifugation, washed three times, and suspended...
In SDS/PAGE sample buffer. The bound proteins were separated by SDS/PAGE and interacting TIR1-Myc was detected by immunoblotting with anti-Myc antibodies.

Statistical analysis
Each experiment was repeated at least three times. Values are expressed as mean ± SE. For all experiments the overall data were statistically analysed using SPSS version 17.0. All comparisons were performed using one-way analysis of variance with Duncan’s test or Tukey’s test for independent samples. In all cases the confidence coefficient was set at \( P < 0.05 \).

Results
Auxin increases the levels of endogenous cGMP in Arabidopsis roots
It has been indicated that cGMP is a secondary signal that acts in response to auxin stimulation and mediates auxin-induced adventitious root formation in mung bean and gravitropic bending in soybean. To further explore the mechanism of cGMP involvement in auxin signalling responses, we measured cGMP production in Arabidopsis roots. Results showed that cGMP levels were markedly induced in 5 \( \mu \)M auxin treatment for 10–120 min, and that they increased to 217.7% of the control after 5 \( \mu \)M auxin treatment for 120 min (Fig. 1A, B). To investigate how auxin increases the endogenous cGMP level, the GC activity was examined. As shown in Fig. 1B, IAA stimulated GC activity, and it increased from 116.2 to 330.7% of the control under 0.1–50 \( \mu \)M IAA treatments for 1h. These results suggested that IAA induces cGMP levels by stimulating the GC activity.

Auxin-induced development of the root system is cGMP-dependent in Arabidopsis
We next used 8-Br-cGMP, a cell-permeable cGMP derivative, and LY38538, a GC inhibitor, to investigate the roles of cGMP in auxin-induced root-system development in Arabidopsis roots, including the LR formation, root hair development, and the inhibition of primary root elongation. As shown in Fig. 2A and C, 0.1–5.0 \( \mu \)M IAA treatment for 5 days markedly increased LR density to 1.4–19 times of the control, respectively. The effects of IAA were obviously strengthened by co-treatment with 100 \( \mu \)M 8-Br-cGMP. In contrast, the LR density induced by auxin was completely suppressed by 20 \( \mu \)M LY38538 (Fig. 2A, C). Interestingly, we also observed that 8-Br-cGMP co-treatment with IAA increased the length of LRs and LY38538 co-treatment with IAA markedly decreased it in comparison with IAA treatment alone (Fig. 2A, D). However, there was no LR formation in seedlings treated alone with 8-Br-cGMP or LY38538 after another 5 days of treatment (data not shown).

To determine whether cGMP promotion of auxin-induced LR formation occurs prior to the emergence of LRs, we employed the DR5::GUS auxin-responsive reporter (Ulmasov et al., 1997) as a molecular probe. DR5::GUS is expressed in the dividing pericycle cells during LR initiation and indicates the initial cells where LR initiation occurs (Benkova et al., 2003). Our results showed that the number of IAA-induced LR primordium sites in DR5::GUS was significantly increased by 8-Br-cGMP and decreased by LY38538 (Fig. 2B). However, the application of 8-Br-cGMP or LY38538 alone did not produce any different effects for the number of DR5::GUS sites compared to controls. Taken together, these results indicate that cGMP is required for auxin-stimulated LR formation as well as LR initiation.

The root hair density and the root hair length also dramatically increased under 0.1 \( \mu \)M 1-naphthaleneacetic acid (NAA) treatments for 24–48 h (Fig. 3). Application of 8-Br-cGMP or LY38538 in combination with NAA treatment dramatically enhanced or inhibited the auxin-induced development of root hairs, including auxin-stimulated initiation and elongation of root hairs compared with the NAA treatment alone (Fig. 3). 8-Br-cGMP treatment alone exhibited a weak increase in root hair density, but not in root hair length (Fig. 3A–C). These results indicate that cGMP is also involved in auxin-regulated root hair development.

