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Biochemical evidence that phytochrome of the moss *Ceratodon purpureus* is a light-regulated protein kinase

Patricia Algarra[a], Stefan Linder[b] and Fritz Thümmler[b]

[a]*Departamento de Ecologia, Universidad de Malaga, Campus de Teatinos, 29071 Malaga, Spain* and [b]*Botanisches Institut der Universität München, Menzingerstr. 67, D-8000 München 19, Germany*

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The phytochrome gene of the moss *Ceratodon purpureus* (*phyCer*) codes for a novel phytochrome polypeptide with a predicted molecular mass of 145 kDa that has a COOH-terminal domain which is homologous to the catalytic domain of eukaryotic protein kinases. In this paper we report the first biochemical evidence that in fact, as predicted from the gene sequence, PhyCer represents an active, light-regulated protein kinase. In vitro phosphorylation experiments with protonemata extracts revealed the existence of a 140 kDa protein, phosphorylated in a red/far-red light dependent manner. The binding of a polyclonal antibody directed to the protein kinase catalytic domain of PhyCer enhanced the phosphorylation of a 140 kDa band when assayed in a renaturation–auto-phosphorylation experiment with nitrocellulose bound protein. These findings strongly implicate that the *phyCer* gene product has protein kinase activity and is capable of auto-phosphorylation. The results of the renaturation–phosphorylation experiments were essentially the same, no matter whether protein extracts from light grown or dark adapted moss protonemata were used. Thus, *phyCer* expression most likely is not light regulated.

Auto-phosphorylation; Photoreceptor; Phytochrome; Plant signal transduction; Protein kinase; *Ceratodon purpureus*

1. INTRODUCTION

With respect to physical, biochemical and molecular properties phytochrome is the best-characterized plant photoreceptor (recent reviews [1,2]). However, in spite of much effort, phytochrome dependent signal transduction pathways are still poorly understood. Recently, we have isolated and characterized the phytochrome gene of the moss *Ceratodon purpureus* (*phyCer*) [3,4]. Interestingly, *phyCer* turned out to code for a novel and unusual phytochrome polypeptide which is composed of a conserved NH_2-terminal chromophore domain and a COOH-terminal domain which, in contrast to all known phytochromes, is homologous to the catalytic domain of eukaryotic protein kinases.

Protein phosphorylation plays an important role in regulatory and signal transducing processes not only in animal systems [5] but also in plants [6–8]. Several genes encoding plant protein kinases have been cloned and characterized (e.g. [9–11]) and like in other eukaryotes, also the plant protein kinase gene family seems to have a great complexity (e.g. [12]). Still little is known about the function of these kinases in signal transduction cascades. Changes in protein phosphorylation patterns in response to phytochrome have been reported [13–17]. The possibility that phytochrome itself could be a protein kinase has been discussed [18,19] but was finally attributed to a contaminant protein kinase within the purified phytochrome samples [20,21].

In this paper we present biochemical evidence that in *C. purpureus* protonemata, as was expected from the nucleotide sequence of *phyCer* [4], the photoreceptor molecule phytochrome itself is an active, light-regulated protein kinase.

Correspondence address: F. Thümmler, Botanisches Institut der Universität München, Menzingerstr. 67, D-8000 München 19, Germany. Fax: (49) (89) 1782 274.

2. MATERIALS AND METHODS

2.1. *Plant material and light sources*

C. purpureus (Hedw.) Brid. protonemata were grown in sterile liquid cultures or on solid medium [4,22] under a light regime of 18 h light:6 h dark. When the protonemata were 5–6 days old, one set of them was transferred to darkness for two days. Harvesting and further handlings were performed under a dim green safelight, for both light and dark cultures. Irradiations were carried out with gallium aluminum arsenide (GaAlAs) LED's from the top in 1.5 ml Eppendorf tubes for two minutes. The distance between LED and sample was about 5 cm. Red light irradiation was carried out with Stanley-LED ER-700-L (Elite electronic, Steinhöring, Germany) with peak wavelength of 660 nm; far-red light irradiations were carried out with Hitachi-LED HE7601SD (Gerhard Franck Optronic, GFO, Hamburg, Germany) with peak wavelength of 760 nm.

