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Role of cAMP in Gibberellin Promotion of Seed Germination in *Orobanche minor* Smith

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Abstract Adenosine 3′,5′-cyclic monophosphate (cAMP) is known as a key second messenger in many living organisms, regulating a wide range of cellular responses. In higher plants the function of cAMP is poorly understood. In this study, we examined the role of cAMP in seed germination of the root parasitic plant *Orobanche minor* whose seeds require preincubation in warm moist environments for several days, termed conditioning, prior to exposure to germination stimulants released from roots of host plants. Accumulation of endogenous cAMP was observed in the conditioned *O. minor* seeds. When the seeds were exposed to light or supraoptimal temperature during the conditioning period, cAMP did not accumulate and the seeds showed low germination rates after stimulation with strigol, a germination stimulant. Addition of membrane-permeable cAMP to the medium restored the germination rates of the seeds treated with light or supraoptimal temperature during the conditioning period, suggesting that cAMP functions during the conditioning period. The endogenous cAMP levels of the seeds conditioned in the light or at a supraoptimal temperature were elevated by treatment of the seeds with gibberellin (GA) during the conditioning period. Uniconazole, a potent inhibitor of GA biosynthesis, blocked elevation of the cAMP level. Furthermore, a correlation between the endogenous cAMP level and GA level was observed during the conditioning period. These results suggest that GAs elevate the cAMP level, which is required for the germination of *O. minor* seeds.

Keywords cAMP · Cyclic nucleotide · Seed germination · Gibberellin · Parasitic plant · *Orobanche minor*

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Introduction

Adenosine 3′,5′-cyclic monophosphate (cAMP) is known to be a key second messenger that regulates a wide range of cellular responses in living organisms such as bacteria, fungi, animals, and algae. However, the presence of cAMP in higher plants was a topic of debate for a long period (Newton and Smith 2004). Recently, the development of a cAMP detection method using mass spectrometric analysis enabled us to detect cAMP in plant cells (Newton and others 1999; Richards and others 2002). cAMP is synthesized from ATP by adenylyl cyclase and hydrolyzed by phosphodiesterase, and adenylyl cyclase activity has been detected in some higher plants (Carricarte and others 1988; Lusini and others 1991; Sato and others 1992; Pacini and others 1993; Cooke and others 1994). Although cloning of the adenylyl cyclase genes has not been accomplished from higher plants, a truncated cDNA encoding a protein homologous to fungal adenylyl cyclase has been isolated from a maize pollen cDNA library (Moutinho and others 2001). Expression of the gene in *Escherichia coli* caused accumulation of cAMP and complemented a catabolic defect in the *E. coli cyaA* mutant. However, adenylyl

cyclase activity of this gene product has not been detected *in vitro* or *in planta*. Similarly, phosphodiesterase activity that specifically hydrolyzes cAMP and is inhibited by methylxanthines has been reported (Newton and others 1999), but plant genes encoding phosphodiesterase which specifically hydrolyzes cAMP have not been identified.

Suggested possible functions of cAMP in higher plants are regulation of the cell cycle in tobacco BY-2 cells (Ehsan and others 1998); contribution to growth and reorientation of pollen tube in *Agapanthus umbellatus* (Moutinho and others 2001); and involvement in defense mechanisms by stimulation of Ca^{2+} influx and regulation of elicitor-induced phytoalexin accumulation (Kurosaki and others 1987; Cooke and others 1989; Kurosaki and Nishi 1993; Cooke and others 1994; Bindschedler and others 2001). Downstream effectors of cAMP in plants have not yet been elucidated. Recently, cyclic nucleotide-gated channels (CNGCs) have been identified from many plant species and are good candidates for the downstream effectors of cAMP (Talke and others 2003). They exhibit similarity to Shaker-type voltage-gated channels and contain a cyclic nucleotide-binding domain in the carboxyl-terminal region. It has been reported that gating of the plant CNGCs crucially depends on binding of cyclic nucleotides and that the CNGCs play a role in heavy-metal homeostasis (Arazi and others 1999; Sunkar and others 2000) and defense response (Clough and others 2000; Balague and others 2003). However, a total view of the cAMP production and the cAMP signaling pathway in plants is not clear. Recently, contribution of a CNGC to seed germination has been suggested in *Arabidopsis thaliana* (Gobert and others 2006). This finding led us to attempt an analysis of cAMP behavior in seed germination.

Orobanche minor Sm. is a root holoparasitic angiosperm that has lost photosynthetic function and depends entirely on host plants for its supply of water and inorganic and organic resources. Its seeds require a preincubation period, termed conditioning, at suitable moisture and temperature levels before treatment with specific chemical germination stimulants (Foy and others 1989; Stewart and Press 1990; Estabrook and Yoder 1998) such as strigolactones secreted by the roots of host plants (Cook and others 1966; Hauck and others 1992; Müller and others 1992; Siame and others 1993). Because carefully prepared *O. minor* seeds absolutely require this chemical stimulation for germination, it may be easier to investigate the germination signaling in these root parasites than in other plants.

