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Spatial distribution and temporal regulation of phytochromes A and B levels in maize seedlings

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Abstract – Phytochrome distribution and putative level in dark- and light-grown maize (*Zea mays* L.) seedlings were analyzed using antibodies against oat phytochrome A (PHYA) and tobacco phytochrome B (PHYB) by immunoblotting. During the course of leaf elongation (4–8 d), the putative level of both PHYA and PHYB declined with age. Exposure of etiolated seedlings to light induced severe down-regulation of PHYA level, and transfer of light-grown seedlings to darkness induced reaccumulation of PHYA. For PHYB, no large effect of dark to light, or light to dark, transitions could be observed. Immunoblotting and spectroscopy of phytochromes along the length of 7-d old leaves showed that the level of both PHYA and PHYB was maximal at the leaf base and in the upper half of the leaf, but their level was low in the lower half of the leaf. In light-grown leaves, PHYB distribution was similar to the dark-grown leaves, though its overall level was lower. In light-grown leaves, PHYA could not be detected by immunoblotting, but when the leaves were transferred to darkness, PHYA reappeared with a distribution pattern more or less similar to that of dark-grown leaves. These results indicate that the temporal regulation and spatial distribution of PHYA and PHYB levels in maize appear to be governed by a common regulatory element during the development of seedlings.

Phytochrome / distribution / maize / leaf / photoreceptor

PHYA, phytochrome A / PHYB, phytochrome B / POR, protochlorophyllide oxidoreductase

1. INTRODUCTION

Plant development is remarkably influenced by several environmental factors; of these, light is the most prominent. The role of light in influencing plant development is best characterized in dark-grown seedlings of angiosperms, where exposure to light initiates a series of metabolic changes leading to photomorphogenesis [14]. Among the photoreceptors regulating photomorphogenesis in plants, phytochrome is the first photoreceptor to be isolated and biochemically characterized. Several lines of physiological and biochemical evidences have indicated that phytochrome is present in nearly all the organs of the plants, including the organs buried in the soil, such as roots, etc. [29]. Studies involving in vivo spectroscopic estimations of phytochrome [5], immunocytochemical staining [27] and immunochemical quantifications [29] have shown that the amount of phytochrome differs in different tissues and organs of plants. In general, more phytochrome seems to be localized in younger and meristematic tissues, while fully differentiated mature cells appear to possess a lower amount.

Discovery of the existence of multiple phytochrome species [11] has necessitated a re-examination of the distribution of phytochrome species in higher plants [7, 13, 26, 33, 38, 39]. It is now established that earlier studies on phytochrome distribution largely represented the distribution of PHYA, a phytochrome species that characteristically accumulates in dark-grown tissues and undergoes a strong down regulation in its in vivo level upon exposure to light. In contrast, other phytochrome species – namely PHYB and PHYC – are relatively stable in vivo and are present at nearly equal levels in both dark- and light-grown plants [33]. Not only are the in vivo levels of phytochromes different, it is now evident that different phytochrome species perform independent, discrete functions in photomorphogenesis [11].

It has been assumed that phytochrome species function in vivo in a cell-autonomous fashion. Neuhaus et al. [24] using tomato *aurea* mutant seedlings, deficient in spectrally active PHYA [30], showed that microinjection of oat phytochrome A restores photomorphogenesis in hypocotyl cells in a cell-autonomous fashion. Further, it is well established that many phytochrome responses are localized at specific cell zones, e.g. phytochrome mediated detection of plant proximity and shade avoidance reactions are localized at the internodes of plants [3]. Since different species of phytochrome may independently or interdependently trigger photomorphogenic responses in a cell-autonomous fashion, knowledge of the intercellular and interorgan distribution of phytochromes is of great significance. Kondo et al. [16] examined the distribution of phytochrome in oat seedlings spectrophotometrically by scanning a single seedling. The availability of polyclonal and monoclonal antibodies against different phytochrome species allowed examination of the distribution of different phytochrome species during development and in different organs [17, 31, 34, 38, 39]. Using polyclonal antibodies, Tokuhisa and Quail [34] studied the accumulation of light-labile and light-stable phytochrome in oat seedlings during initial seedling growth for 72 h. A more systematic and detailed investigation was later done by Pratt and coworkers on oat seedlings, using monoclonal antibodies against three different phytochrome species [37–40]. In oat, three phytochrome species were immunochemically detected in the dry seeds, and their level increased during germination and seedling development.

