cGMP is required for seed germination in Arabidopsis thaliana

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ABSTRACT

Cyclic guanosine 3′,5′-monophosphate (cGMP) is an important second messenger in animals, and is emerging as a player in regulatory functions in plants. In this study, we investigated the role of cGMP in seed germination in Arabidopsis thaliana (Col-0). We demonstrated that both, a membrane-permeant analogue of cGMP (8-Br-cGMP) and the cyclic nucleotide phosphodiesterase (PDE) inhibitor Tadalafil promoted A. thaliana seed germination, whereas the guanylate cyclase inhibitor LY 83583 (6-anilino-5,8-quinolinedione; LY) inhibited it. LY blocked gibberellic acid (GA)-induced seed germination, whereas GA and 8-Br-cGMP co-treatment increased the germination rate and more effectively overcame LY-inhibition than 8-Br-cGMP alone. The giberrellin biosynthesis inhibitor paclobutrazol (PAC) also blocked 8-Br-cGMP and Tadalafil promotion of seed germination. Furthermore, 8-Br-cGMP and Tadalafil decreased abscisic acid (ABA) sensitivity during seed germination. These findings highlight that cGMP is a positive regulator and plays a crucial role in Arabidopsis seed germination. Furthermore, both GA and cGMP are required for seed germination.

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Introduction

Cyclic nucleotide monophosphates (cNMPs) play important roles as signal intermediaries in animals and microorganisms. As a second messenger, guanosine 3′, 5′-cyclic monophosphate (cGMP) regulates complex signaling cascades through immediate downstream effectors. cGMP functions by regulating cGMP-dependent protein kinases, cGMP-regulated phosphodiesterases, and cyclic nucleotide-gated ion channels (Lucas et al., 2000). The cGMP signaling pathway and its feedback regulation are affected by a combination of cGMP synthesis and degradation. Guanylyl cyclases (GCs) are key enzymes in the generation of the cGMP signaling pathway, which converts guanosine-5′-triphosphate (GTP) into cGMP. The GC family comprises both membrane-bound and cytosolic isoforms that are present in nearly all cells. The membrane-bound homodimer, also known as particulate guanylyl cyclase (pGC), serves as a natriuretic peptide (NP) receptor, and the soluble guanylyl cyclase (sGC) is the main receptor of nitric oxide (NO) (Pyriochou and Papapetropoulos, 2005). A GC family member that is also a crucial regulator of cGMP signaling may hydrolyze cGMP. In mammals, cyclic nucleotide phosphodiesterases (PDEs) comprise 11 family members, of which members 5, 6, and 9 specifically degrade cGMP into inactive 5′-nucleotide monophosphate GMP, hence decreasing the endogenous cGMP level (Maurice et al., 2003).

During the last decade, research has revealed that cGMPs also function in higher plants. cGMP has been detected in several plant species, including barley, tobacco, oat, Pharbitis nil and Arabidopsis thaliana (Donaldson et al., 2004; Dubovskaya et al., 2001; Durner et al., 1998; Penson et al., 1996; Szmidt-Jaworska et al., 2004; Wang et al., 2007). Moreover, there are four reports on the cloning of putative cyclases from plants (Kwezi et al., 2007; Ludidi and Gehring, 2003; Szmidt-Jaworska et al., 2009a; Yuan et al., 2008) and some accounts of GC activity (Newton et al., 1984; Szmidt-Jaworska et al., 2008a; 2009b; Volotovski et al., 2003), implying that plant has the capacity to synthesize cGMP. cGMP signaling has also been shown to function in many plant physiological processes and molecular events, such as cGMP-mediated gene expression (Maathuis, 2006), NO-cGMP signal transduction (Prado et al., 2004), modulation of ion channels by cGMP (Hoshi, 1995; Pharmawati et al., 1999), and cGMP-induced stomatal opening (Pharmawati et al., 2001). Furthermore, there is evidence demonstrating that cGMP is involved in phytochrome- and phytohormone-dependent processes (Bowler et al., 1994). cGMP responds to light and is required for anthocyanin biosynthesis; together with Ca2+, it activates the full development of chloroplasts (Bowler et al., 1994). In addition, it has been reported that the phytochrome-generated repressor is regulated by both cGMP and Ca2+ (Neuhaus et al., 1997). Similarly, cGMP has been shown...
to play a role in induction of phytochrome-controlled flowering (Szmidt-Jaworska et al., 2008b).