2.2. *Preparation of protein extracts*

The harvested protonemata (100–400 mg) were ground under liquid nitrogen. The fine powder was suspended in extraction buffer (100 mM Tris-HCl, pH 7.5, 10 mM NaCl, 2 mM DTT, 4 mM PMSF, 2% Triton X-100, 20% glycerine; 100 μl/100 mg protonemata), incubated 20 min on ice and centrifuged at 12 000 × *g* for 10 min at 4°C. The supernatant was directly used or stored at −80 °C.

2.3. *In vitro phosphorylation*

Samples were irradiated according to the different experimental designs. After the light treatments 5 μl samples (about 25 μg of total protein) were incubated in standard kinase assay buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 1 mM Na-orthovanadate, 10 mM NaF, 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham)) for 2 min under green safelight. The final volume was 25 μl. The reaction was stopped by addition of 10 μl sample buffer (110 mM Tris-HCl, pH 6.8, 4% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 20 % (v/v) glycerine and 0.5% (w/v) Bromophenol blue) and immediate boiling for 2 min. SDS-PAGE was carried out according to Laemmli [23] on 8% gels. Molecular markers were obtained from Sigma (SDS-6). The stained gel was dried by vacuum heat drying and exposed to Hyper*film*-MP (Amersham) X-ray film at −80°C using an intensifying screen (Cruix Blue U8, AGFA).

2.4. *Auto-phosphorylation reaction*

About 100 μg total protein from dark and light grown protonemata were electophoresed on a 8% SDS-PAGE as described above. Fractionated proteins were electrotransferred to nitrocellulose (Schleicher and Schuell) in cold transfer buffer [25 mM Tris, 192 mM glycine] according to Celenza and Carlson [24]. The transfer was carried out in a cold room at 400 mA for 2 h. To activate the auto-phosphorylation of the protein kinases, the membrane-bound proteins were denaturated in denaturation buffer (7 M urea, 50 mM Tris, pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 2 mM EDTA) for 10 min at room temperature and renaturated with renaturation buffer (2 mM EDTA, 0.25% (w/v) Blocking Reagent (Boehringer), 0.1% (v/v) 2-mercaptoethanol in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100) overnight in a cold room. The next day the membranes were blocked with 1% (w/v) Blocking Reagent in TBST for 1 hour at room temperature. Subsequently the filters were either incubated with pre-immunoserum (1:5000 dilution) or the purified polyclonal Cerkin1 antibody (1:2500 dilution; see below) in TBST for 1 hour. After 3 times washing with TBST the membranes were equilibrated in kinase standard buffer (as described above but without anti-phosphatases) and incubated for 10 min with 200 μCi [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) per 1 ml kinase buffer. The filters were washed overnight with 100 mM potassium phosphate buffer (pH 7.5, 2 mM EDTA, 0.5% (v/v) Triton X-100). Autoradiography was performed as described above. When desired, the nitrocellulose sheets were incubated subsequently with a second antibody, alkaline phosphatase-conjugated goat antibody to rabbit IgG (Sigma) in a 1/2000 dilution and developed according to Knecht and Dimond [25].

2.5. *Production and purification of the antibody to the PhyCer COOH-terminal domain (Cerkin1)*

Escherichia coli DH5α was transformed with a chimeric expression vector carrying the protein kinase catalytic domain of *phyCer*. The construct was made in the plasmid vector *pMAL-cRI* (Biolabs, New England). The 869 bp (*Bam*HI-*Hin*dIII) fragment coding for the catalytic kinase domain of *phyCer* (Cerkin1) [4] was ligated into the *Bam*HI and *Hin*dIII sites of the polylinker of *pMAL-cRI*. The DNA fragment was inserted between the *malE* gene which encodes maltose-binding protein (MBP) and the *LacZ*α gene resulting in the expression of a MBP fusion protein. The system uses the strong 'tac' promoter and the *malE* translation initiation signals to give high-level expression of the cloned sequence. The transformants were grown in LB medium (standard techniques according to Ausubel et al. [26]) at 37°C with slow shaking for 4 h in the presence of 0.3 mM isopropylthiogalactoside (IPTG) to induce expression of the MBP-Cerkin1 fusion protein. The fusion protein was purified using the purification kit from Biolabs on an amylose resin column using MBP's affinity for maltose. The fusion protein was eluted with free maltose. The purified fusion protein was resolved by SDS-PAGE. The MBP-Cerkin1 gene product was detected as a 77 kDa protein band (not shown).