Recently, the distribution of phytochrome in different organs has also been measured by monitoring expression of phytochrome promoters and also quantifying phytochrome mRNA levels. In transgenic tobacco bearing a PHYA-GUS construct, it was reported that expression levels of the tobacco PHYA gene in young seedlings were determined by a developmental program and in a well-defined organ and tissue-specific pattern [1]. Recently, Adams et al. [2] examined tissue specific expression of the PHYB-GUS gene in transgenic tobacco. In transgenic *Arabidopsis*, the spatial and temporal expression pattern of PHYA-GUS and PHYB-GUS is similar, with a few exceptions [32]. The mRNAs for members of the phytochrome gene family in *Arabidopsis* were found to be expressed in all organs throughout the life cycle of plants [7]. Hauser et al. [13] measured mRNA levels for individual members of a phytochrome gene (PHY) family in tomato during different developmental stages. In dry seeds, PHYB1 mRNA was abundant and in seedlings, PHYB1, PHYB2, PHYE and PHYF mRNAs were abundant in the shoot while PHYA mRNA was abundant in the root. In adult plants, the levels of PHYA, PHYB1 and PHYE mRNAs were relatively uniform in different organs, whereas PHYB2 and PHYF were expressed preferentially in ripening fruits.

In this study, we have examined the distribution of PHYA and PHYB levels in the coleoptile and primary leaf of developing maize seedlings, particularly in the leaf. Though maize leaves have been used in studies concerning photoinduced gene expression and chloroplast development [23], information on the distribution of phytochrome species is not available. Since the maize leaf elongates by generating new cells at the basal meristem, it possesses a gradient of cell maturity, with younger cells near the base and older cells at the tip. Therefore, we also examined whether the cell maturity gradient determines the distribution of phytochromes in the leaf.

2. RESULTS

2.1. Temporal expression of PHYA and PHYB in maize leaf and coleoptile

In the present study, the level and distribution of phytochromes were compared using antibodies against oat PHYA and tobacco PHYB. Thus, the data obtained represent the putative distribution of PHYA- and PHYB-like proteins in maize (see Discussion). Comparison of putative levels of PHYA and PHYB in different organs indicated that these species preferentially accumulate in the leaf and coleoptile (data not shown). Therefore, we analyzed the temporal and spatial distribution of phytochromes in these organs. In 4-d old etiolated seedlings, which possessed a leaf and coleoptile about 2 cm long, PHYA could be detected in both organs. It was observed that while the 4-d old coleoptile possessed a maximal level of PHYA, during further growth of the coleoptile from 2 cm to 5 cm in length, the level of PHYA declined with age (*figure 1* a). No significant level of PHYA could be detected in light-grown coleoptiles. PHYB level was higher in dark-grown coleoptiles than in light-grown coleoptiles, and declined slowly with age; by 8 d, the level of PHYB was below the detectability limit in light-grown coleoptiles (*figure 1* b).

