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REGULAR ARTICLE

ROLE OF INTACT CYTOSKELETON ORGANIZATION IN NUCLEAR IMPORT AND SPECKLE FORMATION OF PHYTOCHROME A AND B IN *ARABIDOPSIS* SEEDLINGS

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SUMMARY

Phytochromes are the primary photoreceptors for red and far-red light in plants and control various aspects of photomorphogenesis. Phytochomes are encoded by five genes (phytochrome A to E) in *Arabidopsis*. Phytochromes have been shown to be transported to the nucleus upon light irradiation and can form a speckled pattern. Nuclear import is the most essential step for the phytochromes to initiate specific photo-sensory transduction pathways. Number and size of these nuclear speckled structures (speckles) have been functionally correlated with the light-responsiveness. In the current study, the role of intact cytoskeleton in nuclear import process and subsequent speckle formation of phytochrome A (phyA) and phytochrome B (phyB) GFP fusion proteins was studied in *Arabidopsis* seedlings. The studies were carried out in three different physiological conditions, where the intact cytoskeleton of the cell is either reorganized or disrupted. These conditions are (i) at lower temperature, where overall cytoskeleton dynamics is altered, (ii) in the protoplasts where tubulin organization is altered and (iii) the application of cytochalasin B, an actin cytoskeleton disrupting drug. The disappearance of the pre-formed speckles in these experimental conditions was also investigated. Here we conclude that the nuclear import kinetics of phyA and B is slower but could not be abolished in all these conditions tested. Moreover, formation and disappearance of speckles from the nucleus is found to be delayed but not completely abolished when the cytoskeleton is disrupted. These result suggest that phytochrome nuclear import, dynamics of nuclear speckles are partly contributed by cytoskeleton components and partly by other protein-macromolecular interaction.

Key words: Phytochrome, *Arabidopsis thaliana*, Cytoskeleton, Speckle formation, Nuclear bodies, Nuclear transport, Cytochalasin B

Abbreviation: phy, Phytochrome; R, Red; FR, Far-red; RT, Room temperature; cytB, Cytochalasin B

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1. Introduction

Plants have evolved a variety of photoreceptor classes to sense the quality, quantity, direction and duration of incidental light in order to execute appropriate physiological and developmental responses throughout their life cycle. Of these, red (R) / far-red (FR) light reversible phytochromes (phy) are well known [1]. Nuclear localization of phyA is triggered by very-low fluences of red, far-red or continuous fluences of far-red light [2]. On the other hand, nuclear localization of phyB is controlled by R/FR reversible low-fluence-rate [3]. The intracellular localization of these photoreceptors has been studied using intracellular partitioning of GFP (green fluorescent protein) fusion proteins of phyA (phyA:GFP) or phyB (phyB:GFP) [4, 5]. It has been shown by this method that intracellular partitioning of these photoreceptors is light regulated [6]. Translocation of photoreceptor fusion proteins into the nucleus is followed by formation of speckles. Responsiveness of phytochromes to excitation by light is

manifested as speckles [7]. Formation and stability of the speckles exhibit different kinetics, wavelength and fluence dependence [8]. The phyA:GFP nuclear transport is rapidly induced by light; even by very short (2-3 seconds) red light pulse. This is preceded by the formation of transient cytoplasmic speckles [4].

Despite this knowledge on sub-cellular partitioning of phytochromes, very little is known about the early events of nuclear import and how it is regulated after light irradiation [9]. Explicitly, FHY1 [10-12] and N-terminal region of phytochrome A [13] regulate the localization of phytochrome A into the nucleus. Unlike Phytochrome A, C-terminal region of phytochrome B alone is able to translocate to the nucleus and cause de-etiolated phenotype [14].

The aim of this study was to understand the role of intact cytoskeleton organization in phytochrome nuclear import and speckle formation. To this end, the *Arabidopsis* seedlings expressing phytochrome A or B GFP fusion proteins were subjected to three experimental treatments where cytoskeleton organization is known to be altered, i.e. incubation at 4 ^{o}C, protoplast formation and treatment with cytochalasin B (ctyB). The effect of these treatments on the nuclear import of phytochrome and phytochrome speckles was studied in two stages, firstly during the initial light irradiation during their first appearance and secondly on the disappearance of preformed speckles in darkness.

