BULG. J. PLANT PHYSIOL., 2003, 29(1–2), 3–12

PHOTORECEPTOR PHYTOCHROME REGULATES THE CYCLIC GUANOSINE 3′,5′-MONOPHOSPHATE SYNTHESIS IN *AVENA SATIVA* L. CELLS

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Received November 12, 2003

Summary. The activity of guanylyl cyclase (GC) responsible for cGMP synthesis was found to be predominantly located in a fraction of plasma membranes in cells of *Avena sativa* seedlings. The GC activity was shown to depend on light conditions of plant cultivation and seedlings age. The reversible regulation of the GC activity in etiolated seedlings by red/far red light was found. This suggests that photoregulation of GC activity is mediated by phytochrome in cells of oat seedlings.

Key words: *Avena sativa* seedlings, guanylyl cyclase, photoregulation, phytochrome signal transduction, red/far-red light, subcellular fractions.

Abbreviations: P – phytochrome, R – red light, FR – far-red light, Pr – red form of phytochrome, Pfr – far-red form of phytochrome, cGMP – cyclic guanosine 3′,5′-monophosphate, GC – guanylyl cyclase, PDE – phosphodiesterase.

Introduction

Light plays a crucial role throughout the life cycle of higher plants activating photosynthesis and modulating plant growth and development from seed germination, stem elongation, leaf expansion, chloroplast biogenesis and induction of flowering to senescence. Plants have developed a complex photosensory machinery that enables them to determine the direction, duration, quality and quantity of light. Photoperception

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occurs using at least several photoreceptors: phytochrome (600–750 nm), cryptochrome and phototropin (320–500 nm) and UV-B receptor (280–320 nm). As concerns phytochrome, it is the best biochemically and physiologically characterized photoreceptor. This photoreceptor exists in two photoconvertible forms denoted P_r ($\lambda_{max} = 662$ nm) and P_{fr} ($\lambda_{max} = 730$ nm). P_{fr} is considered to be the biologically active form, capable to initiate an array of morphological, physiological and biochemical responses in plants (Smith, 2000). It is also agreed that the main targets for phytochrome in plant cell are so-called light-inducible genes.

Recently there is a great progress in the elucidation of *cis*-elements and *trans*-acting factors that mediate the response to phytochrome of genes such as *cab* and *rbcS*. Until recently, however, the molecular transduction events that couple the light perception by phytochrome located in cytosol to the changes in gene transcription in the nucleus were poorly understood. Neuhaus et al. (1993) used the single-cell assays to study phytochrome-deficient tomato mutant and demonstrated that heterotrimeric G-proteins, calcium and calmodulin were involved in phytochrome signalling. While G-protein activation could stimulate full chloroplast maturation and anthocyanin biosynthesis, calcium and calmodulin were only able to stimulate the development of immature chloroplasts lacking cytochrome b_6f and core components of photosystem I. The same group of authors showed that cyclic guanosine 3′,5′- monophosphate triggered the production of anthocyanins and combination of GMP with Ca^{2+} induced the complete maturation of chloroplasts containing all the photosynthetic constituents (Bowler et al., 1994). As a result, the three signalling pathways in phytochrome signal transduction depending on cGMP, Ca^{2+} and cGMP/Ca^{2+} were suggested. Actually, *chs*, *cab* and *fnr* genes are controlled by cGMP, Ca^{2+} and cGMP/Ca^{2+}, respectively.

Our previous data indicated the involvement of cGMP in light-controlled processes in higher plants. We showed that the cGMP concentration in the tissues of oat seedlings depends on the light conditions during plant cultivation. The level of cGMP in the tissue of etiolated oat seedlings is lower than that in the green plants and increased in response to irradiation with red light absorbed by phytochrome (Dubovskaya et al., 2001). Although cGMP is supposed to be a putative intermediate involved in transduction of a signal from phytochrome to the target elements in light-regulated plant genes, its specific position in signal transduction pathways and the mechanism of its action remains unclear. Moreover, there is no information on the localization and levels of activities of plant guanylyl cyclase (EC 4.6.1.2) and phosphodiesterase (3′,5′-cyclic nucleotide 5′-nucleotidehydrolase, EC 3.1.4.17) catalysing the synthesis and breakdown of cGMP. There is insufficient knowledge about the nature of cellular target structures to bind specifically cGMP . Only several studies on the GC and PDE activities in some plant species are published (Brown et al., 1989; Newton et al., 1984; Newton et al., 1999). Furthermore, in contrast to animals, no plant genes encoding GC and PDE have been sequenced and cloned yet.