As shown in Fig. 4, 100 \( \mu \)M 8-Br-cGMP treatment alone for 3 days slightly increased the Arabidopsis primary root elongation (by 122.4%) compared with the control, whereas 2.5 \( \mu \)M LY38538 significantly suppressed it (by 53.3%). 8-Br-cGMP treatment partially reversed the effect of LY38538, suggesting that cGMP is essential for the normal growth of Arabidopsis.
Fig. 2. Effects of cGMP on auxin-induced LR formation. (A) Photographs of LR formation in WT seedlings after 5 days and (B) the number of DR5::GUS-staining sites in the transgenic line DR5::GUS for 2 days under 1 μM IAA, 100 μM 8-Br-cGMP, and 20 μM LY83583 treatments as indicated. (C) Changes in LR density and (D) average LR length under 0.05–5.0 μM IAA, IAA plus 100 μM 8-Br-cGMP, and IAA plus 20 μM LY83583 treatments after 5 days in WT seedlings. WT and DR5::GUS seedlings were grown on medium containing 0.5 μM NPA for 5 days to repress LR initiation, and then they were treated with IAA, 8-Br-cGMP, or LY83583. Mean values and SE were calculated from three independent experiments (n = 12). Within each set of experiments, bars with different letters were significantly different at the 0.05 level. Scale bar in A, 250 mm. This figure is available in colour at JXB online.

Fig. 3. Effects of cGMP on the auxin-induced root hair development in WT seedlings. (A) Photographs of root hairs formed under various treatments for 36 h in the primary root tip. Scale bar, 500 μm. (B) The changes of root hair density and (C) root hair length under various treatments for 24–48 h. The 5-day-old seedlings were transferred onto vertical plates containing 0.1 μM NAA, 100 μM 8-Br-cGMP, or 20 μM LY83583 for the various treatments. Mean values and SE were calculated from three independent experiments (n = 20). Within each set of experiments, bars with different letters were significantly different at the 0.05 level. This figure is available in colour at JXB online.
primary roots (Fig. 4B). The present data also showed that 10–100 nM IAA treatment markedly reduced primary root elongation (86.9–21.8% of the control) and that application of 8-Br-cGMP or LY83583 rescued or aggravated the action of IAA (Fig. 4A, B). Furthermore, responses induced by LY83583 plus IAA were partially reversed by co-treatment with 8-Br-cGMP (Fig. 4B). Together, the pharmacological data revealed that cGMP plays an important role in auxin-induced root-system development in Arabidopsis roots.

Effects of cGMP on the auxin-inhibited primary root elongation in auxin-related Arabidopsis mutants

To evaluate the genetic mechanism of cGMP responses, we compared the primary root growth of WT (Col-0) seedlings and the tir1-1, axr1-3, and axr1-12 Arabidopsis mutants. TIR1 is an auxin receptor, and Arabidopsis tir1-1 mutant shows reduced sensitivity to auxin (Ruegger et al., 1998); axr1 (auxin-resistant 1) mutants also exhibit a severe reduction in auxin response (Lincoln et al., 1990). As shown in Fig. 5, exogenous 2,4-dichlorophenoxyacetic acid (2,4-D) markedly inhibited the primary root elongation (39.1% of the control) in WT (Col-0) seedlings. Furthermore, application of 8-Br-cGMP alleviated (increased by 31.7% of the 2,4-D treatment) the inhibition effect of 2,4-D in primary root elongation while LY83583 reinforced (reduced by 45.2% of the 2,4-D treatment) such inhibition (Fig. 5). Conversely, exogenous 8-Br-cGMP treatment did not relieve 2,4-D-inhibited primary root elongation in these mutants; moreover, these mutants also displayed reduced sensitivity to LY83583. Primary root elongation only decreased by 36.3, 10.7, and 18.1% under 2,4-D plus LY83583 treatment in tir1-1, axr1-3, and axr1-12 mutants, respectively, compared with 2,4-D treatment alone (Fig. 5). TIR1 and AXR1 are the regulatory components of the SCFTIR1/AFB complex (Schenck et al., 2010), suggesting that the effect of cGMP on auxin-dependent primary root elongation might be involved in the process of SCFTIR1/AFB signalling.