The following established facts helped us to design experiments to attain the state of reorganized cytoskeleton. In 4 ^{o}C, the cytoskeleton structure of plant cells gets rearranged [15]. The tubulin cytoskeleton in the protoplasts culture is not normal and it undergoes rearrangements correlating to the number and structure of cell nuclei in the protoplasts [16]. The actin disrupting drug cytochalasin B (cytB) binds to the plus end of F actin and blocks subunit addition [17]. Talin is an intracellular protein which binds to actin and provides a link between actin microfilaments and plasma membrane [18].

2. Materials and Methods

Plant material, growth conditions and light sources

Arabidopsis thaliana transgenic line expressing phyA:GFP was used for the expression analysis of *phyA*. This line was constructed in a *phyA* mutant containing stable transformation of *Arabidopsis PHYA* cDNA fused to GFP, whose expression was driven by *PHYA* promoter [4]. The phyB:GFP expression was studied using *Arabidopsis thaliana* transgenic line containing stable transformation of *Arabidopsis PHYB* cDNA fused to GFP under the control of cauliflower 35S promoter [5]. A transgenic *Arabidopsis* line expressing mouse talin fused to GFP, whose expression was driven by cauliflower 35S promoter was used for the study of cytoskeleton dynamics. This seed line was obtained from Kost et al. [19]. The phyA:GFP and phyB:GFP transgenic lines were obtained from Kircher et al [5].

Seedlings were grown in plastic boxes 85 × 85 × 50 mm on four layers of filter paper. Seeds were pre-soaked in distilled water and were allowed to imbibe in dark for 48 hours (h) at 4 °C. Germination was induced by red light treatment for 2 h, and then transferred to darkness to obtain etiolated seedlings. For all other experiments, after induction for germination, seedlings were initially grown in continuous darkness at room temperature (RT), which was constantly maintained at 25 °C for 4 days followed by the respective light irradiation (either 3 h or over night FR or R) or the three treatments to reorganize the cytoskeleton.

White light source was eight Osram L16/W25 lamps (59 µmol m^{-2} sec^{-1}). Standard red light fields (656 nm) at 37 µmol m^{-2} $sec^{-1}$for red light treatment and far-red fields (730 nm) at 21.4 µmol m^{-2} sec^{-1} for far-red light treatment were used [20]. Incubation in dark in RT was done in constant temperature at 25 °C., All manipulation of plant material was performed in dim-green light according to Schaefer et al. [20] except when indicated.

Incubation at 4 °C

Cold treatment was performed using Colora Messtechnik GmBH Lorch/Württ,

which was set to maintain constant temperature of 4 °C. The plastic boxes containing seedlings were placed on a metal block connected to this cooling device.

Preparation of protoplasts

Protoplasts were prepared directly from seedlings. For a single experiment, 175 to 200 seedlings were incubated in 1 ml of enzyme solution in eppendorf for 5 h. The eppendorf were then centrifuged at a very low speed (i.e. 300 rpm) for 5 min. Experiments were performed directly with this solution containing protoplast. For each result the experiment was repeated twice by analyzing about 20 protoplasts each time. Enzyme solution was prepared by dissolving 1 % cellulase and 0.1 % macerozyme in a solution of 0.4 M mannitol, 20 mM KCl, 20 mM MES and pH was adjusted to 5.7 by KOH. The enzyme solution was filter-sterilized before use.

Cytochalasin B treatment

The concentration of cytochalasin B (cytB) (from Sigma, Deisenhofen) used in the experiments was 15 µg ml^{-1} and it was stored as 1 mg ml^{-1} stock in DMSO at 4 °C. Seedlings were analyzed after 1 h of cytB treatment.