PHYA level was maximal in the first leaf of 4-d old dark-grown seedlings. During the next 4 d, while the

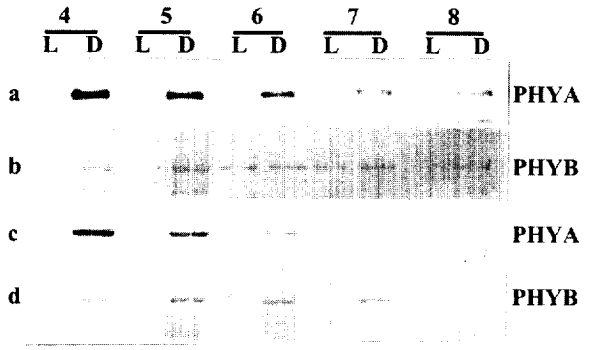

Figure 1. Temporal regulation (4–8 d) of PHYA (**a**, **c**) and PHYB (**b**, **d**) levels in the leaf (**c**, **d**) and coleoptile (**a**, **b**) of maize seedlings grown in dark (D) and red light (L). For the time course study, coleoptiles or leaves were excised from the seedlings at the indicated time points after sowing. Crude extracts (25 µg protein) from maize coleoptile and leaf, after boiling with SDS-PAGE buffer, were subjected to electrophoresis and immunoblotting.

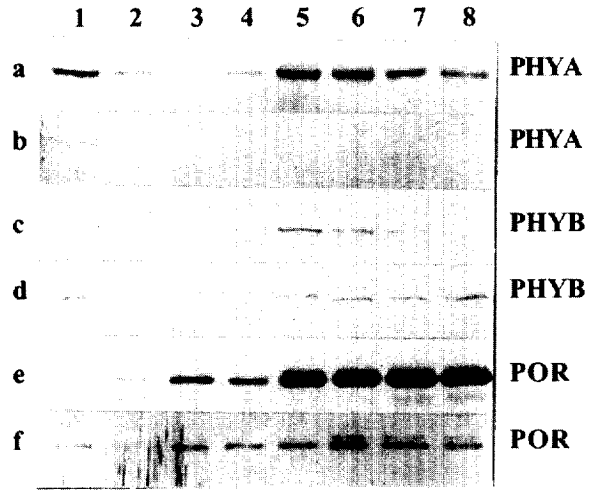

Figure 2. Distribution of PHYA, PHYB and protochlorophyllide oxidoreductase along the length of the maize leaf. The first leaf from 7-d old dark- (**a**, **c**, **e**) and red light- (**b**, **d**, **f**) grown seedlings was excised into 8 segments of 1 cm length from the base to the tip. Extracts (50 µg protein) were subjected to immunoblotting as described in *figure 1* and were probed with PHYA (**a**, **b**) and PHYB (**c**, **d**) antibodies. For protochlorophyllide oxidoreductase (**e**, **f**), crude extracts (2.5 µg protein) were subjected to immunoblotting using polyclonal antibodies raised against barley protochlorophyllide oxidoreductase. The number on top of the gel lane indicates the distance (in cm) of the segment from the leaf base.

leaf elongated from 2 to 8 cm, the PHYA level declined severely with the age of the seedlings, its level being very low in the 8-d old leaf (*figure 1* c). In light-grown leaves, very little PHYA could be seen in young leaves (4–5 d) and thereafter, its level was below the detectability limit. In comparison, the level of PHYB in dark-grown leaves was nearly the same until the sixth day. Though PHYB did not show as large a decline as PHYA, its level also declined with the age of the seedlings. Light-triggered down-regulation of PHYB level was dependent on the age of the leaf: while it was mild in the younger leaves, it was more severe in the older leaves and only a little PHYB was present in 8-d old light-grown leaves (*figure 1* d).

2.2. Spatial distribution of PHYA/PHYB in leaf

Spectral estimation of phytochrome level along the leaf length based on gram fresh weight revealed that in the 7-d old leaf, phytochrome level is higher at the leaf base; it then declines in the middle region of the lower half of the leaf, and is higher again in the upper half of the leaf (data not shown). Immunoblotting of phytochromes confirmed the above pattern of phytochrome distribution in dark-grown leaves. Both PHYA and PHYB (*figure 2* a, c) followed a pattern similar to the distribution of spectrally active phytochrome in the maize leaf with a high level at the leaf base and in the upper half of the leaf. In the light-grown leaf, while the PHYA was below the level of detection (*figure 2* b), the PHYB distribution was essentially similar to that of dark-grown leaves (*figure 2* d).