In many plant species, seed dormancy and germination are regulated by various factors, including gibberellin (GA) and abscisic acid (ABA) (Finch-Savage and Leubner-Metzger 2006). During seed maturation, ABA levels increase and seed dormancy is established. When the dormant seed is exposed to favorable conditions for germination, the level of ABA decreases and GA biosynthesis commences, disrupting dormancy and triggering germination.

In the *Orobanche* species, these plant hormones are involved in regulation of seed germination. It has been suggested that GA biosynthesis takes place during conditioning (Joel and others 1989; Zehhar and others 2002). Treatment of the seeds with inhibitors of GA biosynthesis during the conditioning period reduced the germination rate in several *Orobanche* species (Takeuchi and others 1995; Zehhar and others 2002; Song and others 2005). When exogenous GA was added to the medium during conditioning, the conditioning time required for responding to germination stimulants was shortened (Joel and others 1989; Chae and others 2003). The ABA level in *O. minor* seeds during conditioning was reported to decrease rapidly after imbibition (Chae and others 2003). Despite these observations, the mechanism by which GA and ABA regulate seed germination is unclear.

We have attempted to measure cAMP levels in various plant seeds. Among them we detected cAMP in *O. minor* seeds. In this study we demonstrate that cAMP is functional in *O. minor* seeds and required for the germination process downstream of GAs, an important group of plant hormones.

Materials and Methods

Conditioning and Germination of *Orobanche minor* Seeds

Seeds of *Orobanche minor* Sm. were collected from mature plants that had parasitized red clover, grown either in the Watarase bashi or in an experimental field of Utsunomiya University, Tochigi, Japan. *O. minor* seeds were surface-sterilized by sequential immersions in 70% (v/v) ethanol for 2 min and 1% (v/v) NaOCl containing 0.1% (v/v) Tween-20 for 10 min, then rinsed four times with sterile distilled water and air-dried. The seeds were sown on glass fiber filter papers, which were placed in a 9-cm Petri dish lined with two layers of filter paper moistened with sterile distilled water. In some experiments, 100 μM GA_3, 0.1 or 1 mM dibutyryl cAMP (Sigma, St. Louis, MO, USA), or 5 or 50 μM uniconazole-P (Wako Pure Chemical Industries, Osaka, Japan) were used instead of water. The Petri dishes were sealed with parafilm and incubated at optimal (25°C) or supraoptimal (30°C) temperature in the dark or in the light (fluorescent at 120 μmol m^{-2} s^{-1}). After the conditioning period, the glass fiber filter papers were transferred to a 9-cm Petri dish lined with two layers of filter paper moistened with 10^{-10} M (±)-strigol [synthesized as described by Brooks and others (1985) and Dailey (1987)]

and were incubated at 25°C in the dark for 4 days. The germination was defined as the emergence of the radicle as seen under the microscope.

Quantification of cAMP

The seeds were collected and stored in liquid N_2 until use. The seeds were ground in liquid N_2 and suspended in 6% (w/v) trichloroacetic acid (TCA). After homogenization, the suspension was centrifuged at 10,000*g* for 30 min at 4°C. TCA was removed by extraction with ether saturated with water. After neutralization with Tris base, the sample was dried *in vacuo*. The material left in the tube was dissolved in water. When needed, the samples were treated with 5 mU 3′,5′-cyclic nucleotide-specific phosphodiesterase from beef heart (Sigma) in a final volume of 100 µl of 50 mM MOPS (pH 7.5) at 37°C for 120 min. The reaction was stopped by heat inactivation (100°C, 5 min). The sample was dried *in vacuo*, and the material left in the tube was dissolved in water. The cAMP content was measured by a radioimmunoassay kit (Yamasa Shoyu Co., Chiba, Japan) and an enzyme immunoassay kit (GE Healthcare BioSciences Corp., Piscataway, NJ, USA). The amount of cAMP was routinely determined by the difference between values obtained from samples treated and not treated with phosphodiesterase and shown in pmol of cAMP per gram dry weight of seeds.

In the radioimmunoassay, samples were succinylated by incubation with the succinylation reagents for 10 min at room temperature. The succinylated samples were incubated overnight at 4°C with ^{125}I-labeled succinyl-cAMP and antiserum specific to succinyl-cAMP. The succinyl-cAMP in the sample competed with ^{125}I-labeled succinyl-cAMP to form a complex with a limited amount of the antibody. After absorbing free succinyl-cAMP to dextran-coated charcoal, the radioactivity of ^{125}I-labeled succinyl-cAMP-antiserum complex in the supernatant was measured by a γ-counter. The amount of cAMP was calculated by standard curves obtained for each experiment.