A rabbit was immunized subcutaneously 2 times at 4 weeks interval with 125 μg protein previously dialyzed against 20 mM Tris-HCl, pH 8, 100 mM NaCl and urea at different concentrations, from 7 M to 0 M. The protein was mixed 1:1 with Freund's complete adjuvant. One week after each inoculation the blood was collected (20–30 ml), centrifuged twice at 3000 × *g* and 15 000 × *g* for 10 and 5 min respectively and heated at 56°C for 20 min. In order to remove anti-MBP antibodies from the serum, the immunoserum was passed over an activated Sepharose 4B column (Sigma) to which MBP-LacZα fusion protein was bound covalently. Production and isolation of MBP-LacZα was essentially as described for the isolation of MBP-Cerkin1 using *E. coli* cells transformed with non-recombinant *pMal-cRI* vector. The binding of MBP-LacZα to activated Sepharose 4B was performed according to Ausubel et al. [26]. With this purification step it was not possible to completely remove reactivity of the serum to MBP in Western blot experiments (see Fig. 2Bb, lanes 1 and 2). A second purification step was performed through a MBP-Cerkin1 bound Sepharose column to retain only the specific antibody against the MBP-Cerkin1 fusion protein.

2.6. *Characterization of the specificity of the Cerkin1 antibody*

For testing the specificity of the antibody a second construct was made because, for unknown reasons, the MBP-Cerkin1 fusion protein was not cleavable with factor Xa. Cleavage of MBP-fusion protein with the factor Xa usually separates the maltose binding protein from the protein of interest (see Biolabs manual). In the second case the 1873 bp (*Eco*RV-*Xba*I) fragment (1404 bp of which code for the COOH-terminal domain of PhyCer, the remaining 469 bp represent 3′ non-coding nucleotides [4]) was ligated into the *Eco*RI site (blunted with the Klenow enzyme) and the *Xba*I site of the polylinker of *pMal-cRI* to yield the MBP-Cerkin2 fusion protein. The MBP-Cerkin2 gene product was detected as a 95 kDa protein, together with smaller degradation products, when analyzed by SDS-PAGE (see Fig. 2A, lane 4). The MBP-Cerkin2 protein was purified as described for MBP-Cerkin1. Purified MBP-Cerkin2 and for comparison MBP-LacZα fusion protein were cleaved with factor Xa (according to the Biolabs manual) and subsequently the cleavage products were resolved by SDS-PAGE and blotted onto nitrocellulose (Schleicher and Schuell). Identical filters were incubated either with Cerkin1 antibody (1:2500 dilution), anti-MBP serum (Biolabs) (1:10,000 dilution) or pre-immunoserum (1:5000 dilution) and developed as described above.

3. RESULTS

To prove that in the moss *C. purpureus* phytochrome is in fact a protein kinase, as was suggested by its gene sequence, we performed in vitro phosphorylation experiments with protein extracts from dark adapted *C. purpureus* protonemata after a defined irradiation program. A typical result of such an experiment is shown in Fig. 1A. A short red light pulse (R) leads to changes in the in vitro phosphorylation pattern, i.e. a 50 kDa protein which becomes phosphorylated in the extract kept in the dark (marked with an asterisk in lane 4) is not phosphorylated after R (lane 1). The molecular weight of the deduced *C. purpureus* phytochrome polypeptide (PhyCer) was calculated to be 145 kDa [4]; interestingly, the phosphorylation of a protein with an apparent molecular weight of about 140 kDa is enhanced after R (lane 1). The changes of the phosphorylation pattern induced by R could not be reversed by a subsequent far-red light pulse (F) (lane 2) but F, when given alone, abolished the phosphorylation of the 140 kDa protein completely (lane 3). In order to investigate whether the R/FR dependent phosphorylation of the 140 kDa protein could be related to a light dependent