cGMP enhances the expression of primary auxin-responsive genes

To gain additional insights into the roles of cGMP in auxin signalling responses we employed the transgenic line DRS::GUS, which carries auxin-response elements fused to the β-glucuronidase-encoding gene (GUS). GUS was expressed poorly under a low concentration of IAA (5 nM) treatment as well as with 8-Br-cGMP treatment alone (Fig. 6A). However, the simultaneous application of IAA and 8-Br-cGMP significantly promoted GUS gene expression, and 8-Br-cGMP increased the effect of IAA in a dosage-dependent manner (Fig. 6A). Both LY83583 and ODQ, a GC inhibitor, effectively inhibited the GUS activity induced by 50 nM IAA (Fig. 6B). In addition, the transgenic lines IAA12::GUS and IAA13::GUS (Weijers et al., 2005), in which GUS was driven by native auxin-inducible promoters, were also selected. As shown in Fig. 7, the GUS activity showed a similar profile as in Fig. 6 under different treatments in roots for IAA12::GUS and IAA13::GUS seedlings. Furthermore, the mode of GUS expression was further confirmed by quantification (Figs 6C and 7B).
In order to clarify the role of cGMP in the expression of primary auxin-responsive genes, a set of auxin-response genes (IAA5, IAA11, IAA19, SAUR9, GH3.3, and GH3.5) was selected and analysed in Arabidopsis roots. With 1 μM IAA treatment for 6 h the expression of these genes quickly increased (Fig. 8). The co-treatment of IAA and 8-Br-cGMP led to more marked increase of the expression of these genes in comparison with the IAA treatment alone (Fig. 8). However, the IAA-induced gene expression was effectively inhibited by the addition of LY83583 (Fig. 8). These results suggested that cGMP is indeed required for the expression of primary auxin-responsive genes.

**cGMP stimulates auxin-induced Aux/IAA degradation via the SCFTIR1 complex**

Auxin-mediated gene expression is regulated via degradation of Aux/IAA repressors (Gray et al., 2001). Thus, the stability of the reporter protein AXR3NT-GUS was investigated using the Arabidopsis transgenic line HS::AXR3NT-GUS under various treatments. This reporter is a fusion of the N-terminus of the Aux/IAA protein AXR3/IAA17 (AXR3NT) and GUS under the control of a heat-shock inducible promoter (HS) (Gray et al., 2001). As shown in Fig. 9A, 50 nM IAA caused a decrease in the AXR3NT-GUS stability in both leaves and roots of HS::AXR3NT-GUS plants, which was substantially enhanced by 8-Br-cGMP (Fig. 9A). Moreover, seedlings treated with LY83583 or ODQ in combination with high IAA concentration (1 μM) exhibited much stronger GUS staining (Fig. 9B). To further confirm these results, two control lines, HS::axr3-1NT-GUS, in which the mutation in domain II of AXR3 results in an increased stability of the protein, and HS::GUS transgenic line were used. In contrast to HS::AXR3NT-GUS line, the GUS activity in HS::axr3-1NT-GUS and HS::GUS was unaffected by 8-Br-cGMP or LY83583 treatment in the presence or absence of auxin (Fig. 9C, D). These results suggested that cGMP might be involved in the auxin-mediated degradation of Aux/IAA proteins through SCFTIR1 proteasome pathway.

