J Plant Growth Regul (2006) 25:233–244
DOI: 10.1007/s00344-006-0015-8

J P G R
Journal of Plant Growth Regulation

The Involvement of Cyclic ADPR in Photoperiodic Flower Induction of *Pharbitis nil*

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Abstract

Cyclic adenosine diphosphate ribose (cADPR) is a potent endogenous calcium-mobilizing agent synthesized from NAD^+ by ADP-ribosyl cyclases described for several animal cells. Pharmacological studies suggest that cADPR is an endogenous modulator of Ca^{2+}-induced Ca^{2+} release channels. There is also information about the sub-micromolar concentration of cADPR in plant cells. Whether cADPR can act as a Ca^{2+}-mobilizing intracellular messenger in plant tissue is an unresolved question. Despite the obvious importance of monitoring cADPR cellular levels under various physiological conditions in plants, its measurement has been technically difficult and requires specialized reagents. In the present study a widely applicable sensitivity assay for cADPR is described. We show that *Pharbitis nil* tissue from cotyledons contains a certain cADPR level. To explain the possible roles of this second messenger in photoperiodic flower induction, some physiological experiments were also performed. The exogenous applications of cADPR to *Pharbitis nil* plants, which were exposed to a 12-h-long subinductive night, significantly increased flowering response. Nevertheless 8-Br-cADPR inhibited flowering when these compounds were applied during a 16-h-long inductive night. The effect of ruthenium red, a calcium channel blocker and ryanodine, a calcium channel stimulator, on the photoperiodic induction of flowering was also studied. Ruthenium red, when applied before and during an inductive 16-h dark period, slightly inhibited flowering, whereas ryanodine, when applied before and during a 12-h long subinductive night, stimulated flower bud formation. We also confirmed evidence that Ca^{2+} ions are involved in the photoperiodic induction of flowering. Thus, the obtained results may suggest the involvement of cyclic ADPR-activated Ca^{2+} mobilization in the photoperiodic flower induction process in *Pharbitis nil*.

Key words: Ca^{2+}; cADPR; Flowering; *Pharbitis nil*; Photoperiodic induction.

Introduction

Photoperiodism is the reaction of a plant to the changing duration, over 24 h, and the periodic succession of light and darkness. Photoperiodic induction of flowering is based on the perception

Received: 16 February 2006; accepted: 31 May 2006; Online publication: 26 September 2006
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by the plant of the appropriate photoperiod and the transfer of the light signal to a metabolic reaction leading to the creation of a flower. Phytochromes and cryptochromes fullfil a sensory and a regulatory function in the processes of flower photoinduction. The sensory function involves the perception and interpretation of the light signal, whereas the regulatory function consists of the biochemical transmission of the received signal (transduction chain), which leads to a change in the expression of certain genes and the appearance of a flower inductor in the leaves (cotyledons), which is then transported to the growth shoot apex, where processes of flower evocation and morphogenesis are triggered (Tretyn and others 1990).

The control mechanisms of the photoinduction of flowering are not clear. There are some suggestions that heterotrimeric G proteins (Muschietti and others 1993), calcium, calmodulin (Neuhaus and others 1993), kinase, and phosphatase, which phosphorylate and dephosphorylate specific proteins (Doshi and Sopory 1992), are involved in phytochrome signalling. It was established that as a result of phytochrome activation two separate but cooperating signal transduction pathways are activated (Millar and others 1994). The first of them is dependent on calcium ions and calmodulin; the second one, on changes in the cyclic nucleotides level.

In the past study we confirmed the role of phytochrome (Cymerski and Kopcewicz 1994, 1995; Carter and others 2000), calcium ions (Tretyn and others 1990, 1994; Jaworski and others 2003), and cGMP (Szmidt-Jaworska and others 2004) in photoperiodic flower induction. There are suggestions that these two pathways work together, and that cyclic adenosine-diphosphate-ribose (cADPR) is the intermediary.

The temporal rise of intracellular Ca^{2+} concentration has been well demonstrated to be one of the important factors regulating many processes. At first it was established that $InsP_3$ can elicit increases in the cytoplasmic level of calcium ions in plant cells. Cyclic ADPR, a metabolite of NAD^+, has been reported to have a Ca^{2+}-mobilizing activity in animals (White and others 1993). For a couple of years we have been able to find information about calcium release in response to the sub-micromolar concentration of cADPR in plant cells (Muir and Sanders 1996; Allen and others 1995). However, the physiological function of cADPR in plants has not yet been elucidated. Cyclic ADPR is known to be involved in the abscisic acid signaling pathway in tomato and *Arabidopsis* (Leckie and others 1998; Wu and others 1997). Furthermore, cADPR has been found to play a physiological role in Ca^{2+} mobilization in *Vicia faba*, probably through modulation of vacuolar Ca^{2+} channels (Allen and others 1995).

Cyclic ADPR was first discovered during investigation of $InsP_3$-dependent Ca^{2+} mobilization mechanisms in sea urchin egg preparation (Clapper and others 1987; Dargie and others 1990). It has been suggested that it is the physiological modulator of the ryanodine receptor (Galione and others 1993).

In plants the exact mechanism by which cADPR releases Ca^{2+} is still not known, and the protein(s) with which cADPR interacts to effect calcium release have not been identified.

Muir and Sanders (1996) revealed that in red beet (*Beta vulgaris*) microsomes there are ryanodine receptor homologues. The pharmacology of cADPR-induced Ca^{2+} release bore many similarities to the ryanodine receptor from animal cells, including modulation of release by ryanodine, activation by caffeine and inhibition by ruthenium red. This may suggest that cADPR may act as an endogenous activator of at least one isoform of the ryanodine receptor.