In the enzyme immunoassay, samples were diluted with a sodium acetate assay buffer and acetylated. The acetylated samples were then added to a microtiter plate bearing rabbit cAMP antiserum, and the plate was incubated for 2 h at 4°C. Peroxidase conjugated with cAMP was then added, and the plate was incubated at 4°C for another 1 h. After washing four times with PBS containing 0.05% Tween 20, the reaction was started by adding 1,1′-trimethylene-bis (4-formylpyridinium bromide) dioxime as a substrate. After incubation for 30 min at 25°C, the reaction was stopped by 100 µl of 1 M sulfuric acid and the absorbance at 450 nm was measured. The cAMP concentrations were determined by comparison with the known standards.

cAMP Identification and Quantification by High-Performance Liquid Chromatography Connected to Tandem Mass Spectrometry (LC/MS/MS)

Imbibed seeds (500 mg dry wt) of *O. minor* were ground in liquid N_2 and suspended in an ice-cold mixture of methanol/chloroform/1 M formic acid (12:5:3 v/v). After homogenization, the suspension was centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was collected and dried *in vacuo*. The dried extract was dissolved in 20% methanol and loaded onto a Sep-Pak™ cartridge (100 mg C18, Waters Corp., Milford, MA, USA). The flow-through fraction was collected and dried *in vacuo*. The material left in the tube was dissolved in 100 µl of 0.25% (v/v) acetic acid/20% (v/v) methanol. The resulting solution was filtered through a spin column (Ultra-Free MC, 0.45-µm pore size; Millipore, Billerica, MA, USA) and an aliquot was diluted with a volume of either 0.25% (v/v) acetic acid/20% (v/v) methanol or 0.25% (v/v) acetic acid/20% (v/v) methanol containing known amounts of cAMP. HPLC separation was done with a JASCO U980 HPLC instrument (JASCO, Tokyo, Japan) equipped with an ODS (C18) column (Mightysil RP-18, 2 × 250 mm, 5-µm pore size, Kanto Chemicals Co. Ltd., Tokyo, Japan). The mobile phase was 10% (v/v) methanol in water containing 0.25% (v/v) acetic acid and changed to 100% methanol 20 min after injection. The column was then washed with 100% methanol for 20 min to elute all injected materials. The flow rate was 200 µl min^{-1}. Column temperature was set to 40°C. Mass spectrometry was performed on a Quattro LC mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray source. The MS was operated in electrospray ionization negative mode. Drying gas as well as nebulizer gas was nitrogen-generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). The nebulizer gas flow was set to approximately 100 L h^{-1} and the desolvation gas flow to 500 L h^{-1}. The interface temperature was set to 400°C and the source temperature to 120°C. The capillary and cone voltages were 2.9 kV and 40 V, respectively. MS/MS experiments were done using argon as the collision gas, and the collision energy was set to 28 V. The collision gas pressure was 1.5×10^{-3} mbar. Data acquisition and analysis were performed using MassLynx software (ver. 3.2) running under Windows NT (ver. 4.0) on a Pentium PC. For the quantification of cAMP, multiple reaction monitoring (MRM) was employed, monitoring the transition m/z 328 > 134. The final cAMP concentration was determined by comparing areas of the MRM ion chromatogram in samples diluted with 0.25% (v/v) acetic acid/20% (v/v) methanol or 0.25% (v/v) acetic acid/20% (v/v) methanol containing known amounts of cAMP.

Quantification of Gibberellin

Imbibed seeds (0.6 g dry wt for small-scale experiment or 15 g dry wt for large-scale experiment) of *O. minor* were crushed in a mortar with a pestle, and GAs were extracted with methanol (20 ml for small-scale experiment or 450 ml for large-scale experiment). The dried extract was subjected to solvent fractionation to obtain an acidic ethyl acetate-soluble (AE) fraction. The concentrated AE fraction was dissolved in methanol (3 ml) and loaded onto a Sep-Pak^{TM} cartridge (100 mg C18), which was eluted with methanol (5 ml). Half of the dried eluate from the cartridge was dissolved in 0.5 ml of 0.5% (v/v) acetic acid/20% (v/v) acetonitrile/water (solvent A) and a portion (0.35-0.4 ml) was subjected to HPLC on a PEGASIL^{TM} ODS column (6 mm i.d. × 15 cm; Senshu Scienific Co., Tokyo, Japan), which was eluted with solvent A and 0.5% (v/v) acetic acid/80% (v/v) acetonitrile/water (solvent B) as follows: 0-15 min, isocratic elution with solvent A; 15-35 min, linear gradient of 100% solvent A to 100% solvent B; 35-45 min, isocratic elution with solvent B; flow rate, 1.5 ml min^{-1}. The eluates were collected in fractions every 2 min from the retention time of 5.0 min. An aliquot of each fraction was subjected to immunoassay using antiserum specific for physiologically active gibberellins, as previously described (Nakajima and others 1991). The amounts of gibberellins were calculated by standard curves obtained by serial dilutions of authentic GA_4 and shown in pmol GA_4 eq. per gram dry weight of seeds.