We also analyzed the distribution of protochlorophyllide oxidoreductase level in the maize leaf. Similar to phytochrome, this protein also undergoes down-regulation in light-grown seedlings [10]. In maize leaves, the protochlorophyllide oxidoreductase level increased from the base towards the leaf tip (*figure 2* e, f) in a manner opposite to PHYA and PHYB. It is evident from these results that though light induces a decline in the overall level of PHYB and protochlorophyllide oxidoreductase, it does not alter the distribution profile of these proteins along the length of the leaf.

Since the dark-grown maize leaf showed a distinct pattern of PHYA accumulation, its distribution profile in leaves was analyzed during the course of leaf development. In very young leaves (2 cm long), the basal segment showed a higher level of PHYA, but during the subsequent growth of the leaf, PHYA preferentially accumulated in two different regions viz., the leaf base and the upper half of the leaf (*figure 2* a; *figure 3* A). In contrast to PHYA, protochlorophyllide oxidoreductase, which showed a similar pattern to phytochrome in two segments of 4-d old dark grown

leaves, accumulated preferentially in the tip region of leaf during the course of leaf development (*figure 3* B). Similarly, during the growth of the coleoptile PHYA also showed a preferential accumulation at the tip (*figure 3* C).

2.3. Positional effect on synthesis and degradation of PHYA and PHYB

In leaves of 5-d old dark grown seedlings, red light induced a rapid decline of PHYA within the first 2 h of exposure, followed by a gradual decline to a very low level in the next 24 h (*figure 4* A.a). A similar exposure did not show much effect on the PHYB level in the leaf (*figure 4* A.b). Exposure to red light initiated a similar decline in the PHYA level in the coleoptile (*figure 4* A.c), where its level declined below the level of detectability. We also studied the accumulation of PHYA and PHYB by transferring seedlings grown in continuous light to darkness. *Figure 4* shows that while in the 5-d old light-grown leaf, PHYA level is below the detectability limit, on transfer to darkness, PHYA reaccumulated within 8 h, and thereafter its level increased both in the leaf and coleoptile (*figure 4* B.a, B.c). A similar transfer of the seedlings to darkness did not show much effect on the PHYB level in the leaf (*figure 4* B.b).

PHYA and PHYB distribution in maize leaf followed a typical profile with a large amount of PHYA in the regions near the leaf base and near the tip of the leaf, indicating a likely effect of cell maturity gradient on the phytochrome level. Therefore, we examined the effect of cellular position on PHYA level in maize leaves after transferring 5-d old light-grown seedlings to darkness (*figure 5*). In maize leaves transferred to darkness, PHYA accumulated in the region of the leaf tip and to some extent in the leaf base by 12 h. On further growth in darkness, its level increased in other segments of the leaf too.