This report is an attempt to determine which element of calcium signaling could take part in light/dark-mediated photoperiodic flower induction in cotyledon cells. So, the influence of exogenously applied secondary transmitters (Ca^{2+}, cADPR), a drug known to change the calcium level and release the floral photoinduction processes of *Pharbitis nil*, as well as the cyclic ADPR endogenous content were investigated. It is best to conduct such research on short-day plants because the photoperiodic reactions of these plants are totally unequivocal. One of the most intensively studied plants with respect to the conditions that influence its flowering is the Japanese morning glory (*Pharbitis nil*). *Pharbitis nil* is a short-day plant, which shows a clear response to short day treatment to flower, it keeps its vegetative growth under long days or continuous light conditions and shows complete transition from a vegetative state to a reproductive one over a single 16-h dark treatment (Vience-Prue and Griessel 1984).

Materials and Methods

Plant Material

The investigations were conducted on 5-day-old seedlings of morning glory (*Pharbitis nil* L. Chois),

the Japanese variety Violet (Marutane Seed Co., Kyoto, Japan).

Seeds were soaked in concentrated sulfuric acid for 50 min and then washed in running tap water for 3 h. They were left in distilled water at 25°C overnight. The swollen seeds were planted on a mixture of vermiculite and sand (2:1 w/w) in a plastic pots, covered with Saran Wrap to maintain high humidity and were grown for 5 days at 25°C in continuous light at 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (cool white fluorescent tubes, Polam, Poland) in a growth chamber. There were 15 seeds in each pot.

Depending on the type of physiological experiment, plants were exposed to a 12-h-long subinductive night or 16-h-long inductive night. For endogenous cADPR level analysis, cotyledons were harvested, frozen in liquid nitrogen, and kept at −80°C.

Physiological Experiments

During this time plants were treated with the following substances (depending on the experiment): (1) $CaCl_2$ (10 mM), (2) EGTA (40 mM), (3) cADPR (40 μM), (4) ADPR (40 μM), (5) NAD^+ (100 μM) (6) cADPR (40 μM) and cGMP (0.1 mM), (7) cADPR (40 μM) and Ca^{2+} (10 mM), (8) 8-Br-cADPR (0.1 mM), (9) ryanodine (0.1 mM), (10) ruthenium red (0.1 mM).

All of the tested agents were used at optimal concentrations, that is, the lowest concentration allowing maximum inhibition or stimulation of plant flowering without affecting growth processes (for example, elongation growth, number of nodes and internodes). These concentrations were retained from our preliminary dose/response studies. The chemicals were dissolved in distilled water as a stock solution and then diluted to the required concentrations with distilled water. All solutions of the compounds were applied on cotyledons with a small, soft brush (50 μl per plant).

For a given variant, the particular batch of plants was treated once with a given compound at a particular hour of the night or before night.

EGTA, 8-Br-cADPR, and ruthenium red were used for treatment of plants subjected to complete induction (16 h darkness), whereas the other substances were used for treatment of plants subjected to incomplete induction (12 h darkness). The control plants were treated with water.

After the completion of the experiments, the plants were grown in a growth chamber under continuous light. All seedlings were allowed to grow for 14 days, and then the number of flower buds was examined under a binocular microscope. Flower induction intensity was estimated as the number of flower buds and leaf buds per plant and the percentage of plants exhibiting terminal flowering. Each experiment was repeated 3 times. To compare all treatments, statistical analysis was performed. The results were presented on diagrams and standard errors were calculated. Data were calculated using the Sigma Plot program and are presented as means ± standard error (SE).

There appeared to be a correlation between the number of flower buds and leaf buds, so in all graphs we have only shown the differences in the number of flower buds and the percentage of plants with terminal flower buds.

HPLC Analysis of cADPR

An attempt to extract and detect cADPR was made. The seedlings of *P. nil* were harvested and frozen in liquid nitrogen. Endogenous cADPR was extracted using the procedure described by da Silva and others (1998) with some modifications.

The frozen tissue was ground under liquid nitrogen and the resultant powder was homogenized with $HClO_4$. The extracts were frozen twice in liquid nitrogen. After thawing, the samples were left in an ice-salt bath for 30 min, then centrifuged (15 000 × *g*, 10 min, 4°C). The supernatant was collected and titrated to pH 7 by KOH addition. After incubation, the samples were centrifuged. The supernatants containing nucleotides were collected, lyophilized, and stored at −80°C.

Analysis of cADPR using high performance liquid chromatography (HPLC) (Perkin Elmer series 200) was carried out as follows. A strong anion-exchange HPLC was performed using EC 250/4.6 NUCLEOSIL 100-5 SB (Macherey-Nagel, Germany). Lyophilized samples were dissolved in a total volume of 200 μl of water and filtered through disposable 0.2 μm filters directly before injection. A gradient from 1 mM Tris, pH 8,0 (eluent A) to 150 mM trifluoroacetic acid (TFA)(eluent B) was used at a flowrate of 1 ml/min at 25°C. The gradient was (as % of eluent B): 0 min, 5%; 10 min, 24%; 25 min, 24,4%; 30 min, 50%; 35 min, 5%. Absorbance was measured at the UV detector operating at 254 nm.

In some experiments, part of the analyzed sample was incubated at 80°C for 2 h, to convert endogenous cADPR into ADPR. Subsequently, that sample was analyzed as described above. As a standard, 99.9% pure cADPR and ADPR from Sigma were used.

Table 1. Effect of Different Photoperiods on the Flowering Response in *Pharbitis nil* Seedlings

Light conditions	Number of flower buds per plant	% of plants with terminal flower buds
Continuous light (control)	0	0
Incomplete induction (12 h of darkness)	1 ± 0.2	0
Complete induction (16 h of darkness)	5 ± 0.5	100 ± 2

Results

Effects of Exogenous Application of Chemicals on Flower Bud Formation

Plants growing in different light conditions produced a different number of flower and leaf buds (Table 1). In plants grown under continuous light no flower buds were found. When plants were treated with darkness, the length of the darkness period influenced the number of flower buds; when a 12-h-long subinductive night was used, plants produced about 1 ± 0.2 flower bud per plant. Such darkness periods did not allow the production of terminal flower buds. After a 16-h-long inductive night, plants produced about 5 flower buds, and all plants (100%) had terminal flower buds.