It is obvious that enzymes involved in cGMP metabolism may play an important role in phytochrome signal transduction. The effect of light on cGMP level is thought to be basically due to modulation of the activity of GC rather than PDE. It follows from the suppression of red light-induced effect by GC inhibitor LY 83583 while PDE inhibitor theophylline is ineffective (Dubovskaya et al., 2001). In order to check this possibility the influence of light on the activity of GC was investigated. Thus, the objective of this study was to monitor the GC activity in various subcellular fractions of light- and dark-grown oat seedlings and to elucidate the involvement of this enzyme in phytochrome signalling.

Material and methods

Plants and growth conditions

The experiments were carried out with primordial leaves of 2-6-day-old oat seedlings (*Avena sativa* L. cv. Asilak) grown at 20 ºC under the conditions when 15 h polychromatic white light (30 W.m^{-2}) was alternated with 9 h continuous dark or plants were grown in continuous darkness. All manipulations with seedlings were done under safe green light (15 W incandescent lamp equipped with a glass filter of transmission between 470–605 nm, 0.1 W.m^{-2}).

Red/far-red light treatment

To convert phytochrome either to P_{fr} or P_r forms the homogenate of seedling tissue was irradiated by red light (20 W.m^{-2}) and/or far-red light (20 W.m^{-2}), respectively. To this purpose a tungsten lamp and two R and FR interference filters with transmission maxima at 667 and 730 nm, respectively, and bandwidths of 10 nm were used. The intensity of light source was measured using PMA 2140 photometer (Solar Light Company, USA).

Cell fractionation

The subcellular fractions were obtained using differential centrifugation (Morré et al., 1990). Seedling leaves were homogenized in a medium containing 50 mM Tris-HCl (pH 8.3), 0.3 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol and 2 mM phenylmethylsulfonyl fluoride. The homogenate was filtered and clarified by centrifugation at 1500×g for 10 minutes. The crude mitochondrial fraction was pelleted by centrifugation of the above supernatant at 10 000×g for 10 minutes. To obtain a soluble cytosolic fraction the produced supernatant was centrifuged at 113 000×g for 30 minutes. The pellet was resuspended and used as a crude microsomal fraction

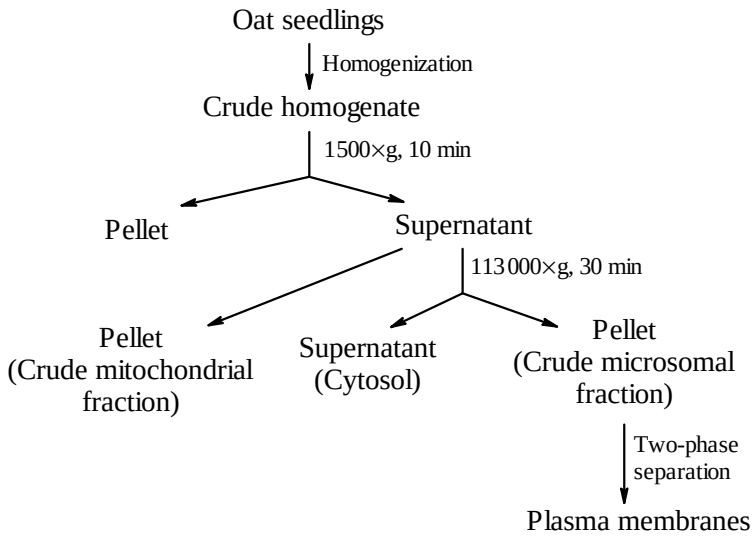

Fig. 1. The scheme of subcellular fractionation of plant material.

immediately after the separation (Fig. 1). Plasma membranes were isolated from the crude microsomal fraction according to the two-phase method designed by Larsson et al. (1987). The obtained fraction of plasma membranes was enriched by marker activity of K^+-stimulated Mg^{2+}-dependent vanadate-inhibited ATPase (Hodges et al., 1972). All operations were carried out at 0–4 ºC.