In addition to the involvement in mediated phytochrome responses, cGMP also functions in hormone signaling. It has been shown that gibberellic acid (GA) can generate a transient increase in cGMP levels and that cGMP is required for GA-induced α-amyrase production in barley aleurone layers (Penson et al., 1996). In addition to GA, cGMP signals interact with another plant hormone, abscisic acid (ABA). For instance, cGMP promotes stomatal opening, which is induced by NP (Pharmawati et al., 2001), whereas the ABA-induced stomatal closure of *T. multiflora* is attenuated by AtPNP-A, which is an NP that has been characterized in *A. thaliana* (Wang et al., 2007). These findings indicate that cGMP signaling may also interact with ABA signaling cascades.

Seed germination, the first stage in the life cycle of plants, is regulated by several environmental conditions that eventually determine the relative levels of phytohormones. Genetic and physiological evidences strongly indicate that GA and ABA exert antagonistic effects on *Arabidopsis* seed dormancy and germination. GA is a positive regulator of seed germination, whereas ABA is essential for the establishment and maintenance of seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Koornneef et al., 2002).

Given the close relationship between seed germination and phytohormones and interactions between the phytohormones and cGMP, it is conceivable that cGMP may be involved in regulation of seed germination by interacting with the phytohormones such as GA and ABA. However, there has been no detailed study to evaluate the role of cGMP in regulation of seed germination in terms of its interaction with GA and ABA in the literature. In the present study, we report that cGMP is a positive regulator of *Arabidopsis* seed germination. Further, we demonstrate that both GA and ABA are involved in the modulation of seed germination by cGMP.

**Materials and methods**

**Plant material**

Seeds of wild-type *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia (Col-0) were used in all of our experiments. Seeds were stratified at 4°C in the dark for 2 d and sown on moist soil in pots covered with plastic wrap and maintained at 22°C in a growth room. After germination, the plastic wrap was removed. Seeds were harvested from plants after about 10 weeks, dried 7 d at room temperature, and stored at room temperature for more than 6 months for full post-harvest after-ripening.

**Germination assays**

In all of our experiments, seeds were surface-sterilized by treatment with Triton X100 (Amresco, Solon, OH, USA) for 10 min and subsequent immersion in 10% NaNCl (v/v) for 12 min, followed by five rinses with sterile water. The seeds were sown on solidified 1/2 MS salts (Murashige and Skoog, 1962) without sucrose, pH 5.95, and 8% agar. The plates were stratified at 4°C in the dark for 2 d to synchronize germination and then placed in a growth chamber at 22°C on a 16 h light/8 h dark regime to facilitate germination.

Controls were germinated on medium containing 0.2% DMSO (v/v). For hormone treatments, GA3 (Sigma-Aldrich, St. Louis, MO, USA) and ABA (Sigma-Aldrich) were dissolved in 95% ethanol (v/v) as a 250 mM stock. The applied concentrations of GA3 were 1, 10 and 100 and 1 μM in the case of ABA. PAC (Sigma-Aldrich) was dissolved in acetone as a 5 mM stock solution and applied as 5 μM.

Tadalafil, 8-Br-cGMP (Sigma-Aldrich) and guanylate cyclase inhibitor 6-anilino-5, 8-quinolinedione (LY 83583; LY; Sigma-Aldrich) were dissolved in DMSO as 10 mM stock solutions. Seeds were plated on medium supplemented with 8-Br-cGMP at 0.1, 1, 10 and 100 μM, Tadalafil at 0.1, 1 and 10 μM and LY at 20 μM. All stock solutions were vacuum-filtered and stored at −20°C. The tested substances were added to the media before use.

Germination, defined as the emergence of a radicle from the seed, was scored at 24, 36, 48, 72, and 96 h, depending on the type of experiment. Each experiment was repeated at least three times, with approximately 25 seeds per treatment.

**Results**

**cGMP promotes seed germination**

To determine whether *A. thaliana* seed germination was affected by cGMP, we grew seeds in different concentrations of 8-Br-cGMP, a membrane-permeable analogue of cGMP. As shown in Fig. 1A, 8-Br-cGMP promoted seed germination; even low concentrations of 8-Br-cGMP (0.1, 1 μM) had a profound effect.

Tadalafil (Cialis), a long-acting competitive inhibitor of PDE5, can elevate the intracellular cGMP concentration (Eardley and Cartledge, 2002). We tested the effect of Tadalafil on *Arabidopsis* seed germination. As shown in Fig. 1B, the germination percentage of seeds treated with a series of Tadalafil concentrations was significantly higher than those of the control after 24 h incubation. Interestingly, the most effective concentration for stimulation of seed germination was found to be 0.1 μM, whereas higher concentrations were less effective.

**GA3 requires cGMP to rescue LY 83583-induced inhibition of seed germination**

As GA plays an essential role in *Arabidopsis* seed germination (Ogawa et al., 2003), we investigated whether GA-induced germination is associated with cGMP by using LY 83583, a SGC inhibitor that can lower the endogenous cGMP level. The inhibitory effect of LY in germination was restored by both 8-Br-cGMP and Tadalafil after 24 h incubation (Fig. 2A, B).