Pretreatment with 50 μM MG132 (the proteasome inhibitor) inhibits the degradation of the AXR3NT-GUS fusion protein and ubiquitin-ligase complex SCFTIR1/AFB-dependent responses (Dharmasiri et al., 2005b; Robert et al., 2010). Hence, we further investigated whether the roles of cGMP on auxin-induced degradation of Aux/IAA proteins were related to the 26S proteasome and then the ATP-dependent proteasome activity was determined using a fluorogenic peptide substrate (Fujinami...
et al., 1994) in Arabidopsis roots. As shown in Fig. 10, proteasome activity was significantly promoted (increased to 129.9%) by 8-Br-cGMP but seriously inhibited (decreased to 41.6%) by LY83583 in the presence of ATP (Fig. 10A). As a positive control, 50 µM MG132 completely repressed the activity of the 26S proteasome (Fig. 10A). These results indicated that cGMP on auxin-induced degradation of Aux/IAA proteins might be achieved by regulating 26S proteasome activity. However, subsequent results showed that application of MG132 reduced auxin-induced LR formation, but it did not alter the effect of 8-Br-cGMP or LY83583 (Fig. 10B). Therefore, we propose that cGMP promotes auxin-induced LR formation by a proteasome-independent mechanism.

The effect of cGMP on the auxin-enhanced TIR1–Aux/IAA interaction

Auxin treatment stimulates the interaction between SCF^{TIR1} and Aux/IAA proteins and promotes their degradation via the 26S proteasome, therefore inducing the expression of primary auxin-responsive genes (Gray et al., 2001; Kepinski and Leyser, 2005). To analyse whether cGMP affects the auxin-dependent TIR1–Aux/IAA interaction, we performed GST pulldown experiments using IAA7/AXR2 and Myc-tagged TIR1 proteins. However, results showed that 8-Br-cGMP was not able to stimulate the interaction of TIR1-Myc and GST-IAA7/AXR2. Furthermore, LY83583 also did not block the IAA-enhanced interaction between TIR1-Myc and GST-IAA7/AXR2 (Fig. 11A). Next, to further verify the above conclusion, we investigated the effect of cGMP on in vivo TIR1–Aux/IAA interactions using the GAL4 two-hybrid system (Bian et al., 2012). In yeast cells 8-Br-cGMP was also unable to influence the interaction of TIR1 with IAA/AUXs (IAA3, IAA7, and IAA17) compared with IAA (Fig. 11B). Altogether, in vitro and in vivo experiments indicated that cGMP is unable to directly influence TIR1–Aux/IAA interactions.
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As reported previously, PKG, consisting of a cyclic nucleotide-binding domain and a protein kinase domain, is a typical downstream cellular target or effector of cGMP modulation in animals and plants (Newton and Smith, 2004; Maathuis, 2006). Therefore, cGMP may affect auxin signalling via PKG action. To address this possibility we used Rp-8-Br-cGMP, a putative inhibitor of PKG. This reagent selectively inhibits the PKG activity in animal cells and auxin-induced stomatal opening in Arabidopsis (Butt et al., 1990; Cousson, 2010). As shown in Fig. 12A and B, we observed that pretreatment with 100 μM Rp-8-Br-cGMP blocked expression – induced by either IAA alone or IAA plus 8-Br-cGMP – of these auxin-responsive genes, including DR5::GUS reporter, IAA5, and IAA11. In addition, it also seriously abolished the degradation – induced by IAA alone or IAA plus 8-Br-cGMP – of the AXR3NT-GUS fusion protein (Fig. 12C). These results strongly suggest that cGMP-mediated modulation of auxin signalling is dependent on the PKG activity in Arabidopsis.

Discussion

Previous studies have demonstrated that the plant growth regulator auxin and the second messenger cGMP coordinate several developmental and physiological processes, including adventitious root development and stomatal opening (Meier and Gehring, 2006). In this study we have provided evidence that cGMP, as a positive modulator, is involved in the auxin-regulated signalling response in Arabidopsis roots, which depends on PKG activity.