Epifluorescence and confocal microscopy

Seedlings were transferred to glass slides and mostly epidermal cells were analyzed using an axioscop (Zeiss, Oberkochem, Germany) for epifluorescence and light microscopy. Excitation of the GFP phytochrome fusion proteins was performed with standard isothiocyanate and GFP filter sets. The exposure time and intensity of the excitation was maintained constant throughout all the experiments (except indicated). In each experiment, 20 nuclei were analyzed for nuclear speckles and each experiment was repeated three times using five different observation fields under the microscope. Documentation of cells was performed during the first 2 minutes of microscopic analysis. The representative cells were documented by photography with a digital Axiocam camera system (Zeiss). Nuclear bodies of phytochrome, described elsewhere [21, 11] were termed here as nuclear speckles. They were named nuclear or cytoplasmic speckles according to their appearance in nucleus or cytoplasm.

The talin:GFP expression in the seedlings was studied using a confocal laser scanning microscope (CLSM) (DB RBE; Leica, Bensheim, Germany) using the 488 nm line of an argon-krypton laser for excitation, a beam splitter at 510 nm and a band pass filter at 515 nm. Each cell layer was optically sectioned into eight individual slices and these individual sections were then projected in to one stack. Each experiment was repeated thrice with five observations each time and the result in each case represents the average of all the observations.

3. Results

Seed lines expressing phyA:GFP or phyB:GFP were grown in darkness for 4 days and then divided into two sets. First set of seedlings (i.e. control set) were irradiated with 3 h far-red (in case of phyA) or 3 h red light (in case of phyB). The second set of seedlings (i.e. experimental set) was subjected to three different treatments (incubation at 4 ^{o}C or preparation of protoplasts or treatment with cytB). After the different treatments, the second set was also irradiated with red or far-red light. The analysis of speckle formation was performed using fluorescence microscopy and compared in both the sets. The untreated seedlings (control set) always showed nuclear GFP fluorescence as well as nuclear speckles. The number of such speckles varied from 3 to 5 per nucleus in most of the hypocotyl cells analyzed (Fig. 1A, Fig. 1B). Cytoplasmic fluorescence or speckles were not observed in any cell in the seedlings of control set (Fig. 1A, Fig. 1B). We tested the effect of various treatments which is described below.

Effect of reorganized cytoskeleton on nuclear import and formation of nuclear speckles of phytochrome A and B : Effect of cold treatment (incubation at 4 °C)

To study the effect of low temperature on the nuclear import of phytochrome, the seedlings were subjected to 3 hours of

respective light irradiation at 4 °C (Fig. 1, 2nd Row). The nuclear import and the pattern of speckle formation were not affected in the seedlings expressing phyA:GFP (Fig. 1C) compared to the control set. However, the phyB: GFP expressing seedlings showed only GFP fluorescence but no nuclear speckles (Fig. 1D). Also these samples showed 4-5 cytoplasmic speckles per cell in 99% of cells analyzed (Fig. 1E), which were not observed in samples irradiated at room temperature (RT).

To confirm that, this altered pattern of speckle formation was a combined effect of the light treatment and 4°C and not the incubation at 4 °C alone, seedling expressing phyA:GFP grown in continuous darkness either at room temperature or at 4 °C were analyzed for GFP fluorescence in the nucleus and cytoplasm. The absence of phyA:GFP fluorescence in nucleus of the seedling samples incubated at 4 °C suggested the requirement of light for the nuclear accumulation of phyA fusion protein.

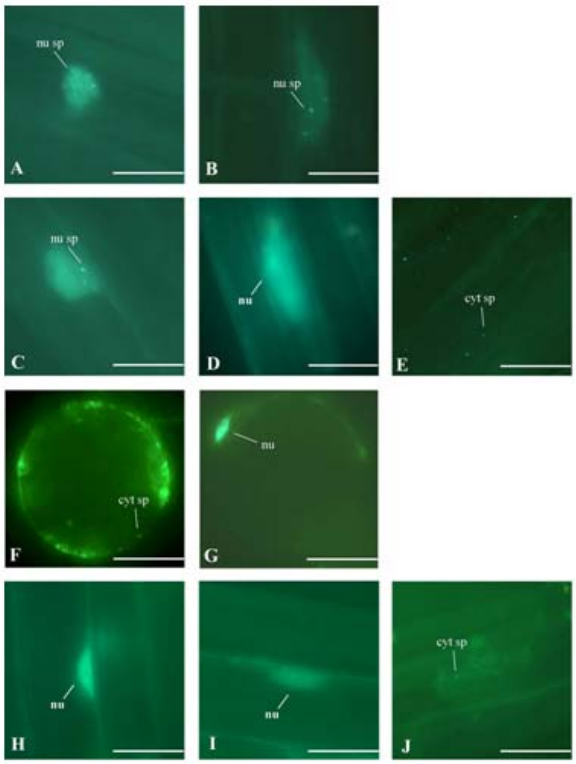
Effect of reorganized cytoskeleton on nuclear import and formation of nuclear speckles of phytochrome A and B: Effect of preparing protoplasts