The effect of Ca^{2+} on the flowering process of *P. nil* was examined by an exogenous application of Ca^{2+} to seedlings subjected to incomplete induction (12-h-long night). As shown in Figure 1A $CaCl_2$ applied on cotyledons before and during a 12-h-long subinductive night, caused an increase in the number of flower buds. The 10 mM $CaCl_2$ solution had a strong influence on flower bud formation and caused an increase in the total flower bud number. The highest stimulation of flowering was observed when the plants were treated at the 4th h of the subinductive night, and those plants produced 6.3 flower buds per plant. Application at the 8th and 12th h of night gave 5.6 and 4.7 flower buds, respectively, and was at least 2 times higher than in control plants. In all cases, plants had terminal flower buds with the highest number at the 4th h.

The influence of cADPR on the flowering process of *P. nil* was examined by an exogenous application of this compound to seedlings subjected to incomplete induction (12-h-long night). As shown in Figure 1B in control plants a small number of flower buds were formed (mean 0.8 flower bud per plant). Application of 40 μM cADPR increased the number of flower buds. The maximum activation was observed when cADPR was applied at the beginning of the subinductive night (4th h; mean 4.3 flower buds per plant). Despite such stimulation, this compound given during the night did not stimulate terminal flower bud formation.

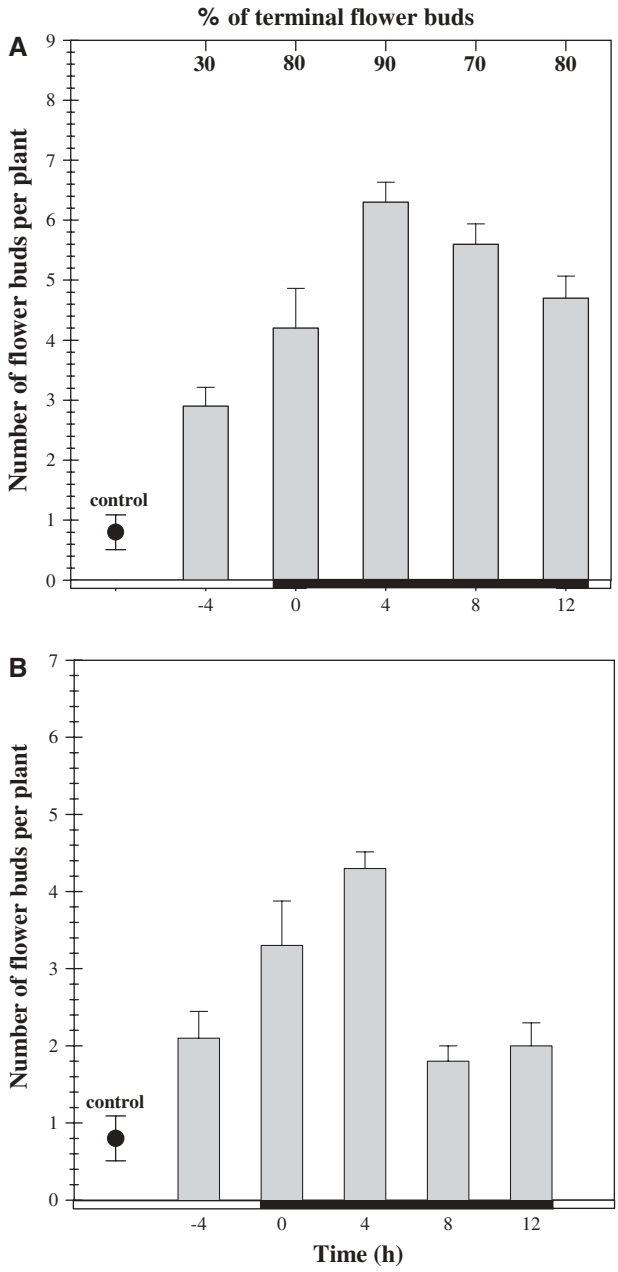

Figure 1. Effect on the flowering response of 10 mM $CaCl_2$ (A) or 40 μM cADPR (B) applied at various times before or during a 12-h-long subinductive night. Terminal flowering shows the percentage of plants with a terminal flower bud formed after $CaCl_2$ or cADPR treatment before or during subinductive night. Bars represent SE.

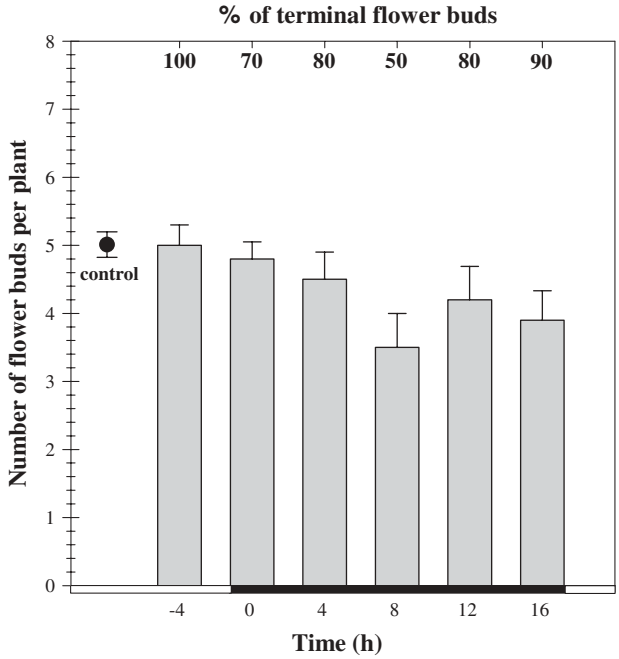

Figure 2. Effect on the flowering response of 100 μM 8-Br-cADPR applied at various times before or during a 16-h-long inductive night. Bars represent SE.