As previously reported in other plant species, our results showed that IAA induces endogenous cGMP accumulation in Arabidopsis roots (Fig. 1A). A recent study using the fluorescent reporter FlincG as an endogenous cGMP sensor also showed that auxin rapidly increased cellular CGMP within a short period of time in Arabidopsis protoplast (Isner et al., 2012), and which was consistent with our results. Moreover, IAA could markedly induce GC activity in a concentration-dependent manner in Arabidopsis roots (Fig. 1B).
A and B seedlings were pretreated with 100 μM Rp-8-Br-cGMP for 0.5 h seedlings. In HS AXR3NT-GUS::IAA11, normalized to genes (IAA5, ACTIN2). Quantitative real-time PCR analysis of the expression of auxin-responsive genes. (B) DR5 protein. (A) GUS activity analysis in 5-day-old WT seedlings, showing 8-Br-cGMP increased and LY83583 was effectively inhibited by treatment with the GC inhibitors ODQ or LY83583 (Fig. 6). Furthermore, this phenomenon was also supported by the native auxin-inducible promoters IAA12::GUS and IAA13::GUS (Fig. 7) and the mRNA levels of primary auxin-induced genes (Aux/IAAs, GH3s, and SAURs) in Arabidopsis roots (Fig. 8). Therefore, in agreement with the physiological data, these results clearly indicated that cGMP is also required for expression of auxin-responsive genes. It has been reported that Arabidopsis F-box proteins TIR1/AFB are auxin receptors that mediate degradation of Aux/IAA repressors, and therefore cGMP may act by affecting Aux/IAA protein degradation, thus explaining the effects of cGMP on the expression of primary auxin-responsive genes. To test this possibility we used the HS::AXR3NT-GUS transgenic line, which strongly expresses an AXR3/IAA17 translational fusion protein under the control of a heat-shock promoter, to analyse the effect of cGMP on auxin-induced degradation of Aux/IAA proteins (Gray et al., 2001). We found that 8-Br-cGMP accelerated the degradation rate of the Aux/IAA fusion protein caused by IAA treatment (Fig. 9A). In contrast, treatment with LY83583 or ODQ abolished degradation of the fusion protein in the presence or absence of IAA (Fig. 9B). These results suggested that cGMP might regulate the expression of primary auxin-responsive genes by activating auxin-induced Aux/IAA degradation.

It has been reported that cGMP accumulation was attributable to activation of GC rather than 3′,5′-cyclic-cGMP phosphodiesterase in ABA-mediated stomatal movement (Dubovskaya et al., 2011). These results suggest that auxin could increase the endogenous cGMP levels by affecting GC activity in Arabidopsis roots.

Auxin is a key phytohormone involved in a broad spectrum of developmental and physiological processes in plants, where it notably contributes to the regulation of root-system architecture remodelling (Woodward and Bartel, 2005; Vanneste and Friml, 2009). In this study, the LR density induced by auxin was obviously strengthened by 8-Br-cGMP and completely suppressed by LY83583, suggesting that cGMP is required for auxin-stimulated LR formation (Fig. 2). This was further confirmed by the assay of stained LR sites using GUS in the transgenic line DR5::GUS (Fig. 2B). These results agreed with the previous conclusion that cGMP is involved in auxin-mediated adventitious root formation (Pagnussat et al., 2003; Bai et al., 2012). Furthermore, we presented evidence that cGMP is also involved in auxin-regulated root hair development (Fig. 3) and primary root growth (Figs 4 and 5). The root-elongation assay showed that cGMP could rescue the inhibition of primary root growth by exogenous auxin while LY83583 exacerbated it. However, the phenomenon was not obviously observed in the tir1-1, axr1-3, and axr1-12 mutants (Figs 4 and 5). Moreover, axr1-3 and axr1-12 mutants exhibited fewer LRs and reduced root hair formation (del Pozo et al., 2002; Swarup et al., 2002), suggesting that cGMP might modulate auxin-induced root growth by affecting the auxin signal response.