Protoplast system was implemented in order to understand the effect of reorganized cytoskeleton on nuclear import of phytochrome [16]. To this end, protoplasts were prepared from dark grown seedlings expressing phyA:GFP or phyB:GFP, followed by 3 h R or FR light irradiation respectively. In both the transgene expressing protoplast samples nuclear speckles were not formed and only a diffuse nuclear fluorescence was observed (Fig. 1, 3rd row). In addition, 2-3 cytoplasmic speckles in most of protoplasts analyzed and GFP fluorescence was observed in all protoplasts (Figs. 1F; 1G). These results indicated that this disorganized cytoskeleton in protoplast was not sufficient to inhibit nuclear import but was sufficient to prevent nuclear speckle formation. More over it indicated that, the cytoskeleton components are required for the nuclear import of phytochrome or other interactors that would have been transported to nucleus and helped in formation of nuclear speckles were possibly retarded in the cytoplasm.

Effect of reorganized cytoskeleton on nuclear import and formation of nuclear speckles of phytochrome A and B : Effect of cytochalasin B application

The actin cytoskeleton in the seedling expressing phyA:GFP or phyB:GFP was disrupted by treating with appropriate concentration of cytochalasin B for 1 h followed by fluorescence microscopy (Fig.1, 4th Row). The viability of the seedlings was confirmed before microscopy by staining with methylene blue. Diffusive GFP fluorescence in the nuclei was noticed in both the seedling samples expressing PhyA and PhyB fusion proteins. However, nuclear GFP speckles were not observed (Figs. 1H; 1I). Additionally, the seedlings expressing phyB:GFP showed the presence of 4-5 cytoplasmic speckles per cell in almost all the cells analyzed (Fig. 1J). The similar experiments with DMSO alone showed nuclear speckles of phyA and phyB:GFP, which was similar to localization in the untreated seedling. These observations further strengthen the finding that the altered pattern of phytochrome localization was due to cytB treatment.

[Figure.1. Effect of reorganized cytoskeleton on the nuclear import and formation of nuclear speckles of phyA and phyB:GFP



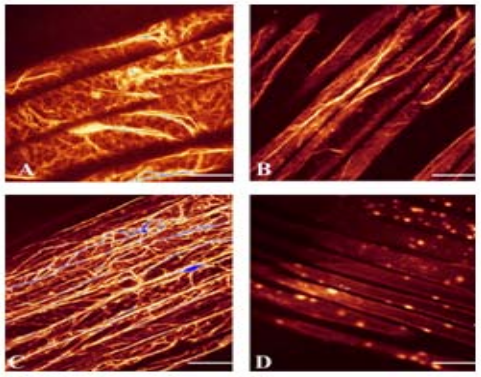
Arabidopsis seedlings expressing phyA:GFP or phyB:GFP were grown in darkness for 4 days followed by irradiation with 3 h of FR or R light respectively. Localization of the photoreceptors in hypocotyl cells was analyzed using fluorescence microscopy by analyzing the GFP fluorescence or speckles. The pictures are the microscopic images of hypocotyl cells of untreated seedlings (1st row A; B), seedlings incubated at 4 ^{o}C (2nd row C; D; E), protoplasts of seedlings (3rd row F; G) and seedlings treated with cytochalasin B (4th row H; I; J). First column represents the images taken from seedlings expressing phyA:GFP and 2nd, 3rd column represents the images taken from seedlings expressing phyB:GFP.