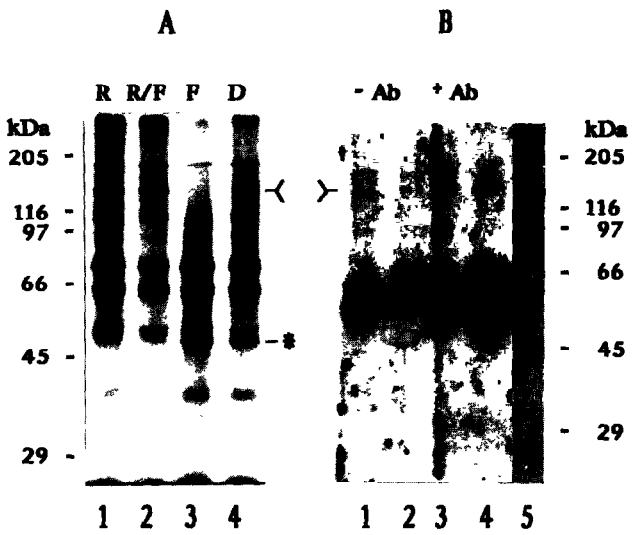

Fig. 1. In vitro phosphorylation and immunocharacterization of *C. purpureus* protonematal proteins. (A) SDS-PAGE analysis. The proteins contained in extract from dark grown protonemata (25 μg per lane) were incubated with [γ-^{32}P]ATP for 2 min at room temperature (RT) under green safe light after different light treatments and separated by SDS-PAGE using a 8% gel. After staining with Coomassie blue the gel was autoradiographed for 1 day using an AGFA intensifying screen. Irradiation was as follows: lane 1, 2 min red light (R) followed by 2 min darkness; lane 2, 2 min red light followed by 2 min far-red (R/F); lane 3, 2 min far-red light (F) followed by 2 min darkness; lane 4, dark control (D). (B) Auto-phosphorylation and immunoblot analysis. Protein contained in extracts from light grown (lanes 1 and 3) and from 2 days dark adapted (lanes 2 and 4) protonemata (100 μg per lane) were separated by SDS-PAGE using a 8% gel and transferred to nitrocellulose at 4°C for 2 h. The filter bound proteins were denaturated for 10 min with buffer containing 7 M urea at RT and renaturated overnight at 4°C with buffer without urea. Subsequently the filters were first incubated either with pre-immunoserum (lanes 1 and 2) or with PhyCer antibody (lanes 3 and 4) at RT for 1 h and finally incubated in the presence of [γ-^{32}P]ATP for 10 min at RT. After washing the filters were submitted to autoradiography for 6 days using an AGFA intensifying screen. After autoradiography lane 2 was incubated with PhyCer antibody and a second antibody, conjugated to alkaline phosphatase and developed (lane 5). The presumed PhyCer band is marked with an arrow head, the protein which is phosphorylated only in the dark control and in the sample irradiated solely with far-red light is marked with an asterisk.

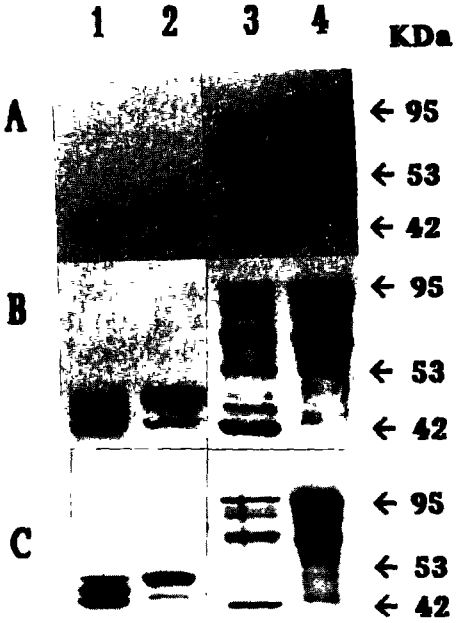

Fig. 2. Immunoreactivity of MBP-Cerkin1 antibody against the *phyCer* gene product. Proteins were resolved by 10% SDS-PAGE. Identical gels were either (A) stained with Coomassie blue or blotted to nitrocellulose and (B) immunostained with Cerkin1 antibody or (C) anti-MBP serum (Biolabs). Lane 1: purified MBP-LacZα fusion protein (10 μg) partially cleaved with factor Xa. Lane 2: purified MBP-LacZα fusion protein (10 μg). Lane 3: purified MBP-Cerkin2 fusion protein (13 μg) partially cleaved with factor Xa. Lane 4: purified MBP-Cerkin2 fusion protein (13 μg). Molecular weights of MBP-Cerkin2 fusion protein (95 kDa), pure MBP (42 kDa) and pure Cerkin2 protein (53 kDa) were derived from the respective gene sequences and are given on the right.