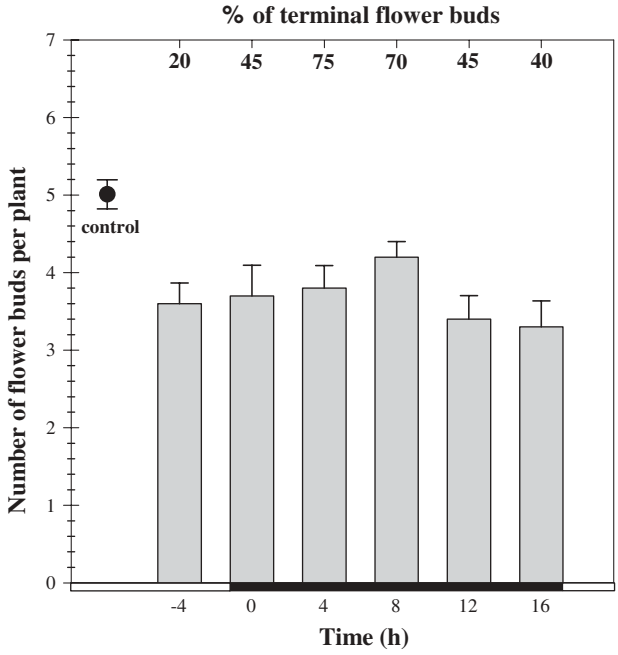

Figure 3. Effect on the flowering response of 40 mM EGTA applied at different times before or during a 16-h-long inductive night. Bars represent SE.

ADPR and NAD^+ applied on *P. nil* cotyledons in subinductive conditions did not influence the photoinduction process and plants in those experiments had the same number of flower buds as controls (data not shown).

The specificity of the response was confirmed using the antagonist of cADPR (8-Br-cADPR). Plants subjected to complete induction (16-h-long night) were treated with a 100 μM solution of 8-Br-cADPR. The control plants grown in such conditions had 5 flower buds per plant. Treatment with that antagonist caused a reduction in the number of flower buds (Figure 2). The strongest reduction (mean 2.4 flower buds per plant) was observed when applications were done at the 8th h of the inductive night. 8-Br-cADPR also inhibited terminal flower bud formation, most strongly, by as much as 50%, when this compound was applied at the 8th hour.

The effect of the calcium chelator on the induction process was also analyzed. Plants subjected to complete induction (16-h-long night) were treated with a 40 mM solution of EGTA. The control plants grown in such conditions had 5 flower buds per plant. Treatment with EGTA caused a maximal reduction of 34% in the number of flower buds, but no close relation between the time of chelator application and the degree of flowering was observed (Figure 3). EGTA also inhibited terminal flower bud formation, especially when plants were treated either at the beginning or at the end of the inductive night.

Co-application of cADPR and Ca^{2+} caused a strong stimulation of flowering (Figure 4), especially when both compounds were applied at the 4th h of the subinductive night. Application at that hour induced terminal flower bud formation in all plants.

Also co-application of cADPR with cGMP caused an increase in the number of flower buds when compared with cADPR application only (Figure 5). The strongest stimulation was observed after application at the 4th h, whereas treatments before and at the beginning of the night only slightly induced terminal flower bud formation.

The effect of RyR-channels activator and inhibitor on the induction process was analyzed. Treatment with a ryanodine solution (100 μM) stimulated flowering of plants grown under subinductive conditions (12-h-long night; Figure 6), and the intensity of stimulation was dependent on the application time. When treatment was performed before darkness the effect was more evident (mean 3.8 flower buds per plant). Also, 50% of plants formed terminal flower buds. Application during the night also caused a slight stimulation of flowering.

The inhibitor of cADPR regulated channels (ruthenium red) at 100 μM concentration applied during the induction night caused a reduction in the

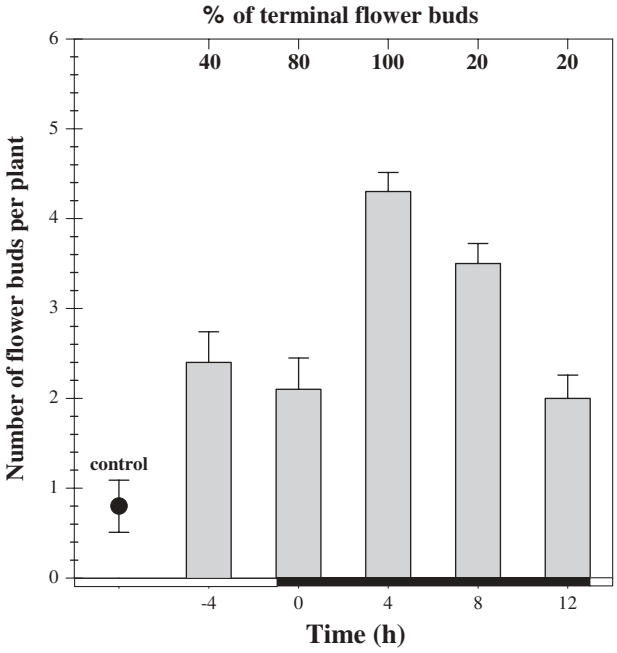

Figure 4. Effect on the flowering response of 40 μM cADPR and 10 mM $CaCl_2$ coapplied at various times before or during a 12-h-long subinductive night. Bars represent SE.