Note: Nuclear speckles in (A; B; C), diffuse GFP fluorescence in nucleus in (D; F; G; H; I), cytoplasmic speckles in (E; J). Nu: nucleus; nu sp: nuclear speckles; cyt sp: cytoplasmic speckles; scale bars: 10 μm.]

Visualization of reorganization of the actin cytoskeleton

The orientation of talin:GFP was analyzed in the hypocotyl cells incubated after exposure with 3 h FR at 4 °C or treated with cytB in comparison with the untreated seedlings in presence or absence of light, using confocal microscopy (Fig. 2). In the dark grown seedlings at RT, a dense cortical meshwork of actin filaments could be observed (Fig. 2A). After exposure to continuous FR light, the actin filaments were mostly orientated longitudinally (Fig. 2B). In the seedlings exposed to FR light at 4 °C, the re-orientation of actin filaments was in longitudinal fashion and mostly in the form of bundled strands (Fig. 2C). In the seedlings treated with cytB, actin cytoskeleton was drastically disintegrated (Fig. 2D) and was observed as dots (20-30 per section in all sections analyzed). These results demonstrated that the cold or cytB treatment could alter the orientation of actin filaments drastically. The altered organization cytoskeleton could not be demonstrated in live protoplast cells because the randomly floating nature of protoplasts in the nutrient medium made it impossible to visualize and photograph using confocal microscopy.

[Figure.2. Reorganization of actin cytoskeleton after incubating at 4 °C or upon treatment with cytochalasin B using confocal leaser scanning microscopy (CLSM)



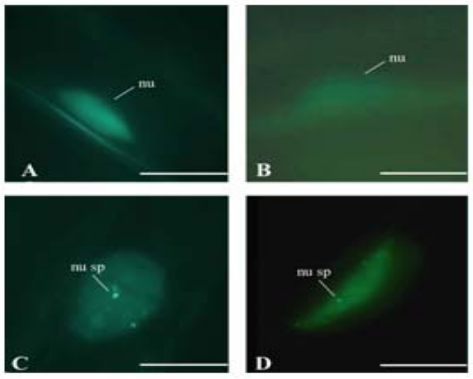
Arabidopsis seedlings expressing talin: GFP were grown in darkness for 4 days. The pictures are microscopic images of representative cells of seedlings in darkness (A), seedlings irradiated with 3 h FR at room temperature (B), seedlings irradiated with 3 h FR at 4 °C (C), seedlings irradiated with 3 h FR after treatment with cytB (D).

Note: Actin organization in the form of dense cortical meshwork in (A), longitudinal orientation in (B), longitudinally bundled orientation in (C), disorganized actin network in the form of dots in (D). Scale bars: 10μm.]

Effect of reorganized cytoskeleton on disappearance of pre-formed speckles of phytochrome A and B: Effect of incubation at 4°C

We also studied its effect on the pre-formed speckles of phytochrome. For this purpose, initially the nuclear speckles of phyA or phyB were induced to form by irradiating the seedlings at 3 h FR or R (as done previously in Fig. 1A; B). These seedlings with speckles in the nucleus (3-4 per nucleus in all cells analyzed) were incubated in darkness to induce the disappearance of the speckles either at 4 °C or at RT (Fig. 3). During incubation, the kinetics of the disappearance of the speckles in RT versus 4 °C was analyzed. In the cold treated samples, the disappearance of nuclear speckles took longer period of time. In case of phyA:GFP the nuclear speckles disappeared completely after 30 min of incubation in RT (Fig. 3A) in all the cells analyzed. In the 4 °C, one to two phyA:GFP nuclear speckles were observed in all the cells analyzed (Fig. 3C), which disappeared after 1.5 hours. In case of phyB:GFP, the nuclear speckles disappeared completely only after 13 h of incubation in darkness at RT (Fig. 3B), whereas in the 4 °C treated samples, 1-2 speckles per nucleus were still observed (Fig. 3D), which disappeared completely in ~16 hours.