Auxin regulates plant development by inducing rapid cellular responses and changes in gene expression (Gray et al., 2001). Here, we found that 8-Br-cGMP enhanced auxin-induced expression of the auxin reporter gene DR5::GUS; this was effectively inhibited by treatment with the GC inhibitors ODQ or LY83583 (Fig. 6). Furthermore, this phenomenon was also supported by the native auxin-inducible promoters IAA12::GUS and IAA13::GUS (Fig. 7) and the mRNA levels of primary auxin-induced genes (Aux/IAAs, GH3s, and SAURs) in Arabidopsis roots (Fig. 8). Therefore, in agreement with the physiological data, these results clearly indicated that cGMP is also required for expression of auxin-responsive genes. It has been reported that Arabidopsis F-box proteins TIR1/AFB are auxin receptors that mediate degradation of Aux/IAA repressors, and therefore cGMP may act by affecting Aux/IAA protein degradation, thus explaining the effects of cGMP on the expression of primary auxin-responsive genes. To test this possibility we used the HS::AXR3NT-GUS transgenic line, which strongly expresses an AXR3/IAA17 translational fusion protein under the control of a heat-shock promoter, to analyse the effect of cGMP on auxin-induced degradation of Aux/IAA proteins (Gray et al., 2001). We found that 8-Br-cGMP accelerated the degradation rate of the Aux/IAA fusion protein caused by IAA treatment (Fig. 9A). In contrast, treatment with LY83583 or ODQ abolished degradation of the fusion protein in the presence or absence of IAA (Fig. 9B). These results suggested that cGMP might regulate the expression of primary auxin-responsive genes by activating auxin-induced Aux/IAA degradation.
strongly suppressed the ATP-dependent proteasome activity in *Arabidopsis* roots, respectively (Fig. 10A). These results further confirmed that cGMP acted on the auxin signalling pathway through the SCF<sup>TIR1</sup>-mediated degradation of Aux/IAAs. However, we found that repression of the proteasome activity using MG132 could not block the action of cGMP on IAA-induced LR formation in *Arabidopsis* roots, suggesting that cGMP promotes auxin-induced LR formation by a proteasome-independent mechanism (Fig. 10B). These seemingly paradoxical results of cGMP action are similar to nitric oxide (NO), which was reported to be involved in the auxin signalling through Aux/IAA degradation (Terrile et al., 2012), whereas it promotes reduction of PIN1 protein levels by a proteasome-independent mechanism (Fernández-Marcos et al., 2011). In addition, we also noticed that the inhibition of proteasome activity could not entirely repress the effect of auxin on LR formation (Fig. 10B), suggesting that the effect of cGMP on auxin-induced LR formation might be involved much more complicated mechanisms. Taking into account all these findings, we propose that cGMP might operate in multiple ways, including the dependent as well as independent regulation of proteasome-dependent Aux/IAA ubiquitination and subsequent degradation.

TIR1 as the auxin-recognition component of the SCF complex that interacts with Aux/IAA proteins to target them for proteolysis has been illustrated in detail (Gray et al., 2001; Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Recent work has shown that the amount of endogenous TIR1 protein appeared to be rate-limiting for auxin response and excess TIR1 protein in 35S::TIR1 even led to the degradation of Aux/IAAs in the absence of auxin treatment (Maraschin et al., 2009). In addition, the discovery that inositol hexakisphosphate is associated with the TIR1 protein (Tan 2009) has shown that TIR1 is a member of a small gene family between TIR1 and its ligand, auxin (Fig. 11). Previous studies have shown that TIR1 is a member of a small gene family that contains five additional AFB proteins that all function as auxin receptors (Dharmasiri et al., 2005b; Greenham et al., 2007). The functional defects of TIR1 protein evoke the reduction in auxin response. Moreover, the SCF complex mutants axr1-3 and axr1-12 also showed alteration of responses to auxin (del Pozo et al., 2002; Swarup et al., 2002) and Aux/IAA proteins exhibit increased stability in axr1 and tir1 mutants (Gray et al., 2001). In addition, our results showed that the tlr1-1, axr1-3, and axr1-12 mutants displayed reduced sensitivity to LY83583 and 8-Br-cGMP on auxin-inhibited primary root elongation (Fig. 5), suggesting that cGMP might be involved in SCF<sup>TIR1/AFB</sup> signalling. Thus, it is possible that cGMP alters the interaction of other AFB proteins with Aux/IAA proteins or TIR1–Aux/IAA interaction via downstream effectors of cGMP signalling, such as PKG.