auto-phosphorylation of PhyCer, we performed experiments as described by Celenza and Carlson [24]. This method consists in the renaturation of protein kinase activity on protein blots followed by an auto-phosphorylation reaction with radioactive labelled [γ-^{32}P]ATP. This is a rapid and powerful method which was used to demonstrate that a gene with sequence homology to protein kinase does in fact encode a functional protein kinase [24]. The auto-phosphorylation reaction revealed the existence of a phosphorylated band with a molecular weight around 140 kDa (Fig. 1B, lanes 1 and 2; marked with an arrowhead). The main phosphorylation products, most likely the results of the activities of renaturated protein kinases, were in the range between 50 and 60 kDa. To test whether the high molecular weight phosphorylated band could be related to the *phyCer* gene product, the blot was subsequently incubated with a polyclonal antibody directed to the protein kinase catalytic domain of PhyCer. This antibody was prepared by immunizing rabbits with a purified bacterial fusion protein composed NH_2-terminal of a maltose binding protein and COOH-terminal of the protein kinase catalytic domain of PhyCer (Cerkin1). The fusion protein was produced with the help of an *Escherichia coli* chimeric expression vector (see Materials and Methods). Upon immunostaining of the filter we were not able to detect the PhyCer polypeptide at around 140 kDa; only a cross-reacting protein with an apparent molecular weight of about 90 kDa (not detected with pre-immunoserum) was visible (Fig. 1B, lane 5), a position where no radioactive labelled band could be detected (see Fig. 1B, lanes 1 to 4). Nevertheless, when an identical blot was incubated with the antibody prior to the auto-phosphorylation reaction, the radioactivity of the 140 kDa band was enhanced significantly (Fig. 1B, lanes 3 and 4). In the literature it is reported that in immunocomplexes the phosphorylating activity of protein kinases can be stimulated [24] (probably the bound antibodies can serve as substrates for the respective kinases).

In Fig. 2 the reactivity of the Cerkin1 antibody against the *phyCer* gene product is demonstrated. MBP-LacZα (lanes 1 and 2) and MBP-Cerkin2 (lanes 2 and 3; see Materials and Methods) fusion proteins were cleaved with the factor Xa. Factor Xa separates the maltose binding protein (42 kDa) from the LacZα fragment (8 kDa) and from the Cerkin2 protein (53 kDa) respectively. Western blot analysis of the cleavage products revealed that: (i) MBP-Cerkin1 antibody recognises MBP (Fig. 2B, lanes 1 and 2) as well as Cerkin2 (the 53 kDa band in Fig. 2B, lane 3) and (ii) that anti-MBP serum does not recognise Cerkin2 (the 53 kDa band is missing in Fig. 2C, lane 3). These data demonstrate that the rabbit had produced antibodies not only against the maltose binding protein domain but also against the COOH-terminal domain of PhyCer. No staining was observed when pre-immunoserum was used in the experiment (not shown). From these experiments we conclude that the auto-phosphorylating 140 kDa polypeptide is a light regulated protein kinase representing the *phycer* gene product.