[Figure.3. Effect of incubation at 4 °C on disappearance of pre-formed speckles of phyA and B:GFP



Arabidopsis seedlings expressing phyA:GFP or phyB:GFP were grown in darkness for 4 days followed by irradiation with 3 h of FR or R light respectively (as done previously Figure1A; Figure1B). Localization of the photoreceptors in hypocotyl cells was analyzed using fluorescence microscopy by analyzing the GFP fluorescence or speckles. Seedlings irradiated with FR (from Figure1A) were incubated in darkness for 30 minutes at RT (A) or in 4 °C (C). Seedlings irradiated with R (from Figure1B) were incubated in darkness for 13 h at room temperature (B) or in 4 °C (D).

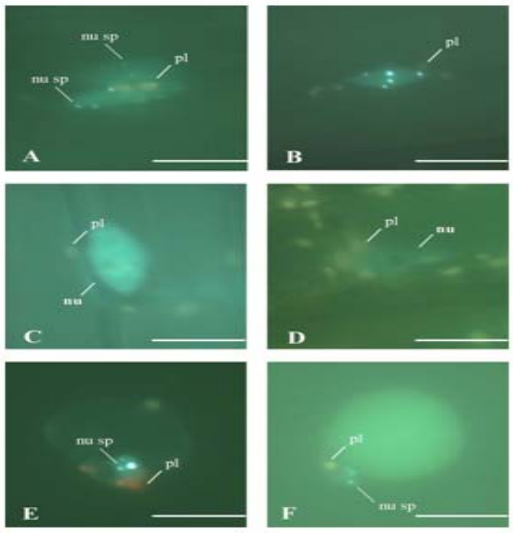
Note: Diffuse GFP fluorescence in (A; B) and nuclear speckles in (C; D). Higher intensity of excitation was applied to visualize the nuclear speckles in (D). Nu: nucleus; nu sp: nuclear speckles; scale bars: 10 μm.]

Effect of reorganized cytoskeleton on disappearance of pre-formed speckles of phytochrome A and B: Effect of preparing protoplasts

To study the disappearance of the pre-formed speckles in the protoplasts, first nuclear speckles of phyA:GFP or phyB:GFP were induced to form by irradiating the transgenic seedlings with overnight FR or R light respectively (Figs. 4A; 4B). These seedlings containing nuclear speckles (5-7 per nucleus) were either kept as such or protoplasts were prepared from them. The number and size of the nuclear speckles were not affected by preparing protoplasts. This

was followed by incubation in darkness for 9 h for inducing the disappearance of the speckles. Microscopy results showed that in the seedlings expressing phyA:GFP, nuclear speckles had completely disappeared and only GFP diffused fluorescence was observed Fig. (4C), whereas in the protoplasts from the same batch of seedlings showed nuclear speckles (1-2 per nucleus in all the protoplasts analyzed) (Fig. 4E). In a similar pattern in case of the phyB: GFP, only the protoplast samples (Fig.4F) still had the nuclear speckles (1-3 per nucleus in all the protoplasts analyzed) as compared with the seedlings (Fig. 4D).

[Figure.4. Effect of preparing protoplasts on disappearance of pre-formed speckles of phy A and B:GFP



Arabidopsis seedlings expressing phyA:GFP or phyB:GFP were grown in darkness for 4 days followed by overnight irradiation with FR or R light respectively. Localization of the photoreceptor in hypocotyl cells was analyzed using fluorescence microscopy by analyzing the GFP fluorescence or speckles. The pictures are the microscopic images of hypocotyl cells of seedlings expressing phyA:GFP (A) and phyB:GFP (B). Seedlings irradiated with FR (from A) were incubated in darkness for 9 h (C) or used for preparing protoplasts prior to the incubation in darkness (E). Seedlings irradiated with R (from B) were incubated in darkness for 9 h (D) or protoplasts were prepared from those seedlings prior to the incubation in darkness (F).

Note: Nuclear speckles in (A; B; E; F), diffuse GFP fluorescence in (C; D). Nu: nucleus; nu sp: nuclear speckles; pm: plasma membrane; pl: plastid; scale bars: 10 µm.]