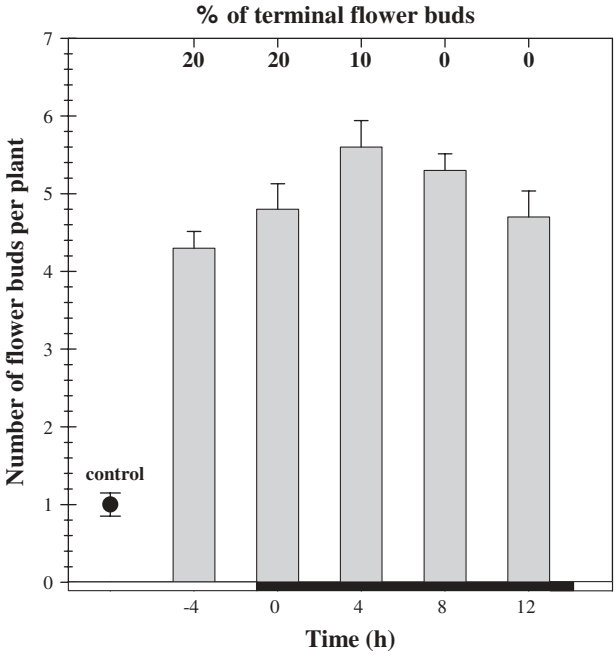

Figure 5. Effect on the flowering response of 40 μM cADPR and 0.1 mM cGMP coapplied at various times before or during a 12-h-long subinductive night. Bars represent SE.

number of flower buds (Figure 7). The strongest reduction in the number of flower buds (mean 2.6

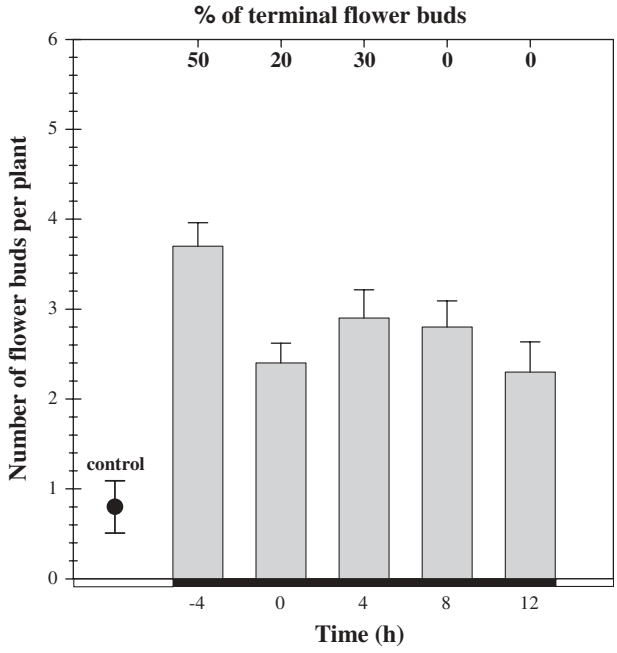

Figure 6. Effect on the flowering response of 100 μM ryanodine applied at various times before or during a 12-h-long subinductive night. Terminal flowering shows the percentage of plants with a terminal flower bud formed after treatment before or during subinductive night. Bars represent SE.

flower buds per plant) was observed when the chemical was applied at the 4th h of the inductive night. Ruthenium red also completely inhibited terminal flower bud formation.

Quantification of Intracellular Level of cADPR by HPLC

To determine if the cADPR level could be detected in *P. nil* cotyledons in various light/dark conditions we used the elegant isolation and detection system developed to measure the cADPR level in animal tissue. After some modifications we were able to isolate nucleotides from plant tissue using $HClO_4$ as an extraction reagent.

A HPLC method was used for analysis of the endogenous level of cADPR in cell extracts. The method was validated in terms of within-day and between-day reproducibility of retention times and peak areas of standard nucleotides.

Separation of standard cADPR on the strong anion-exchange column resulted in a single symmetrical peak with a retention time of 17.4 min (Figure 8A). Cyclic ADPR is unstable at high temperature, and after heating at 80°C for 2 h, it converts to ADPR, which, after separation, was

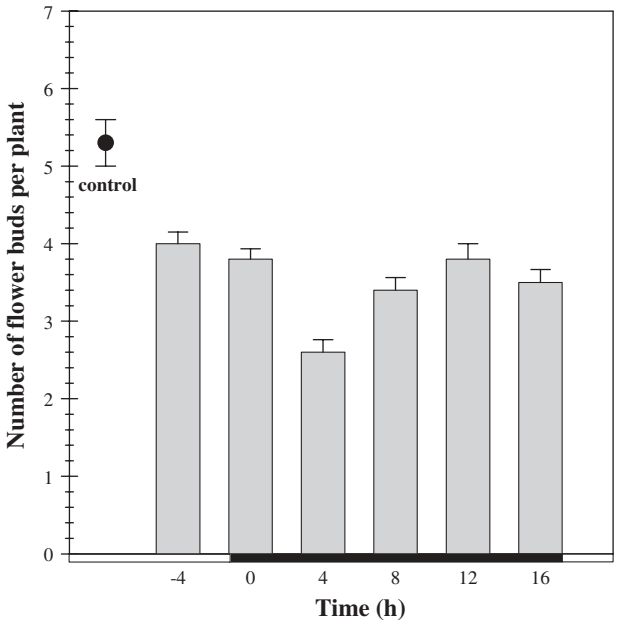

Figure 7. Effect on the flowering response of 100 μM ruthenium red applied at various times before or during a 16-h-long inductive night. Terminal flowering shows the percentage of plants with a terminal flower bud formed after treatment before or during subinductive night. Bars represent SE.

eluted from the column with a retention time of 21.2 min (Figure 8B).