While cGMP rescued LY-induced germination inhibition, GA3 did not mitigate the LY-induced inhibition of germination (Fig. 2C). In contrast, treatment of seeds with 8-Br-cGMP and GA3 together remarkably alleviated the inhibitory effect of LY (Fig. 2D). The effect was better than treatment with 8-Br-cGMP alone. This finding indicated that GA can rescue LY-induced germination inhibition and promote seed germination in the presence of 8-Br-cGMP.

**The promotion of seed germination by cGMP also requires GA**

Since exogenous GA requires a basal level of cGMP to rescue LY-inhibited germination, we wondered if the same is true for endogenous molecules. Hence, we tested the abilities of GA3, 8-Br-cGMP, and Tadalafil to reverse the germination inhibition induced by paclobutrazol (PAC), a GA biosynthesis inhibitor (Hedden and Graebe, 1985).

Based on previous experimental results, we chose concentrations of 0.1 μM 8-Br-cGMP and 1 μM Tadalafil for incubations. Incubation with 5 μM PAC for 24 h almost completely inhibited seed germination. In contrast, treatment with GA resulted in a
35% increase in germination percentage, but neither 8-Br-cGMP nor Tadalafil had a promoting effect (Fig. 3). However, after 36 h of incubation, PAC-induced germination inhibition was alleviated by the accumulation of endogenous GA, and both 8-Br-cGMP and Tadalafil showed significant promoting effects again at that time.

cGMP reduces the sensitivity of seed germination to ABA

ABA plays a crucial role in seed dormancy and suppression of seed germination, and acts as an antagonist of GA (Koornneef et al., 2002). Having demonstrated that cGMP stimulated Arabidopsis seed germination, we next examined the effect of cGMP on seed germination in the presence of ABA. As shown in Fig. 4, ABA at 1 μM reduced the germination percentage from 57% to 10%, whereas the addition of 100 μM GA₃, 0.1 μM 8-Br-cGMP, or 0.1 μM Tadalafil increased the germination percentage approximately to 23%, 36%, and 25% compared with ABA treatment alone. Incubation for 48 or 72 h with GA₃, as well as 8-Br-cGMP or Tadalafil also resulted in a higher germination percentage than that observed for ABA-treated seeds.

**Discussion**

In this study, we demonstrated that seed germination in Arabidopsis was regulated by cGMP such that exposure to 8-Br-cGMP and Tadalafil increased seed germination (Fig. 1). We found that seed germination was inhibited by exposure to LY and that this inhibition was partially reversed by 8-Br-cGMP and Tadalafil (Fig. 2A, B), suggesting that the inhibitory effect of LY on Arabidopsis seed germination is specific and functions mainly by
indicates significance at $P < 0.05$ ($t$-test).

Fig. 3. Effect of GA$_3$/8-Br-cGMP/Tadalafil on paclobutrazol (PAC)-induced inhibition of Arabidopsis thaliana seed germination, scored 24, 36 and 48 h after transfer to a growth chamber. Seeds were incubated with PAC (5 $\mu$M), PAC (5 $\mu$M)/GA$_3$ (100 $\mu$M), PAC (5 $\mu$M)/8-Br-cGMP (0.1 $\mu$M) and PAC (5 $\mu$M)/Tadalafil (1 $\mu$M). Bars=mean ± SD. Asterisk indicates significance at $P < 0.05$ ($t$-test).

Fig. 4. Effect of GA$_3$ and cGMP on ABA-induced inhibition of Arabidopsis thaliana seed germination, scored 24, 36 and 48 h after transfer to a growth chamber. Seeds were incubated with ABA (1 $\mu$M), ABA (1 $\mu$M)/GA$_3$ (100 $\mu$M), ABA (1 $\mu$M)/8-Br-cGMP (0.1 $\mu$M) and ABA (1 $\mu$M)/Tadalafil (0.1 $\mu$M). Bars=mean ± SD. Asterisk indicates significance at $P < 0.05$ ($t$-test).

inhibiting cGMP synthesis. Thus, these results suggest that cGMP is required for seed germination process.

Previous studies have demonstrated that GA is required for seed germination, which is controlled by the balance of GA and ABA (Koornneef et al., 2002). In this study, we found that GA-induced seed germination was associated with cGMP. Although GA is an important hormone in regulating seed germination (Koornneef et al., 2002), we did not find the reversal of LY-induced germination inhibition by GA (Fig. 2C). This result may be explained as the blockage by LY of GA-induced seed germination, with cGMP being involved in GA-induced seed germination.