4. Discussion

This study provides an insight on the effect of disrupting or reorganizing cytoskeleton on the nucleo-cytoplsmic partitioning of phyA and phyB in presence of light. In the first attempt, when the nuclear import of phyB:GFP was analyzed in the cells incubated at 4 °C, GFP fluorescence was diffuse but no speckles were formed in the nucleus. On the other hand, speckles were observed in the cytoplasm, which is observed mostly in transgenic lines where phytochrome is over-expressed like ABO (Arabidopsis phytochrome B over expresser) in presence of red light [22]. It is known that the number and size of phyB:GFP speckles is directly proportional to the amount of Pfr form (active form of phyB) [8]. Moreover, it is known that the action of phytochromes is reflected in the degree of speckle formation [5]. So this altered pattern of speckle formation of phyB:GFP after incubation at 4 °C can be interpreted as lowered nuclear import of phyB:GFP, which after 3 hours of red light ultimately leads to a comparatively bigger pool of active Pfr form of phyB in cytoplasm than in nucleus. So these cytoplasmic speckles are significant here to account the decreased nuclear import as an effect of reorganized cytoskeleton. However, this pattern was not observed in the case of phyA:GFP expressing lines since there was no difference in the speckle formation pattern in the lower temperature treated seedlings. This difference is probably because at 4 °C, the cytoskeleton dynamics is only lowered down, but not completely disrupted. The nuclear import of phyA is a fast process and reaches to its maximum within ~2 h [7]. This after 3 h of light treatment, lowered but probably adequate Pfr form of phyA could be transported in to the nucleus for formation of nuclear speckles. Although we didn't observe any difference in the pattern of speckle formation in phyA:GFP at 4 °C after 3h FR light irradiation, the possibility of lowered nuclear transport cannot be excluded.

As a further support for involvement of tubulin cytoskeleton, in the protoplast cells, neither the phyA nor the phyB:GFP nuclear

speckles were formed even after 3 hours of respective light irradiation.

The inability of cytB treated samples to form nuclear speckles in the respective irradiation in the seedlings indicated some role of actin network in the nuclear import and speckle formation of phytochrome. Further, the predominance of cytoplasmic aggregation of phyB:GFP indicated that cytoplasmic speckles are in fact a consequence of lowered nuclear import. Diffuse GFP fluorescence in nucleus in all our treatments and also the appearance of cytoplasmic speckles indicated that probably the phytochrome pool in the speckles includes an aggregation of active Pfr form along with other unidentified proteins. This supports the results demonstrated recently by Chen [23, 24]. It is striking to note that even when the actin cytoskeleton is completely disrupted (treatment with cytB), the phytochrome nuclear import is still possible as we observed diffuse GFP fluorescence in the nucleus in cytB treated seedlings. Moreover, it indicated that disruption of actin cytoskeleton could not prevent the formation of cytoplasmic speckles. Absence of diffuse fluorescence in the cytoplasm could be explained by the formation of cytoplasmic speckles as these speckles could include phytochrome self aggregation [25].

It would be interesting to understand the disappearance of pre-formed speckles when actin cytoskeleton is completely disrupted. In the current study, this couldn't be addressed in the cytB treated seedlings as the viability of seedlings may be adversely affected by treating with cytB long.

Taken together, all the results demonstrate that the cytoskeleton organization plays two different roles in phy intracellular partitioning at two different stages. The intact cytoskeleton organization plays a positive role in import of phytochrome to the nucleus during the initial light irradiation. Consequently, when the cytoskeleton organization is altered, the nuclear import of phyA and B is lowered. The second role is in the initial dark periods after prolonged light irradiation. Here the cytoskeleton plays a negative role in maintaining the integrity of the already formed speckles in absence of light by targeting the active Pfr form for degradation. In summary, intact cytoskeleton organization can neither block the nuclear import and formation of phytochrome speckles nor can it completely check their disappearance in darkness, but it can lower the kinetics of both the processes. The approach here to show the involvement of cytoskeleton organization in phy nuclear import is a new step. Further researches such as analysis of nuclear transport in a mutant of a cytoskeleton component can reveal where exactly the role of cytoskeleton is in phy nuclear transport.

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