Plant extracts upon separation on the strong anion-exchange column were resolved into several relatively broad peaks (Figure 8C). Taking into account that small differences in retention times might occur, the identity of this endogenous material was further confirmed by co-chromatography of the analyzed sample with standard cADPR (Figure 8D) and by its ability to be converted to ADPR upon heating the cell extract at 80°C for 2 h (Figure 8E). Concentration was demonstrated by analyzing peak areas obtained for cADPR alone or in cell samples containing a different amount of this nucleotide. In *P. nil* tissue the concentration of cADPR was 1.519 μmol/mg of fresh weight.

Finally the experiments in which the endogenous cADPR level was measured after isolation from plants grown in different photoperiodic conditions were performed. Results have shown that cADPR is present in all plant tissue but just after moving plants to the darkness the cADPR level remained the same as in light grown cotyledons. However, at the end of the induction night the concentration of cADPR was 2.839 μmol/ mg of fresh weight.

Discussion

In the present work we seek to define a role for cADPR in the photoperiodic flower induction of *Pharbitis nil*. The shift from vegetative to reproductive growth is a process that is critical to plants and is tightly regulated metabolically. Flowering time in many species is environmentally regulated by photoperiod (Vience-Prue 1994). Photoinduction processes have been the subject of decades of study. Despite this, the mechanisms involved in photoinduction processes are largely unknown. There is some evidence indicating that the second messengers including cAMP, cGMP, Ca^{2+}, $InsP_3$ and DG are involved in these processes (Shacklock and others 1992).

Pharbitis nil is a short-day plant induced to flowering by a single, 16-h-long dark period. Phytochrome is involved in this induction (Vience-Prue and Griessel 1984; Carter and others 2000). Roux (1994) suggested that the mechanism of phytochrome action is linked with the regulation of calcium ion metabolism. With time, there appeared strong evidence for the involvement of calcium ions in the photoperiodic induction of flowering in long- and short-day plants (Love and others 2004). Friedman and others (1989), Tretyn and others (1990) and Takeno (1993) found that Ca^{2+} stimulated flowering of *Pharbitis nil* when it was applied to the cotyledons before a dark period, whereas EGTA, a specific chelator of Ca^{2+} ions, inhibited flowering under the same conditions.

Flowering in the present study was inhibited by EGTA at a slightly higher concentration (40 mM) than doses that were effective in previous studies (Friedman and others 1989; Tretyn and others 1990). In our experiments there was only a 16% reduction when EGTA was applied before or during the first half of the night and 34% at the end of the night, when compared with the control. Such a discrepancy may be explained by differences in EGTA application. Spraying the seedlings to obtain full coverage (Friedman and others 1989) might cause changes in the shoot apex, making it impossible to change from vegetative to generative. Takeno (1993) also used EGTA and a perfusion method that resulted in 50% inhibition, similar to our results.

Pharmacological studies and direct measurement of [Ca^{2+}] support a role for alteration in calcium concentration in the transduction pathway regulating floral induction in response to changes in photoperiod. An important unresolved question is how changes in the endogenous Ca^{2+}