Results

cAMP Levels of *O. minor* Seeds During Conditioning

To investigate cAMP levels in *O. minor* seeds during conditioning, we established a method for quantifying cAMP in this tissue. Validation of the procedure was done by the standard addition method (Brown and Newton 1992). When a known amount of cAMP was added to samples prepared from *O. minor* seeds conditioned for 3 days at 25°C in the dark, the cAMP levels of these samples (Fig. 1A, right bar, total height indicates the result of measurement) were higher than that of the original samples (left bar) by the amount added to the samples (indicated by the gray bar) (Fig. 1A). The model samples were treated with phosphodiesterase (Fig. 1B). Under conditions that allow complete degradation of 0.025 pmol cAMP (indicated by "cAMP"), the compound from the model sample was hydrolyzed efficiently by the enzyme to give a value close to zero (sample). A mixture of the model sample and authentic cAMP gave a similar result (sample + cAMP). The heat-denatured enzyme after heat treatment did not decrease the cAMP levels. These results suggest that the cAMP levels determined here indeed reflected the cAMP levels in the samples. Two types of cAMP detection systems (see Materials and Methods) eventually yielded the same results, reconfirming our analysis.

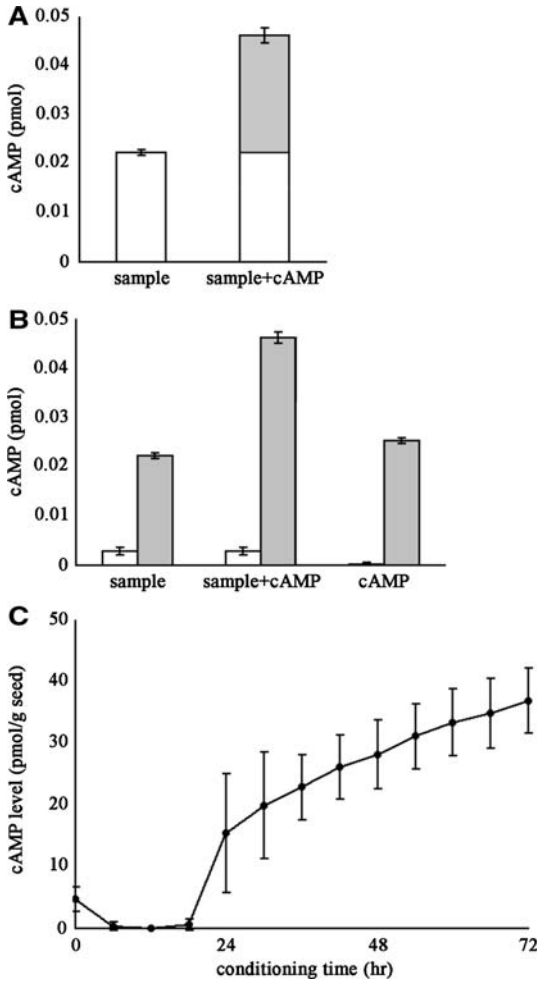

Fig. 1 cAMP levels in *O. minor* seeds during the conditioning period. **A** Confirmation of cAMP content measurement method in a sample extracted from *O.* minor seeds. A model sample prepared from *O. minor* seeds was divided into two tubes equally, and 0.025 pmol of cAMP was added to one tube. The cAMP levels were measured with the two kinds of cAMP assay kits, yielding similar results. The results from the radioimmunoassay are shown. **B** Hydrolysis of cAMP with phosphodiesterase. Authentic cAMP (0.025 pmol) and the model samples mixed with or without authentic cAMP (0.025 pmol) were treated with active phosphodiesterase (white bars) or heat-denatured phosphodiesterase (gray bars). The cAMP levels were measured with the two kinds of cAMP assay kits. **C** cAMP level of *O. minor* seeds during the conditioning period. *O. minor* seeds were conditioned for the indicated period at 25°C in the dark. The cAMP level per 1 g of seeds was measured with the two kinds of cAMP assay kits. Experiments were independently conducted at least three times, producing similar results each time. ±SD values were determined from triplicate measurements

The immunologic methods used for identification and quantification of cAMP were validated by LC/MS/MS. cAMP levels in seeds conditioned for 36 h or 72 h were 23 pmol/g and 37 pmol/g, respectively, determined using the immunologic methods**.** Similar samples gave values of 28 pmol/g and 39 pmol/g, respectively, by LC/MS/MS, which were very close to those obtained by the immunologic methods, suggesting that the results of the immunologic methods are valid.

cAMP levels during the conditioning period were analyzed by the immunologic method described above. cAMP was undetectable at the beginning of the conditioning period and its level gradually increased until it reached about 37 pmol/g after an incubation period of 3 days (Fig. 1C). The cAMP level remained unchanged for an additional 7 days (data not shown). The protein level of the seeds did not change appreciably during conditioning (Nun and Mayer 1993) (data not shown). These results suggest that the cAMP level in *O. minor* seeds is regulated before germination.

cAMP Is Required for Germination of *O. minor* Seeds

It has been reported that exposure of *O. minor* seeds to light or supraoptimal temperature during conditioning reduces the germination rate (Takeuchi and others 1995; Chae and others 2003). We therefore tested whether such treatments affected the cAMP level. The germination rates of the seeds were determined after an additional incubation in the presence of 10^{-10} M strigol, a germination stimulant, at 25°C in the dark. As shown in Figure 2A, the cAMP level in seeds conditioned for 3 days at the optimal temperature (25°C) in the dark was 39 pmol/g (control). The cAMP levels were much lower in seeds exposed to light or supraoptimal temperature (30°C), being 15 and 11 pmol/g, respectively. There was a good correlation between the cAMP level and the germination rate (Fig. 2A). The cAMP level of these seeds did not change significantly after stimulation with strigol (data not shown).