Guanylyl cyclase activity

GC activity was measured by estimating the rate of cGMP formation from Mn^{2+}-GTP in the reaction mixture containing 175 mM Tris-HCl, 100 mM $MnCl_2$, 20 mM theophylline, 1 mM GTP and 0.06 mg of total protein in final volume of 0.25 ml, pH 7.9. The modulators were introducedd directly to the incubation medium with the final concentrations being equal to 5 mM of Ca^{2+} and 10 μM of GTPγS and GDPβS. The samples containing all reagents except protein were preincubated at 25 ºC for 20 s before initiation of the reaction by adding the protein preparation. After the start of reaction the samples were incubated at 25 ºC for 10 min at constant shaking. The reaction was terminated by adding to each sample 0.25 ml 0.2 M zinc acetate solution followed by placing them in ice-bath. After termination of the reaction, 0.25 ml of 0.2 M Na_2CO_3 was added, and the solution was mixed, frozen at –20 ºC or –70 ºC, thawed, mixed again and centrifuged in order to obtain a tightly packed precipitate containing nucleotides GMP, GDP and GTP coprecipitated with $ZnCO_3$ (Garbers et al., 1974). The concentration of formed cGMP in the obtained supernatant was measured by RIA-([^{125}I]-cGMP)-kit produced by the Institute of Bioorganic Chemistry of the National

Academy of Sciences of Belarus (Minsk, Belarus). The minimal concentration of cGMP estimated using this kit was 0,05 nmol l^{-1}. Protein content in the individual fractions was determined according to the method of Bradford (1976).

Statistical analysis

The assays were performed in duplicate. The figures and tables represent the means and their standard deviations (n = 3).

Results and discussion

The data on GC activity in individual fractions obtained from leaves of 5-day-old oat seedlings showed that the GC activity was detected in all subcellular fractions, however, the highest GC activity was observed in plasma membranes (Table 1). Therefore, GC in oat cell is likely to occur predominantly bound to membranes. Actually, an endogenous activator of membrane-bound intestinal GC in animal systems guanylin (Currie et al., 1992) increased the GC activity in plant tissue homogenate almost 5-fold (data not shown).

Under optimum conditions of incubation the GC activity in the fraction of plasma membranes isolated from green oat seedlings was as high as 55.7±03 fmol cGMP mg^{-1}(protein) s^{-1} (Table 1), which is more than 2-fold higher than the specific GC activity (20 fmol cGMP mg^{-1}(protein) s^{-1}) in intact chloroplasts from *Phaseolus vulgaris* measured by Newton et al. (1984) using the conversion of labelled [^{32}P]-GTP into [^{32}P]-cGMP.

Table 1. The guanylyl cyclase (GC) activity in subcellular fractions of tissue homogenate of 5-day-old *Avena sativa* L. seedlings. The GC activity was determined by RIA after incubation of samples at 25 ºC for 10 min in medium of pH 7.9 containing 1 mM GTP and 0.06 mg of protein and is expressed as cGMP formed by milligramme of total protein per one second. The assays were performed in duplicate. The values are means ±SD (n = 3).

Fraction	Guanylyl cyclase activity (fmol(cGMP) mg^{-1}(protein) s^{-1})	
	Light-grown plants	Dark-grown plants
Crude homogenate	30.8±1.7	0.84±0.5
1500g pellet	23.7±0.8	1.05±1.7
Crude mitochondrial fraction	21.5±0.3	1.11±2.0
Crude microsomal fraction	28.8±1.0	1.28±1.3
Plasma membranes	55.7±0.3	1.78±0.5
Cytosol	12.5±1.3	0.56±0.8

The values of apparent $K_m = 4{,}2$ mM and $V_{max} = 252$ fmol mg^{-1}(protein) s^{-1} were comparable with those estimated in homogenates of animal tissues (Krishnan et al., 1976). Taking of these results into account, it is possible to believe that cellular amount of cGMP is sufficient to consider this cyclic mononucleotide as a messenger in higher plants.

To answer the question whether the cGMP synthesis in plant cell is under light control, the GC activity in homogenates of leaf tissue of green and etiolated *Avena sativa* seedlings of different age were compared. The data in Fig. 2 indicate that the activity of this enzyme in oat seedlings was dependent on light conditions during plant

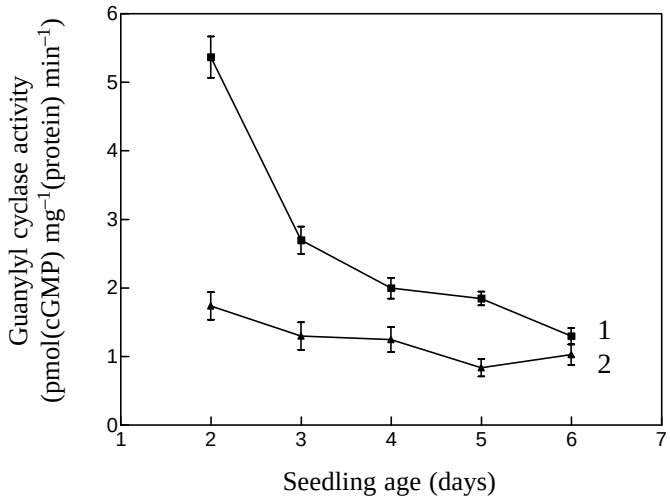

Fig. 2. The guanylyl cyclase activity in homogenate of green (1) and etiolated (2) *Avena sativa* seedlings of different ages. Plants were grown at 20 ºC under conditions of 15 h polychromatic white light (30 $W.m^{-2}$)/9 h complete dark (1) or grown in complete darkness (2). The assays were performed in duplicate. The values are means ±SD (n = 3).