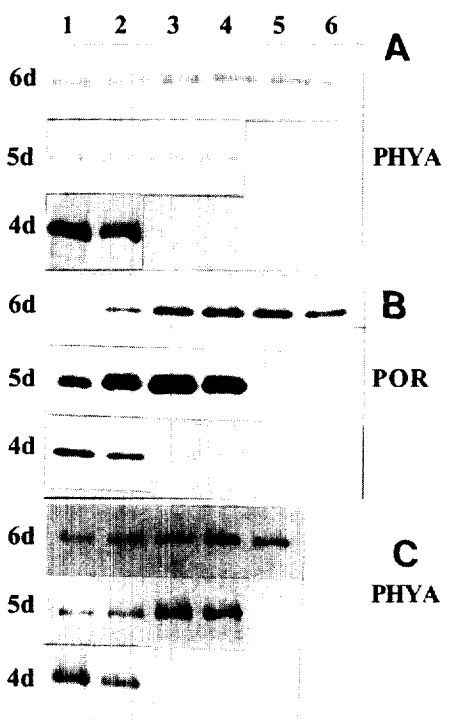

Figure 3. Spatial distribution of PHYA level in the leaf and coleoptile excised from 4-d to 6-d old dark-grown seedlings. Seedlings were harvested daily and PHYA level was analyzed in 1 cm long segments excised from the first leaf (**A**) and coleoptile (**C**). Spatial distribution of protochlorophyllide oxidoreductase (**B**) was also analyzed in the leaf excised from 4-d to 6-d old dark-grown seedlings. Extracts (25 μg protein) were subjected to immunoblotting as described in *figure 1* and were probed with PHYA antibodies. Extracts of protochlorophyllide oxidoreductase were subjected to immunoblotting using its antibodies as described in *figure 2*. The number on top of the gel lane indicates the distance (in cm) of the segment from the organ base.

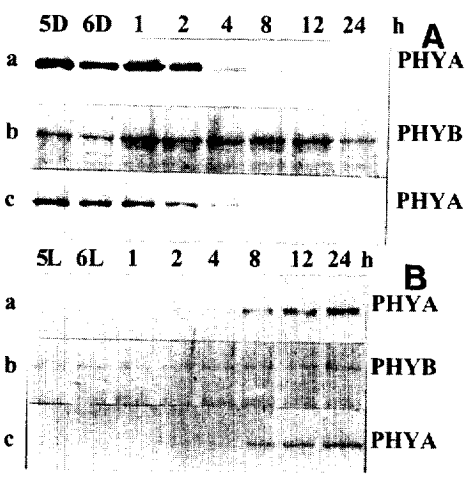

Figure 4. Time course of PHYA and PHYB levels after dark-to-light or light-to-dark transitions. For dark-to-light transition (**A**), 5-d old dark-grown (5D) seedlings were transferred to red light for 24 h or kept in darkness (6D) as control. For light-to-dark transition (**B**), 5-d old light-grown (5L) seedlings were transferred to darkness for 24 h or kept in red light (6L) as control. At the time points (h) indicated, PHYA and PHYB levels were analyzed in the leaves (a, b). Similarly, in coleoptile (c), the level of PHYA was analyzed at the indicated time points (h). Extracts were subjected to immunoblotting as described in *figure 1*.

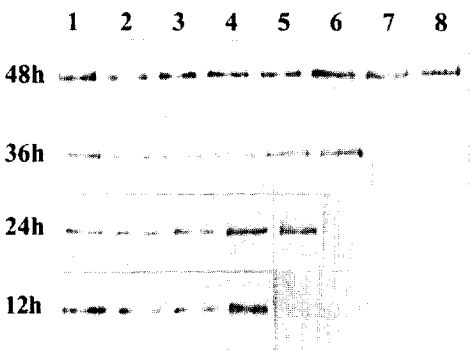

Figure 5. Profile of reaccumulation of PHYA level along the length of maize leaf. Five-d old red light-grown seedlings were transferred to darkness for 48 h. At the time points (h) indicated, PHYA level was analyzed in 1 cm long segments excised from the first leaf. Extracts (25 μg protein) were subjected to immunoblotting as described in *figure 1* and were probed with PHYA antibodies. The number on top of the gel lane indicates the distance (in cm) of the segment from the leaf base.

3. DISCUSSION

3.1. Specificity of antibodies against PHYA and PHYB

A comparison of the gene sequences of phytochrome species has shown that while the sequences of PHYA diverge about 50 % between monocots and dicots, those of PHYB are more conserved between angiosperm groups [8, 25]. PHYB of *Arabidopsis* and rice [8], and also of rice and tobacco [15], share amino acids identity close to 77.6 and 76.4 %, respectively. In addition, monocot and dicot PHYB possess very similar hydropathy profiles over the entire length of their amino acid sequences [8] and therefore may also possess antigenically similar epitopes.