4. DISCUSSION

To investigate the phosphorylating capacity of the *phyCer*gene product we chose to use an in vitro phosphorylation approach for the following reasons: (i) because no interference occurs with light dependent, electron transfer coupled phosphorylation activities found in intact chloroplasts [8]; (ii) much higher rates of incorporation of labelled phosphorus into proteins are achieved compared to in vivo labelling methods; and (iii) because of the observation that blue-light dependent phosphorylation takes also place with isolated pea membrane fractions (e.g [27]). The fact that R dependent changes in phosphorylation can not be reverted by subsequent F does not contradict the proposal that phytochrome is responsible for the light dependent changes in phosphorylation. As was recently pointed out by Short et al. [27], the observed phosphorylation of an in vitro kinase experiment is the result of many parameters such as the presence of amino acid residues which are capable of being phosphorylated (and are not already blocked by cold phosphorus) and the state of activity of the protein kinase after extraction. With respect to *C. purpureus* protonemata the situation could be interpreted as follows: only PhyCer in the Pfr form (the physiological active form of phytochrome which is present in the light) and not in the Pr form (physiological inactive form which is synthesized in the dark, also formed after F treatment of Pfr) can be phosphorylated. The 140 kDa band in Fig. 1A, lane 4, could be the result of residual, stable Pfr (the protonemata were grown in the light and kept in the dark for two days prior to protein extraction). This is likely because PhyCer has the clear features of light stable (type II) phytochrome [4]. In vitro photoconversion of PhyCer with R probably results in a Pfr species which cannot be reverted back to Pr under the experimental conditions. In contrast, F before R treatment probably is capable to quantitatively revert residual Pfr back to Pr which is not phosphorylated in vitro (Fig. 1A, lane 3). From the immunoblot-kinase experiments we conclude that PhyCer is in fact a protein kinase capable to auto-phosphorylate. That the increase of phosphorylation of the 140 kDa band (Fig. 1B) is not the result of an unspecific binding of labelled ATP to the immuncomplex is strengthened by the observation that the immunostained band at 90 kDa (Fig. 1B, lane 5) is not labelled in the experiment (Fig. 1B, lanes 3 and 4). This 90 kDa protein probably represents another, abundant protein kinase, cross-reacting with the Cerkin1 antibody, which is not active under the experimental conditions or might represent an unknown protein reacting with the anti-MBP antibody also present in the serum. The incubation of an identical filter with [α-^{32}P]ATP did not result in any labelled band (data not shown) which further demonstrates that the radiolabelled bands on the filters are not the results of unspecific binding of ATP but are rather due to phosphorylating activities of the respective proteins. The experiment shown in Fig. 1B also implicates that PhyCer expression is not effected by light. The phosphorylation of the 140 kDa band is the same irrespective if protein was extracted from light grown or from dark adapted protonemata; also on the mRNA level *phyCer* is expressed in a light independent manner [4].

Thümmler et al. [4] speculate that *phyCer* codes for a protein-tyrosine kinase because of the high sequence conservation between *phyCer* and recently characterized protein-tyrosine kinases of *Dictiostelium discoideum* [28]. Because of the low level of phosphorylation we were not able to perform phospho-amino acid analysis. For such an experiment we would like to use a phosphorylation product as shown in Fig. 1B because it is the result of an auto-phosphorylation event. In a kinase experiment as shown in Fig. 1A we cannot exclude the possibility that other protein kinases are involved in the phosphorylation of the presumed PhyCer band of 140 kDa. Unfortunately, the fusion proteins composed of the COOH-terminal maltose binding protein and the NH_2-terminal Cerkin1 respectively Cerkin2 domain did not show any phosphorylating activity probably due to wrong folding of the protein, thus, we have to rely on other systems expressing active PhyCer polypeptide. Such experiments are already in progress.

From our experiments it became evident that PhyCer is expressed in only very low amounts in *C. purpureus* protonemata. With the polyclonal antibody against the PhyCer protein kinase catalytic domain we were not able to detect PhyCer in crude protein extracts. Only radioactive labelled PhyCer could be detected (Fig. 1B). Also with a polyclonal oat PhyA antibody, which clearly reacted with the chromophore domain of

PhyCer expressed in *E. coli* (not shown), we could not detect the PhyCer polypeptide in crude protein extracts. Since *C. purpureus* protonemata grow slowly even under optimum incubation conditions, the achievement of large amounts of plant material is nearly impossible. Large amounts of protonemata material are necessary for the further characterization of the properties of PhyCer. Therefore we developed a protocol to transform *C. purpureus* protoplasts [22] with the aim to obtain transgenic moss cultures over-expressing their own phytochrome gene and the hope to substantially increase the amount of the PhyCer polypeptide for partial purification and further characterization of PhyCer and the PhyCer dependent transduction pathway.

The finding that PhyCer most likely represents a light regulated protein kinase again opens up the discussion whether the phytochromes in higher plants are also protein kinases and, as a consequence, the triggers of light-regulated phosphorylating cascades. Spermatophyte and fern phytochromes are clearly not homologous to eukaryotic protein kinases because they do not exhibit the conserved features of a eukaryotic protein kinase catalytic domain (serine-threonine and tyrosine kinases); nevertheless, as was first pointed out by Schneider-Poetsch et al. [29] these phytochromes exhibit sequence similarities to the catalytic domain of bacterial receptor protein-histidine kinases.

The biochemical verification that *phyCer* in fact encodes a light-regulated protein kinase opens new perspectives to understand the regulatory potential of phytochrome.

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