cultivation. In green seedlings the GC activity was higher than that in etiolated plants. The data presented in Table 1 showed that this was the case for all subcellular fractions isolated from both etiolated and green seedlings. Besides, the GC activity in oat seedlings, especially grown under light conditions was inversely related to their age. In 2-day-old plants the GC activity was much higher than that in 6-day-old seedlings (Fig. 2). This fact is likely to explain that higher cGMP content observed in tissue of green seedlings as compared with etiolated plants decreased with seedling age as shown earlier in our laboratory (Dubovskaya et al., 2001). Thus, these data mean that cGMP anabolism in oat cells is controlled by light.

In order to clarify whether relationship between the GC activity and phytochrome exists experiments on R/FR-reversibility were performed. It is known that far-red light

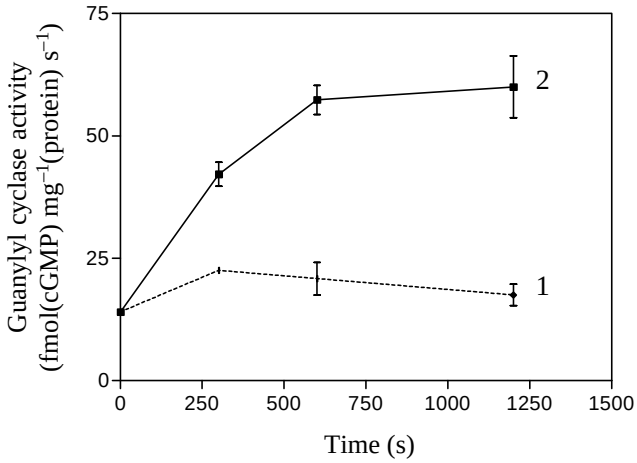

Fig. 3. Effect of red light (R; $\lambda = 667$ nm, $\Delta\lambda = 10$ nm; 20 W.m^{-2}) (2) on the guanylyl cyclase activity in homogenate of 5-day-old etiolated *Avena sativa* seedlings. 1 – untreated control. The assays were performed in duplicate. The values are means ±SD (n = 3).

reverses the red light-induced effects if it is used just after red light illumination, as it was found for the phytochrome-dependent swelling and Ca^{2+} accumulation in protoplasts isolated from etiolated wheat (Bossen et al., 1988) and oat (Sokolovsky et al., 1996) plants. Similarly, the R/FR reversibility of phytochrome-dependent increase in cGMP concentration was revealed (Dubovskaya et al., 2001). This increase was explained by both red light activation of GC and PDE inhibition.

The experiments on light influence on the GC activity showed that red light-induced effect was developed within about 600 s after the beginning of homogenate irradiation (Fig. 3). When tissue homogenate was illuminated during the time exceeding 600 s, the enzyme activity did not increase any more. It is evident from Fig. 4 that red light considerably enhanced the GC activity (4-fold), while FR was almost ineffective. However, red light irradiation followed by far-red light-treatment suppressed the stimulating effect of red light. This did not occur while darkness instead of far-red light was used. Thus, the photocontrol of cGMP content in oat plant cell is related to photostimulation of the GC activity by red light.

It is tempting to assume after Bowler et al. (1994) that similarly to many phytochrome-dependent phenomena the photoregulation of the GC activity in oat seedlings is likely to associate with GTP-binding proteins. Actually, non-hydrolysable GTP analogue guanosine 5′-[β-thio]diphosphate (GTPβS) inhibiting the activity of G-proteins in animals was found to suppress both the GC activity itself and red light-induced increase in the GC activity in oat tissue homogenates (Fig. 5). On the contrary, the activator of animal G-proteins guanosine 5′-[γ-thio]triphosphate (GTPγS) exerted an opposite effects. This means that the activity of membrane-bound GC is likely to be