A temperature shift to 30°C after conditioning under optimal conditions has been shown to inhibit germination of the seeds (Chae and others 2003). In this study the germination rate of the seeds conditioned at 25°C for 3 days was 56% and decreased to 25% after additional conditioning at 30°C for 1 day (Fig. 2B). A reduction of the cAMP level from 40 to 13 pmol/g was observed during this procedure. In addition, the cAMP level and the germination rate were restored to the levels before the temperature shift by an additional incubation at 25°C for 2 days. These results suggest that cAMP functions during the conditioning period and may be required for the germination.

The effect of exogenous cAMP on germination of the seeds exposed to light or supraoptimal temperature was tested. The seeds were conditioned in the presence of membrane-permeable cAMP (dibutyryl cAMP: dbcAMP). The germination rates of the seeds were determined after an additional incubation in the presence of 10^{-10} M strigol at 25°C in the dark. Although a relatively high concentration of dbcAMP was required, the addition of dbcAMP to the conditioning medium restored the germination efficiency of the seeds treated with light or supraoptimal temperature to a rate close to that of control seeds (Fig. 2C). The need for a high dose of dbcAMP may be due to its inefficient penetration into the seeds. To test this hypothesis, the dbcAMP treatment was repeated in the presence of Tween 20 or Triton X-100. However, the addition of these detergents to the medium with dbcAMP did not change the efficacy of cAMP for induction of seed germination under inhibitory conditions (data not shown).

Regulation of cAMP Levels by Gibberellin

It has been reported that GAs can restore seed germination from the inhibition imposed by light or high temperature in *O. minor* (Takeuchi and others 1995; Chae and others 2003). Therefore, the effect of GA treatment on the cAMP level was examined. As shown in Figure 3A, no effect was observed on the cAMP level after GA_3 treatment under optimal conditions (25°C, dark). However, the cAMP level was increased by GA_3 treatment in the *O. minor* seeds exposed to light or supraoptimal temperature during conditioning. The relationship between GAs and cAMP was further examined in experiments using uniconazole, a potent inhibitor of GA biosynthesis. Seed germination of *O. minor* was decreased by uniconazole treatment (Fig. 3B, white bars). Importantly, cAMP accumulation was also inhibited by this treatment. Addition of exogenous GA_3 with uniconazole to the medium restored the cAMP accumulation as well as the seed germination to the level it was without uniconazole treatment (Fig. 3B, gray bars).

We examined whether the endogenous GA level was regulated during conditioning of *O. minor* seeds (Table 1). $GA_{1/3}$ was detected in seeds conditioned at 25°C in the dark for 48 h (Table 1, Sample 2A) but undetected in seeds conditioned at the supraoptimal temperature (Table 1, Sample 2B). The amount of GA was decreased to an undetectable level by incubation at the supraoptimal temperature for an additional 24 h (Table 1, Sample 3B). There was a good correlation between the cAMP level and the $GA_{1/3}$ level. When conditioning of the seeds was completed under optimal conditions (25°C, dark) and then the temperature shifted to 30°C, the GA level was undetectable**,** although a low level of cAMP was detected. The results