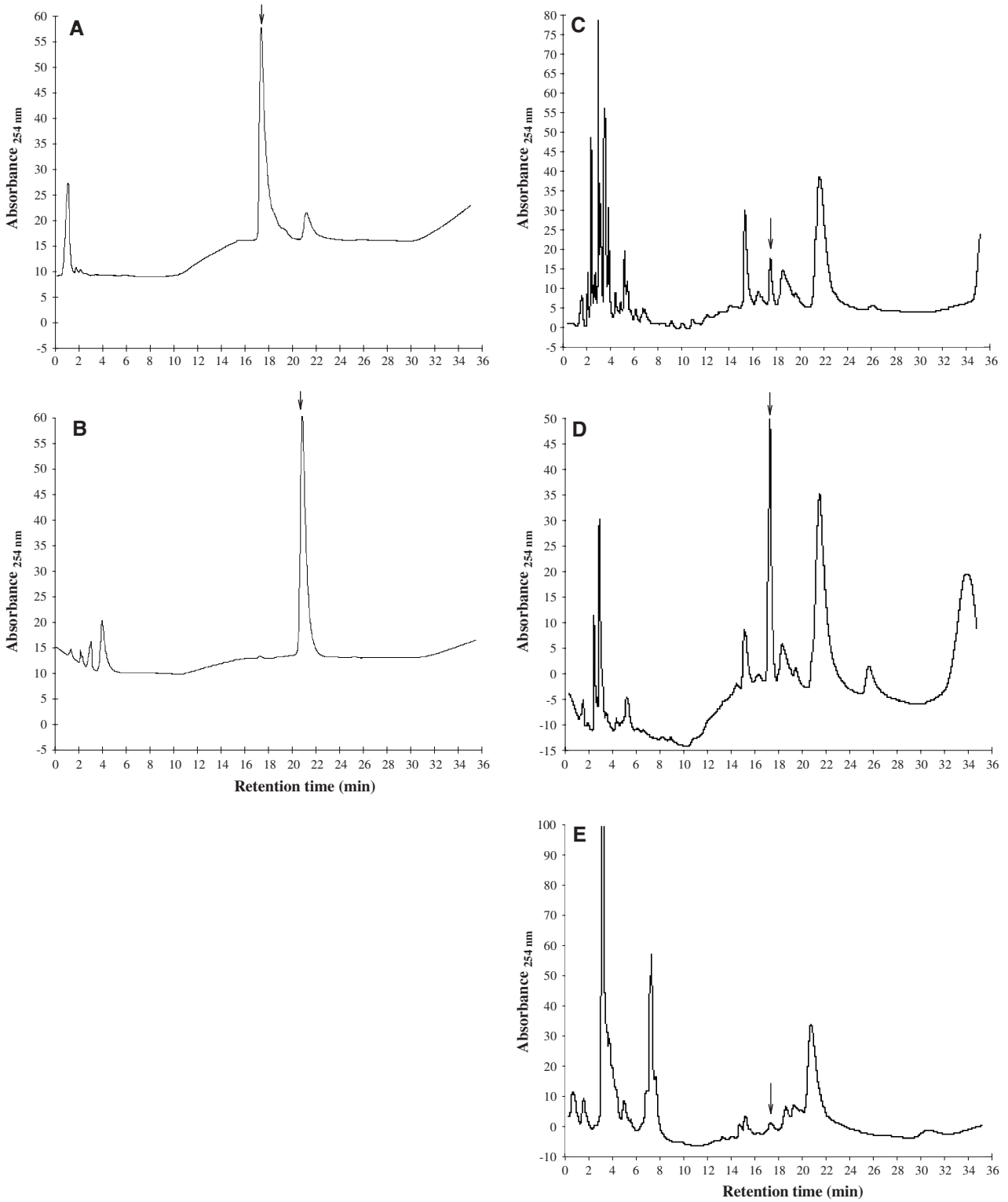

Figure 8. High performance liquid chromatography analysis of cADPR. Elution was monitored by HPLC on an anion-exchange column described in the text. (A) Standard cADPR; (B) ADPR received after heating cADPR at 80°C for 2 h; (C) crude extract as the starting material; (D) extract obtained after cADPR addition; (E) extract obtained after heating at 80°C for 2 h.

level appear and how they influence photoinduction processes.

In animals, cADPR is known to act as a transmitter in the regulation of a diverse array of physiological processes (Lee and others 1994). The widespread occurrence of cADPR in mammalian cells and the ubiquitous presence of the enzymes that synthesize and hydrolyze it indicate the generality of this Ca^{2+} release system (Galione and Sethi 1996). By contrast, whether cADPR plays any role in activating various plant processes has been less clear. As was revealed, cyclic ADPR elevates free Ca^{2+} in plants and thereby plays a central role in signal transduction pathways evoked by the drought and stress hormone abscisic acid (Navazio and others 2001; Sanchez and others 2004). cADPR is known to be involved in the abscisic acid signaling pathway in tomato and *Arabidopsis* (Leckie and others 1998; Wu and others 1997, 2003). It has also been found to elicit Ca^{2+} release from beet vacuoles (Allen and others 1995) and to play a physiological role in Ca^{2+} mobilization in *Vicia faba*, probably through modulation of vacuolar Ca^{2+} channels (Allen and others 1995). In cauliflower (*Brassica oleracea*) inflorescences, cADPR-gated Ca^{2+} release was detected in the heavy-density fractions associated with rough endoplasmatic reticulum (Navazio and others 2001).

In the present study the presence of endogenous cADPR and its ability to stimulate flowering has been demonstrated. Results have shown that the increase in the cellular concentration of cADPR, by exogenous application, may cause flowering in plants exposed to a 12-h dark period and that cellular cADPR may be primarily involved in the control of photoinduction processes. It was also revealed that neither NAD^+ nor ADP-ribose was capable of stimulating flower bud formation. It is known that both NAD^+ and ADPR have no Ca^{2+}-mobilizing activity (Galione and Sethi 1996; Lee and Aarhus 1993). In red beet microsomes, Ca^{2+} release was specific for the cyclic isomer only, because NAD^+ and ADPR were not effective in releasing Ca^{2+} (Allen and others, 1995).

Most compounds synthesized so far are antagonists of cADPR-mediated Ca^{2+} signaling pathways. In human T cells and invertebrate cells one such antagonist is 8-Br-cADPR (Graeff and others 1998; Walseth and Lee, 1993). In our experiment that compound was used to confirm the observation that cADPR can stimulate flowering. 8-Br-cADPR inhibited flower bud formation when it was applied before and during a 16-h-long inductive night. So from these experiments we can speculate that cADPR only is involved in calcium release and the flowering process.

The roles of cADPR in calcium signaling in plants are still discussed. The first is as a classical second messenger where its level is controlled by extracellular stimuli, and the second mode of cellular regulation is that the level of intracellular cADPR may set the sensitivity of rianodine receptors to activation by an influx of calcium in excitable cells. Its main signaling function is modulation of Ca^{2+}-induced Ca^{2+} release. Evidence shows that cADPR may in fact be responsible for mediating the Ca^{2+}-mobilizing activity of cyclic GMP (Durner and others 1998), especially that a soluble ADP-ribosyl cyclase is sensitive to cGMP (Graeff and others 1998).

In our previous experiments, we revealed the role of cGMP in photoperiodic flower induction (Szmidt-Jaworska and others 2004). In this work, we coapplied cADPR and cGMP. That combined application efficiently stimulated flowering, and the results obtained were similar to those observed after cGMP application only, with one exception: some plants were able to produce terminal flower buds. This result may suggest that both second messengers are involved in flowering processes.

In calcium signaling, little is known about the mechanisms of calcium release from intracellular stores during an inductive night. It is known that the endomembrane possesses release pathways for Ca^{2+}, for example, gated by voltage (Ward and others 1995), by $InsP_3$ (Allen and Sanders 1994a, 1994b) and by cADPR (Allen and others 1995). Calcium release through cyclic-nucleotide gated channels seems very important, because it could be an alternative means of calcium release, and this pathway seems independent of membrane voltage and $InsP_3$.

Cyclic ADPR has been found to mobilize Ca^{2+} in more then 20 animal cell types. It has been suggested as being the physiological modulator of the ryanodine receptor (Galione and others 1993), although there is increasing evidence that cADPR may not act directly on the ryanodine channel but that its agonistic action is mediated by an accessory protein (Walseth and others 1991), suggested as being calmodulin (Lee and others 1994).