In view of the above observations, we ascertained the possibility of detecting maize PHYB by tobacco anti-PHYB monoclonal antibodies. The fact that the percentage of homology of *Arabidopsis* PHYB to tobacco (77.6 %) is nearly similar to the homology between tobacco and rice (76.4 %) [15], and that the tobacco monoclonals against PHYB specifically detect *Arabidopsis* PHYB in wild type but not in a PHYB deficient mutant [21, 22], strengthens the possibility of detection of monocot PHYB. Such a possibility is further supported by the fact that the PHYB sequence between *Arabidopsis* and rice shares a homology of 73 %. Moreover, monoclonals against rice PHYB can specifically recognize *Arabidopsis* PHYB protein and do not recognize *Arabidopsis* PHYA and PHYC apoproteins [36]. Wang et al. [40] have also observed that a monoclonal antibody against light-stable pea phytochrome can recognize a phytochrome species abundant in light-grown oat leaves.

So far tobacco anti-PHYB monoclonals have been used to detect PHYB in *Arabidopsis* [21], cucumber [19] and tomato [30]. The results obtained in this study indicate that the above antibodies likely recognize PHYB-like apoproteins in maize. Immunoblots of PHYB show an immunostained band in the region of the observed molecular mass of PHYB. Since the PHYB sequence diverges by 50 % from other phytochrome species, the chance of detection of a species other than PHYB is small. In fact, most oat monoclonals against phytochrome purified from green oat shoots fail to crossreact with the 124 kDa phytochrome species which are abundant in etiolated oat seedlings [28]. Taken together, the evidence above favors the view that the tobacco monoclonal antibody mixture is likely to detect PHYB present in maize seedlings.

Since maize and oat PHYA also possess 88 % homology in their amino acid sequence [25], we used an oat PHYA antibody against maize PHYA. The crossreactivity of an oat PHYA antibody against maize PHYA was established by light-mediated down-regulation of PHYA, and also by the susceptibility of the Pr form of PHYA to endogenous proteases (data not shown). Furthermore, the Mr of the immunoblotted band for PHYA matched the reported Mr of maize PHYA [4] and is close to the reported Mr of maize [6].

Although arguments presented above favor the view that the antibodies used in this study recognize PHYA- and PHYB-like proteins in maize, the possibility that these antibodies recognize a phytochrome species other than PHYA and PHYB also merits consideration. For example, Tokuhisa et al. [35] observed that rabbit polyclonal antibodies against PHYA recognized two phytochromes and similarly certain monoclonals against oat PHYA can detect a second phytochrome. Since the molecular masses of PHYA and PHYB on immunoblots are different, it is unlikely that the PHYA antibodies used in this study recognized PHYB or vice versa. Nevertheless the possibility of recognition of an additional phytochrome species other than PHYA or PHYB by the antibodies can not be unequivocally excluded. The current lack of knowledge about all of the phytochrome species in maize and the lack of specific antibodies against each of them preclude testing the above possibility.

3.2. Effect of light-to-dark and dark-to-light transitions on PHYA and PHYB levels

Several lines of physiological and biochemical evidence have indicated that phytochrome species consist of a light-labile and a light-stable pool [11], which have been shown to belong to PHYA and other green-tissue specific phytochromes, respectively. Immunoblotting of PHYB and PHYC from *Arabidopsis* [32], and PHYB in tomato [30] and cucumber [19] showed nearly identical levels in etiolated and green tissues. In this study, PHYA drastically declined on exposure to light, but PHYB was rather stable in maize seedlings. Dark adaptation of continual light-grown seedlings elevated the PHYA level in the maize leaf after 8 h, whereas PHYB level was not influenced. Likewise, though continual-red-light-grown maize coleoptiles were virtually devoid of PHYA, upon dark adaptation, PHYA reaccumulated after 8 h.