Further investigation showed that, in the presence of cGMP, GA reversed LY-induced germination inhibition, implying that cGMP is required for GA-induced germination (Fig. 2D). These data are consistent with other reports showing that cGMP is a component of GA-induced physiological processes (Gomez-Cadenas et al., 2001; Penson et al., 1996). Similar results have been demonstrated in barley aleurone cells, in which LY reduces GA-treated $\alpha$-amylase production in both aleurone layers and protoplasts. This inhibition can be reversed by either dibutyryl-cGMP (db-cGMP) or 8-Br-cGMP, which are membrane-permeable analogue derivatives of cGMP (Penson et al., 1996). Moreover, LY represses GA induction in GAMYB and $\alpha$-amylase reporter constructs in both wild-type and slender aleurone layers (Gomez-Cadenas et al., 2001; Penson et al., 1996). Furthermore, LY also blocks the GA-dependent induction of isocitrate lyase (ICL) activity (Eastmond and Jones, 2005), which is essential for gluconeogenesis from storage lipids. This is consistent with the observation that cGMP has effects in human fat cells on stimulating lipolysis through a cGMP-dependent protein kinase (PKG) signaling pathway (Eastmond et al., 2000; Eastmond and Jones, 2005; Lafontan et al., 2008). Together, these findings imply that cGMP is necessary for GA-induced germination.

Some researchers have concluded that cGMP alone cannot substitute for GA regulation of $\alpha$-amylase synthesis (Gomez-Cadenas et al., 2001; Penson et al., 1996). Our results also indicate that both 8-Br-cGMP- and Tadalafil-induced seed germination require GA (Fig. 3). Based on our results and other research, we conclude that cGMP plays an important role in the GA signaling pathway. cGMP is involved in GA-induced Arabidopsis seed germination, and on the other hand, cGMP effects on seed germination require an accumulation of GA.

In addition to these results, other observations complement the finding that cGMP signaling interacts with ABA responses, including the putative role of cGMP in ABA-mediated protoplast volume regulation and stomatal opening, as well as cGMP involvement in some ABA responses (Wang et al., 2007).

Interestingly, ABA does not affect the transient increase in cGMP levels in barley aleurone cells (Penson et al., 1996; Wang et al., 2007), whereas GA generates an accumulation of endogenous cGMP (Penson et al., 1996; Wang et al., 2007). These observations are consistent with results obtained using Arabidopsis protoplasts (Penson et al., 1996; Wang et al., 2007). Our results also indicate that 8-Br-cGMP and Tadalafil can rescue GA-induced seed germination inhibition, but have no effect on alleviating PAC-induced germination inhibition after 24 h incubation (Figs. 3, 4). These data imply that the interactions between cGMP and plant hormones, especially GA and ABA may differ.

When the environment is suitable for seed germination, GA begins to accumulate and may generate a transient increase in the endogenous cGMP level. Then, cGMP promotes seed germination by regulating an unknown complex of signaling cascades. Notably, GA is required for cGMP to exert an effect on Arabidopsis seed germination. Meanwhile, cGMP also greatly reduces the sensitivity of seed germination to ABA. Although we did not examine intracellular concentrations of cGMP, our data strongly support the conclusion that cGMP promotes Arabidopsis seed germination.

Genetic evidence shows that ABA is a negative regulator of germination (Koornneef et al., 2002). Recent studies have shown that NO, which is an important signaling molecule, plays a key role in reducing dormancy and promoting germination of Arabidopsis seeds. NO may reduce the sensitivity of Arabidopsis seeds to ABA and also participate in GA-induced seed germination (Bethke et al., 2006; 2007). Some evidence also indicates that an NO/cGMP signaling pathway may exist in higher plants, as it does in animals (Prado et al., 2004). Therefore, we suppose that NO may break ABA-induced germination inhibition in part because of NO-induced cGMP accumulation. Our results also show that both 8-Br-cGMP and Tadalafil can reverse the germination inhibition of ABA (Fig. 4), which further support this hypothesis. Another group of plant hormones, brassinosteroids (BRs), also promote seed germination in Arabidopsis (Kucera et al., 2005; Steber and McCourt, 2001). An antagonistic effect of ABA versus BR in seed germination has been shown in tobacco (Lebner-Metzger, 2001). Moreover, the BR receptor AtBR1 shows positive GC activity in vitro (Kwezi et al., 2007). This implies that BR-mediated regulation of germination may be correlated with cGMP. Although much direct and indirect evidences show that cGMP is related to phytohormones in regulating germination, the mechanism by which cGMP affects Arabidopsis seed germination is still unknown. This question requires further elucidation of cGMP functions in higher plants.
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