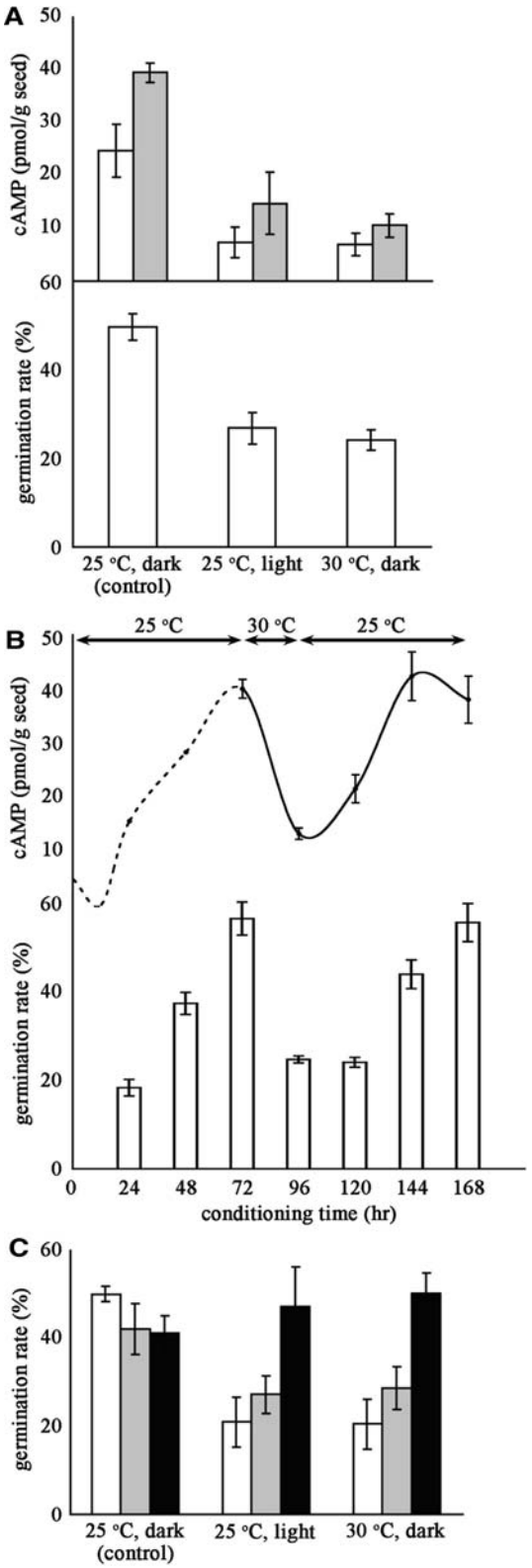

Fig. 2 cAMP regulates germination of *O. minor* seeds. **A** cAMP levels and germination rates after treatment with light or supraoptimal temperature. *O. minor* seeds were conditioned at 25°C in the dark (control), at 25°C in the light, or at 30°C in the dark. cAMP levels were measured after conditioning for 36 h (white bars) or 72 h (gray bars) (upper panel). The seeds conditioned similarly for 72 h were treated with 10^{-10} M strigol, a germination stimulant, and further incubated at 25°C in the dark for 4 days to determine the germination rate (lower panel). **B** Recovery of cAMP level and germination rate by additional conditioning. After conditioning for 3 days at 25°C in the dark, *O. minor* seeds were incubated for 1 day at 30°C in the dark and then at 25°C in the dark. cAMP levels at the indicated times were measured and shown by the line in the upper part. At the same time, seeds that had been treated similarly to the ones used for cAMP measurement were stimulated with strigol (10^{-10} M) and further incubated at 25°C in the dark for 4 days; germination rates are shown by the bars in the lower part. The broken line indicates the change of cAMP level before the temperature shift to 30°C (as shown in Figure 1C). **C** The effect of exogenous membrane-permeable cAMP on the germination rates of the seeds treated with light or supraoptimal temperature. In the presence of 0 (white bars), 0.1 (gray bars), or 1 (black bars) mM of dibutyryl cAMP, *O. minor* seeds were conditioned at 25°C in the dark (control), at 25°C in the light, or at 30°C in the dark for 3 days. Then strigol (10^{-10} M) was given and the germination rates were determined as described in **A**. Germination rates were expressed by the number of germinated seeds divided by the total number of seeds used in the experiments (ca. 200 seeds) × 100. Experiments were conducted independently at least three times, producing similar results each time. ±SD values were determined from triplicate measurements

support the hypothesis that GA regulates cAMP production, a possible signal transduction mediator of conditioning. These results suggest that the cAMP level, which is positively related to the germination rate, is regulated by GAs.

Discussion

cAMP is one of the best characterized second messengers in many organisms such as bacteria, fungi, animals, and algae. Although the presence of cAMP in plant cells was confirmed in recent years, knowledge of physiologic functions of cAMP in higher plants is limited. The demonstration of a role for cAMP in physiologic processes will help discover new cAMP functions in higher plants. However, the cAMP content of plant cells is very low compared with that of other organisms so that it is difficult to analyze the function and behavior of cAMP *in planta*. To overcome this problem, most studies of cAMP function in plant cells involved the use of membrane-permeable cAMPs and/or cultured cells.

In this study we established a method for determining the cAMP level in *O. minor* seeds and showed that cAMP plays a role in seed germination. Germination of *O. minor* seeds is unique because it requires not only incubation at suitable moisture and temperature conditions, but also stimulation with germination stimulants. The regulation mechanism of seed germination in these seeds is not well studied. Analysis of the cAMP behavior in *O. minor* seeds in the present study demonstrated that the cAMP level increased dramatically during the conditioning period (Fig. 1C) and correlated with the efficiency of the germination induced by a germination stimulant (Fig. 2A, B).