3.3. Temporal regulation of PHYA and PHYB levels

Higher levels of PHYA and PHYB appear to be associated with younger organs, and the PHYA level, which was maximal in the 4–5-d old leaf and coleoptile, declined significantly during further growth. A similar age-dependent decline is noticeable in the level of PHYB in the leaf. It is obvious from the profiles of PHYA and PHYB levels that the massive accumulation of PHYA is distinctively restricted to the shoot of younger etiolated seedlings. However, the aging of seedlings does not preclude reaccumulation of phytochromes, and on seedling dark-adaptation, the PHYA level augments significantly.

3.4. PHYA and PHYB distribution in maize leaves

The profile of PHYA distribution in the coleoptile supports the notion that in cereals, it specifically accumulates in the coleoptile tip [27]. The observed uniform distribution of PHYA in maize shoots by Pratt and Coleman [27] appears to be true for very young seedlings only. In older maize seedlings, the level of PHYA in the leaf is higher in the upper half of the leaf and in the region close to the meristematic base. This is similar to the accumulation profile of PHYB. The above profile of phytochrome accumulation is at variance with protochlorophyllide oxidoreductase which also preferentially accumulates in dark-grown leaves but follows an acropetal gradient with maximal concentration towards the tip.

A monocot leaf consists of cells of differing maturity as the leaf elongates by continual cell division at the basal meristem resulting in a gradient of cell maturity with youngest cells at the leaf base and the oldest cells at the tip of the leaf [23]. It is likely that the profile of phytochrome distribution in maize leaves may be determined by the existing cell maturity gradient in leaf. On transferring the light-grown seedlings to darkness, PHYA reappears with a profile more or less similar to that of the seedlings grown in continuous darkness. The fact that light does not seem to alter this pattern is evident from the observation that the distribution pattern of both PHYB and protochlorophyllide oxidoreductase [10] remain the same in the light-grown leaf. In view of the close similarity in the distribution of PHYA and PHYB levels along the length of maize leaves, we speculate that the expression of these two phytochromes may be regulated to some extent by the same gradient of cell maturity, at least in leaves. However, the above speculation is conjectural in nature as the cell and tissue level distribution of these phytochromes is still to be elucidated.

A comparison of the profiles of phytochrome distributions in this study and phytochrome mediated induction of several plastidic and cytosolic enzymes along the length of maize leaves [23] indicated a clear lack of correlation between the distribution of these photoreceptors and photoinduced response in monocot leaves. In the past, such absence of correlation between phytochrome level and photoresponse was attributed to the existence of multiple pools of phytochrome and was termed as a paradox. Results obtained in this study do not correlate photoreceptor distribution with known photoresponses in maize leaves. In fact, recent studies indicate that though a photoreceptor such as phytochrome functions in a cell-autonomous fashion [24], a photoresponse would be indirectly connected to the photoreceptor level and would rather result from a change in the level, or the presence/absence, of trans-acting transcriptional factors controlling gene expression [9].

In summary, the results obtained in the present study indicate that the profile of distribution and temporal regulation of the PHYA/PHYB levels appears to be controlled by a common mechanism in maize seedlings. The observed semblance between accumulation and distribution of the phytochrome species possibly results from the co-regulation of synthesis of these phytochrome species during development. A detailed molecular-genetic analysis of the distribution and function of all phytochrome species is nevertheless

needed to understand the respective roles and interaction between different phytochrome species in controlling plant development.

4. METHODS

4.1. Plant materials and growth conditions. Maize seeds (*Zea mays* L., cv. Ganga-5, Andhra Pradesh State Seed Corporation, Hyderabad, India) were first soaked for 12 h in distilled water, and were then sown in plastic boxes on moist Crepe Kraft seed germination paper (Jayshree Paper Traders, New Delhi, India). The time point of soaking was considered as the time point of onset of germination. Seedlings were grown either in continuous red light (λ_{max} 650 nm, 2.8 μmol) or in continuous darkness at 25 °C and were watered using distilled water. At the indicated time intervals, the seedlings were harvested and dissected into different organs. Phytochrome distribution in the first leaf of the seedling was analyzed by dissecting the leaf into 1 cm long segments and numbered from the base to the tip of the leaf. Similarly, the coleoptiles were dissected into 1 cm long segments and numbered from the base to the tip.