In plants, studies on beet tap root vacuoles (Allen and others 1995) and Ca^{2+} flux measurements with microsomes have demonstrated cADPR-induced release of Ca^{2+} from plant endomembranes (Muir and Sanders 1996). The pharmacology of cADPR-induced Ca^{2+} release and sensitivity to cADPR bears many similarities to that of Ca^{2+} release via the ryanodine receptor, including modulation of the release by ryanodine and inhibition by ruthenium red (Galione and others 1991; Allen and others

1995; Muir and Sanders, 1996). At present there is no molecular evidence for the existence of a ryanodine-line Ca^{2+} release channel in plants. Only in the nucleotide database GenBank is there information that, in *Arabidopsi thaliana* and *Oryza sativa* genomes, small fragments with homology to ryanodine receptors from *Homo sapiens* exist.

To investigate the role of ryanodine and ruthenium red, we tested these compounds for the ability to stimulate the flowering process. We demonstrated that the flower bud formation process is sensitive to modulation by a ryanodine receptor agonist and antagonist in concentration ranges comparable with that observed in animal cells. The inhibition caused by ruthenium red has shown a concentration-dependent profile, and the maximum effect was achieved at a concentration of 0.1 mM. This dose dependence is like those reported in other types of cells (White and others 1993). Ruthenium red was applied to animal cells at 5–200 μM or 1 mM to the medium. We applied 100 μM to the surface of cotyledons, so it would not cause toxic effects when compared to experiments on single-cell assays and cell suspension cultures. Ruthenium red completely inhibited the cADPR-induced Ca^{2+} release in *Euglena* microsomes (Masuda and others 1997), as well as in sea urchin eggs (Galione and others 1991). Navazio and others (2001) observed that Ca^{2+} release evoked by cADPR at the endoplasmic reticulum was fully inhibited by ruthenium red. So it was a confirmation that the Ca^{2+} release pathway activated by cADPR exists in higher plants.

Ryanodine is a plant alkaloid that acts as a modulator of the ryanodine receptor in animal cells (Zimanyi and Pessah 1994). The complexity of ryanodine binding explains why, depending upon the receptor isoforms and concentration, this chemical can either activate or inhibit Ca^{2+} release. We revealed that ryanodine, at a relatively high concentration of 0.1 mM, is capable of stimulating flower induction, but a higher concentration inhibited that process. These results may suggest both the involvement of a calcium-release process in photoperiodic induction and a degree of conservation in the calcium ion release mechanism. Also, our finding that application of ryanodine and ruthenium red changed the flowering process allows us to postulate the existence of a ryanodine-like receptor in *Pharbitis nil* cells. Similar conclusions were drawn by Bauer and others (1998) and Navazio and others (2001) on the basis of the effect of ruthenium red and ryanodine on Ca^{2+}-spiking in algal cells and in cauliflower, respectively.

As already mentioned, despite information about the existence of cADPR, the precise mechanism of its action and its distribution are incompletely understood. Further insight into the signaling function of cADPR is likely to be obtained by direct analysis and quantification of the endogenous level of this compound in cellular samples.

So far the method most often used for analysis of the cADPR content of plant tissue is a bioassay based on measurements of the tissue extracts to release Ca^{2+}. In such bioassays one needs to make sure that, apart from endogenous cADPR, no other Ca^{2+}-release activators or inhibitors are present in the sample, which would strongly interfere with the bioassay. For animal tissues, a few studies on column chromatographic procedures, radioimmunoassay, and HPLC methods have been described (Kuemmerle and others 1995; Takahashi and others 1995; da Silva and others 1998). In view of this history, we have used an HPLC method for the sensitive and specific determination of the intracellular level of cADPR. Applications of this method to assays of endogenous cADPR in *P. nil* cotyledons were presented and show that the method used in our experiments for the isolation of cADPR is reasonably effective. Da Silva and others (1998), using the same method of isolation and chromatography on strong anion-exchangers for cADPR level analysis, demonstrated that the calibration curves obtained for cADPR showed a quasi-linear relationship between the peak area and the amount of the compound injected in the range of 10 pmol–2.0 nmol. In our experiments the assayed cADPR level in *P. nil* tissue was about 0.281 μmol per cotyledon. A similar level was described for *E. gracilis*, where the concentration of cADPR in cytosol is thought to be higher than 0.2 μM (Masuda and others 1997).

The recovery of the method was evaluated by quantification of the cADPR in two samples, one of which was spiked with a known amount of standard cADPR. This procedure led to recovery values of 54% ±10%. These values are in the range of previously reported recovery data, obtained with a similar extraction procedure (da Silva and others 1998). It must be mentioned that further investigation on cADPR identification in plant tissue by mass spectrometry (GC-MS) is under way.

To clarify the participation of cADPR in the induction of flowering, the level of cADPR in cotyledons during the induction night was investigated. After moving plants to darkness, no significant differences were observed. When the cADPR level was analyzed at the end of the long night its concentration was two times higher. When measurements were made at a particular time of night (4, 8, 12 h) we were not able to see any differences. There can be several explanations for this: first, cyclic ADPR is

a second messenger which, by definition, occurs for a short time and then disappears, so it is difficult to speculate when it may be present. Also, it is possible that some cADPR was lost during the extraction procedure. Finally, cADPR may act only in the part of the tissue that is connected with stimulus synthesis. Results suggest, however, that at the time when plants pass from the vegetative to the generative state, a higher level of cADPR is present. This would also explain the stimulation of flowering after exogenous application of cADPR during subinductive conditions.

In summary, *Pharbitis nil* photoperiodic flower induction is associated with changes in the level of calcium ions. Cyclic ADPR- and cADPR-regulated channels are involved in this process. To our knowledge, the existence and the level of cADPR in plant tissue have rarely been reported. The work presented here describes the method of isolation and identification of cyclic ADPR in plant tissue. Further studies may help to elucidate the molecular mechanism of light signal transduction in this system.

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