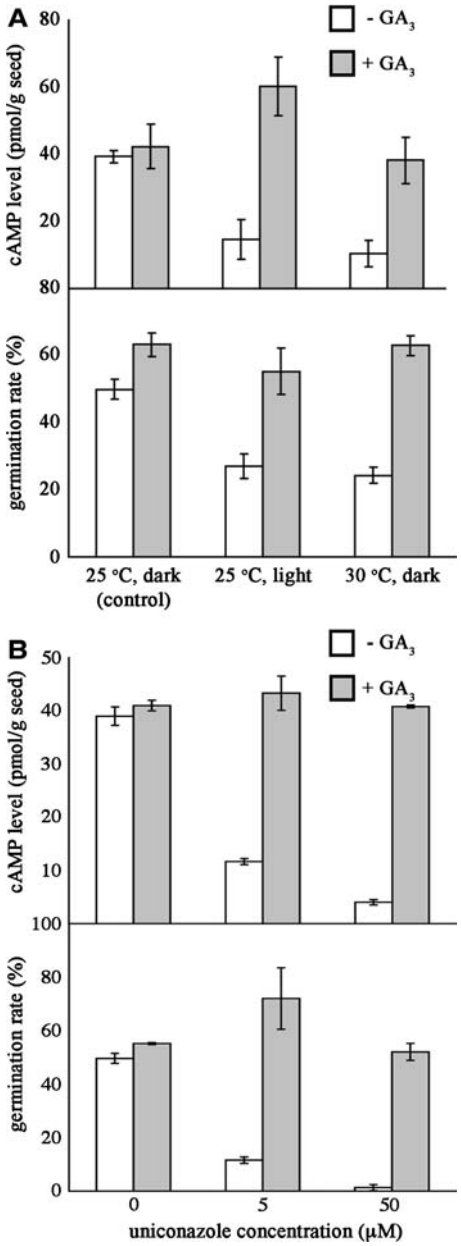

Fig. 3 Regulation of cAMP level by gibberellin. **A** Effect of GA_3 on cAMP levels and germination rates in *O. minor* seeds exposed to light or supraoptimal temperature. *O. minor* seeds were conditioned at 25°C in the dark (control), at 25°C in the light, or at 30°C in the dark in the presence (gray bars) or absence (white bars) of 100 μM GA_3 for 3 days and the cAMP levels were measured (upper panel). The seeds conditioned similarly to the ones used for cAMP measurement were stimulated with strigol (10^{-10} M), and the germination rates were determined after additional incubation at 25°C in the dark for 4 days (lower panel). **B** Effect of uniconazole on cAMP level and germination rate in *O. minor* seeds. cAMP levels of *O. minor* seeds were measured after incubation with uniconazole (0, 5, 50 μM) for 3 days at 25°C in the dark in the presence (gray bars) or absence (white bars) of 100 μM GA_3 (upper panel). The germination rates of the seeds incubated similarly were determined as described in **A** (lower panel). Germination rates were expressed as described in Figure 2. Experiments were conducted independently at least three times, producing similar results each time. ±SD values were determined from triplicate measurements

There is no information about the role of cAMP in seed germination. These findings led to further analysis of cAMP function in seed germination.

Treatment of *O. minor* seeds with light or supraoptimal temperature during the conditioning period inhibited the elevation of the cAMP level (Fig. 2A). A temperature shift to the supraoptimal temperature after conditioning at optimal temperature decreased the cAMP level, which returned to the high level when the temperature was shifted back to an optimal one (25°C) (Fig. 2B). These observations suggest that the seeds monitor environmental conditions and reflect the information in the cAMP level. The fact that membrane-permeable cAMP is effective in promoting germination induced by a germination stimulant in *O. minor* seeds, in which cAMP levels are kept low by exposure to light or supraoptimal temperature (Fig. 2C), suggests that cAMP is required in some steps of the germination process. The role of cAMP can be either maintaining seed sensitivity to germination stimulants or the signal transduction induced by germination stimulants that results in germination. Although downstream effectors of cAMP functioning in regulation of seed germination have not been well characterized, cyclic nucleotide-gated channels (CNGCs) are good candidates. Indeed, it has recently been shown that CNGC3 functions in seed germination in *A. thaliana* (Gobert and others 2006), supporting this idea. It is possible that CNGCs are involved in the germination of *O. minor* as well.

In many plants seed germination is regulated by the balance of GA and abscisic acid (ABA). Therefore, cAMP levels may be regulated by these plant hormones in *O. minor* seeds. The ABA level in *O. minor* seeds during the conditioning period was reported to decrease rapidly after imbibition and did not increase with a temperature shift to supraoptimal temperature (Chae and others 2003). On the other hand, cAMP levels increased after imbibition and decreased with a temperature shift to supraoptimal temperature in our experiments (Fig.s 1C, 2B). Therefore, the ABA level does not correlate with the cAMP level. In contrast, GA levels correlated with the cAMP level in these seeds (Table 1). Furthermore, cAMP accumulation in the seeds was promoted by GA_3 and blocked by uniconazole, a GA biosynthetic inhibitor (Fig. 3). Hence, it is likely that cAMP is not regulated by ABA but by GA. It has been suggested that GA synthesis occurs during the conditioning period and is required for the response to germination stimulants (Joel and others 1989). Our results support this idea and imply that GA functions as a sensor or a signaling