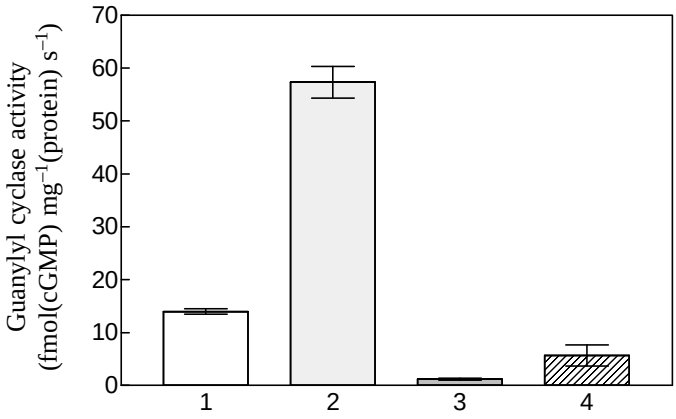

Fig. 4. Effect of red light (R; λ = 667 nm, Δλ = 10 nm; 20 W.m^{-2}, 600 s) (2), far-red light (FR;λ = 730 nm, Δλ = 10 nm; 20 W.m^{-2}, 600 s) (3) and far-red light used immediately after red light illumination (4) on the guanylyl cyclase activity in homogenate of 5-day-old etiolated *Avena sativa* seedlings. 1 – untreated control. The assays were performed in duplicate. The values are means ±SD (n = 3).

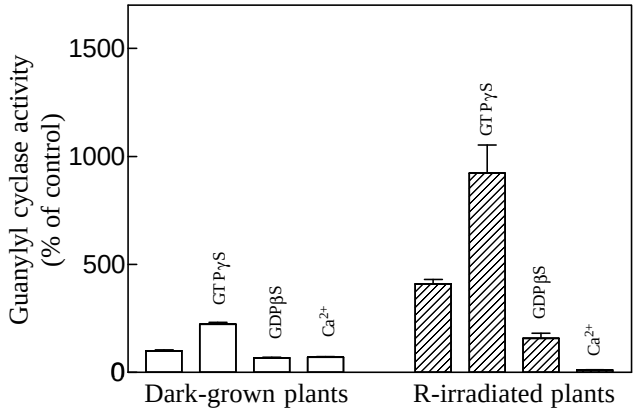

Fig. 5. Effect of GTPγS (10 μM), GDPβS (10 μM) and Ca^{2+} (5 mM) on the red light-stimulated (R; λ = 667 nm, Δλ = 10 nm; 20 W.m^{-2}, 600 s) guanylyl cyclase activity in homogenate of 5-day-old dark-grown *Avena sativa* L. seedlings. The GC activity in untreated homogenate was taken as 100%. The assays were performed in duplicate. The values are means ±SD (n = 3).

controlled by G-proteins in plant cell and G-proteins are involved in the regulation of GC activity and the increase in cGMP concentration in plant cell by light. The above data are rather in accordance with those by Okamota et al. (2001) who have shown that overexpression of the heterotrimeric G-protein alpha subunit enhances phyto-

chrome-mediated inhibition of hypocotyls elongation. In contrast to that however, Jones et al. (2003) have found that single- and double-null mutants for the *GPA1*, *AGB1* genes encoding the alpha and beta subunits of heterotrimeric G-protein, respectively, have wild-type sensitivity to R and FR.

Calcium and calmodulin play the role of messengers in phytochrome signalling acting in the pathway downstream G-proteins. Bowler et al. (1994) found that close relationship among three possible pathways of phytochrome transduction in plant occurred. Actually, the increase in cGMP concentration inhibits signalling along Ca^{2+} and Ca^{2+}/cGMP-dependent pathways. Conversely, Ca^{2+} suppresses cGMP-dependent pathway thus indicating a cross-talk within the system of phytochrome signalling.

We revealed that calcium ions inhibited both GC activity and red light-induced increase of GC activity (Fig. 5) in accordance with negative feed-back relations of cross-talk. The most attractive point was the action of Ca^{2+} that eliminated completely the activating effect of red light thus suggesting a possible cGMP involvement in light-induced modulation.

Therefore, one can conclude that functionally active GC in *Avena sativa* plant cells has the characteristics comparable with those of animal enzyme. These data testify that phytochrome is involved in photoregulation of the GC activity in oat seedlings. Functional relationship between the plant photoreceptor phytochrome and GC responsible for GMP synthesis is established. The GC activity depends on light conditions of plant cultivation and is controlled by R/FR-illumination.

Acknowledgment: The work was supported by the Belarussian Republican Foundation of Fundamental Research (project no. Á02M-081).

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