4.2. Preparation of PHYA antibody. Phytochrome A was purified to homogeneity from 4.5-d old dark-grown oat (*Avena sativa* L.) seedlings using the procedure outlined by Grimm and Rudiger [12]. Polyclonal antibodies against purified oat phytochrome A were raised in rabbits using standard protocol. The crossreactivity of antibodies to maize PHYA was ascertained by using some characteristic features of phytochrome A, such as light-induced in vivo down-regulation and the preferential in vitro proteolysis of the Pfr and Pr forms of phytochrome, respectively.

4.3. Phytochrome spectral assay. The primary leaves from dark-grown maize seedlings were harvested under green safe light and transferred onto ice. Phytochrome distribution in the primary leaf was analyzed by dissecting the leaf into 1 cm long segments numbered from the base to the tip of the leaf. In vivo amount of phytochrome in the leaf segments was quantified by difference spectroscopy of the harvested tissue (1 cm light path) after packing it in a 3-mL plastic cuvette at 4 °C after red light and far red light irradiation in a Hitachi-557 dual wavelength spectrophotometer. One relative unit of phytochrome is equal to a value of $\Delta(\Delta A)(A_{666} - A_{730}) = 0.001$ A.

4.4. Blotting of phytochromes and protochlorophyllide oxidoreductase. Tissues frozen in liquid nitrogen were homogenized with a semifrozen (–20 °C) extraction buffer consisting of 100 mM Tris-Cl (pH 8.3), 140 mM ammonium sulfate, 10 mM EDTA, 10 % (w/v) insoluble polyvinylpolypyrollidone and 50 % (v/v) ethylene glycol. Before homogenization, protease inhibitors were added to a final concentration of 18 mM iodoacetamide, 4 mM PMSF, 0.75 $\mu g \cdot mL^{-1}$ leupeptin, 20 mM sodium bisulfite and 14 mM 2-mercaptoethanol. After centrifugation (20 000 × *g*, 0 °C, 20 min), the supernatants were boiled after mixing with an equal amount of 2x SDS-PAGE sample buffer. The amount of protein was estimated by the Lowry method [20]. SDS-PAGE was carried out in 8 % gel for phytochrome and in 10 % gel for protochlorophyllide oxidoreductase as described by Laemmli [18]. At the end of PAGE, the gel was electroblotted using a semi-dry blotting method to a polyvinylidenedifluoride membrane (Immobilon P, Millipore) following the procedure described elsewhere [19]. Anti-PHYA polyclonal antibodies raised in rabbit for oat phytochrome A and a mixture of monoclonal antibodies (mAT-1, mAT-2, mAT-5) raised against tobacco PHYB [19] were used to detect the corresponding antigens.

4.5. Evaluation of data. Experiments were repeated at least three times independently with fresh extractions. The gels were loaded on the basis of equal proteins and the degradation of phytochrome was minimized by working quickly and also by inclusion of protease inhibitors. The extraction buffer employed in this study extracted close to 90 % of the phytochrome present in the tissue during first homogenization. There was no significant loss of phytochrome during extraction by proteolysis. The relative amount of phytochrome on immunoblots was also evaluated by densitometric scanning by using a Molecular Dynamics laser scanning system (data not shown). The relative amount of phytochrome on temporal scale and its distribution within the leaf was compared on the basis of equal protein. Using oat PHYA as the standard protein, we could detect up to 0.5 ng of PHYA on immunoblot as established by serial dilution. In contrast to Wang et al. [37] who examined the data on a per organ basis, we evaluated the data on the basis of equal protein, as our study entailed comparisons between different parts of the same organ. The immunoblots presented show the representative results.

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