Table 1 Relationship between Endogenous cAMP and Endogenous Gibberellin in Imbibed *Orobanche* Seeds

	$GA_{1/3}$ level[a] (pmol GA_4 eq/g dry wt)	cAMP level (pmol/g dry wt)
Sample 1	n.d.[b]	n.d.
Sample 2A	22 ± 2.1[c]	28 ± 6
Sample 2B	n.d.[c]	n.d.
Sample 3A	47 ± 3.1[b]	42 ± 5
Sample 3B	n.d.[b]	13 ± 1

Sample 1, 0.5 day after imbibition (DAI) at 25°C; sample 2A, 2 DAI at 25°C; sample 2B, 2 DAI at 30°C; sample 3A, 4 DAI at 25°C; sample 3B, 3 DAI at 25°C and then 1 day at 30°C. Experiments were independently conducted at least twice, producing similar results each time. ±SD values were determined from triplicate measurements

n.d. = not detected

[a] $GA_{4/7}$ was undetectable in all the samples

[b] 0.6 g dry wt of seeds was used for extraction of GA, and a portion (0.05 g dry wt eq) was subjected to immunoassay

[c] 15 g dry wt of seeds for extraction of GA, and a portion (0.66 g dry wt eq) was subjected to immunoassay

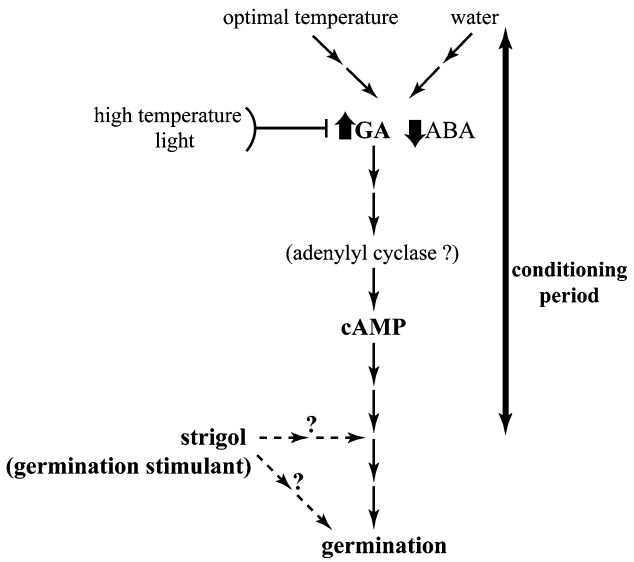

Fig. 4 A model for cAMP-mediated signaling pathway in seed germination

molecule for environmental stresses by regulating the cAMP level in the seeds and preventing the germination under unfavorable conditions.

It has been shown that GA promotes seed germination in many plant species. Recent studies revealed that DELLA proteins function in the early stages of GA signal transduction (Olszewski and others 2002; Peng and Harberd 2002; Gomi and Matsuoka 2003). However, events downstream of DELLA proteins have yet to be determined. Our results imply that cAMP mediates GA signals in seed germination, at least in *O. minor*. The relationships between cAMP and GA in seed germination were studied in some laboratories in the 1970s. cAMP and GA_3 synergistically promoted germination of light-sensitive lettuce seeds (Kamisaka and Masuda 1971; Hall and Galsky 1973). Germination of mannitol-treated seeds of a common weed was promoted by red light, GA, and cAMP (Holms and Miller 1972). In addition, cAMP shows an effect similar to GA_3 in stimulating α-amylase synthesis in barley seeds (Duffus and Duffus 1969; Galsky and Lippincott 1969). These results can be explained by our hypothesis that the cAMP level is positively regulated by GA.

Based on our results, we propose a model for cAMP-mediated signaling pathway in seed germination (Fig. 4). When seeds are incubated at suitable moisture and temperature conditions, ABA is reduced and GA is produced in the seeds as has been shown in other plants. In *O. minor*, GA induces elevation of endogenous cAMP levels, possibly by activating adenylyl cyclase by unknown mechanisms. The role of cAMP could be either to maintain sensitivity of the seeds to germination stimulants or to act in a signaling pathway that induces germination distinct from that of germination stimulants. The GA level can be downregulated by unfavorable conditions such as exposure to light or high temperature during conditioning, which decrease the cAMP level to prevent germination.

Although we cannot exclude the possibility that cAMP regulates GA production, our results strongly suggest that GA regulates cAMP levels and that cAMP may act as a mediator of GA signaling for germination in *O. minor* seeds. As described in the Introduction, possible functions of cAMP in higher plants have been suggested in other physiologic processes. Therefore, it is possible that cAMP can also be regulated by other mechanism other than GA. Because cAMP is known to be a multifunctional second messenger in a wide variety of organisms, it is possible that cAMP functions in many physiologic processes in plants. To understand the whole picture of the function of cAMP, one of the best characterized second messengers in plants, will require considerable effort.

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