It is well known that PKG is also a regulator of protein activation. Thus, it is plausible that cGMP influences TIR1–Aux/IAA interaction through the PKG and that PKG is able to modify TIR1 activity, although findings from this study cannot directly prove this issue. However, it is worth addressing in a future work. In order to further test the hypothesis, the putative inhibitor of PKG, Rp-8-Br-cGMP, was used to examine whether the cGMP-mediated expression of auxin-responsive genes and degradation of the AXR3NT-GUS fusion protein is dependent on PKG activity. As expected, our data showed that the inhibition of PKG activity strongly blocked the auxin responses (Fig. 12), suggesting that cGMP might influence auxin signalling in *Arabidopsis* roots through PKG activity. This conclusion is in agreement with previous findings that cGMP as a mediator participates in photoperiodic flowering induction in *Pharbitis nil* (Szmidt-Jaworska et al., 2009) and auxin-induced stomatal opening in *Arabidopsis* via its influence on PKG activity (Cousson, 2010). In addition, it has been demonstrated that AGC kinases are required for auxin-related processes such as auxin-mediated root development and organogenesis in *Arabidopsis* (Galván-Ampudia and Offringa, 2007; Cheng et al., 2008). PKG is a component of AGC kinases, which further complement our conclusion that the cGMP-mediated auxin response is dependent on PKG activity.

In summary, our results have revealed a new aspect of cGMP signalling, and explained the mechanism of the
involvement of cGMP in the auxin signalling pathway in Arabidopsis root development. We propose one possible model in Fig. 13. According to this model, although the direct cellular targets of cGMP action remain unknown, several lines of evidence indicate that treatment with auxin in the roots can rapidly induce the accumulation of cGMP by stimulating GC activity; cGMP then influences the auxin-dependent SCF\textsuperscript{TIR1} complex through PKG action, which results in Aux/IAA degradation, facilitates activation of gene expression, and finally affects auxin-regulated root growth. It is well known that cGMP is an important component of NO signalling and a number of NO-regulated physiological processes may be mediated by GC (Wu \textit{et al.}, 2013). Recently, similar to cGMP, Terrile \textit{et al.} (2012) reported that NO and its S-nitrosylation are involved in auxin signalling and regulate auxin-dependent gene expression, Aux/IAA protein degradation, and TIR1–Aux/IAA interaction (Fig. 13). However, whether they share the signalling pathway or have more complicated regulation mechanisms in auxin signalling needs future investigation.

Supplementary material

Supplementary material is available at \textit{JXB} online.

Table S1. Sequences of primers used in the study.

Table S2. The OD values of the control group in cGMP detection.

Table S3. The values of the standard curve in cGMP detection.

Figure S1. The standard curve used for cGMP detection.

Figure S2. Change of endogenous cGMP levels after various treatments in roots of 7-day-old WT seedlings: 100 μM 8-Br-cGMP, 20 μM LY83583, and 5 μM IAA were used for various treatments for 1 h. For IAA plus LY83583 treatment, seedlings were pretreated with LY83583 for 10 min and then treated with IAA plus LY83583 for 1 h. Mean values and SE were calculated from three independent experiments.

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