BIOLOGIA PLANTARUM 55 (2): 253-260, 2011

Responses of transgenic *Nicotiana tabacum* seedlings expressing a *Cucurbita pepo* antisense *PHYA* RNA to far-red radiation

T.A. GAPEEVA*, T.V. ANTSIPAVA[1], A.N. PUNDIK and I.D. VOLOTOVSKI

Institute of Biophysics and Cellular Engineering, National Academy of Sciences of Belarus, 27 Akademicheskaya Str., 220072, Minsk, Belarus

Abstract

The *Nicotiana tabacum* transgenic plants expressing a *Cucurbita pepo* antisense *PHYA* RNA were obtained. The seedlings of transgenic tobacco with reduced phytochrome A (PHYA) content displayed decreased sensitivity to continuous broad-band far-red radiation (λ > 680 nm). Under far-red irradiance transgenic seedlings showed less elongation of the hypocotyls, more rapid plastid development, more chlorophyll accumulation, less repression of light-dependent NADPH:protochlorophyllide oxidoreductase than wild-type plants that was in accordance with PHYA control of plant development. Dynamics of the far-red radiation dependent changes in low temperature chlorophyll fluorescence spectra for the transgenic and wild-type seedlings were consistent with the more rapid formation of photosynthetic apparatus in the seedlings with reduced PHYA.

Additional key words: antisense expression, chlorophyll, chloroplast, phytochrome-deficient tobacco, high-irradiance response.

Introduction

The plants are known to have the special photoreceptors (phytochromes, cryptochromes, phototropines, *etc*.) for monitoring the red, far-red, blue and UV radiation signals (Jiao *et al*. 2007). Red (R) and far-red (FR) radiation is detected by the phytochrome adopting two major photoconvertible red (λ_{max} = 660 nm) and far-red (λ_{max} = 720 nm) form (Jiao *et al*. 2007). Far-red absorbing form is thought to be mainly responsible for mediating the phytochrome signal activity (Bae and Choi 2005). This activity manifests itself in the activation of some genes and is responsible for a number of morphogenetic effects such as induction of seed germination, flowering, deetiolation of the seedlings, hypocotyl elongation and others.

The functioning of the phytochrome system is due to the action of different types of phytochromes encoded by a small family of nuclear genes. For example, two phytochromes were reported for *Nicotiana tabacum* (PHYA, PHYB) (Adam *et al*. 1997, Intrieri and Buiatti 2008) and five for *Arabidopsis thaliana* (PHYA, PHYB, PHYC, PHYD, PHYF) (Sharrock and Quail 1989). All the phytochromes are subdivided into two main types: light-labile (phytochrome A) and other light-stabile forms. All the phytochromes accumulate in the darkness in red form, but PHYA is significantly more abundant in the etiolated plants. Its far-red form is sensitive to radiation and it is rapidly depleted to the level of other phytochromes in the greening seedlings. This phytochrome was established to remain activity under a strong shift of the photodynamic equilibrium to the side of the red form, as it takes place in the case of high irradiance response (HIR) observed under continuous far-red irradiance (Casal *et al*. 1997, Shinomura *et al*. 2000).

The functions of different phytochromes of the higher plants were investigated by using photomorphogenetic mutants deficient in phytochrome apoproteins, chromophore and by using transgenic plants (Whitelam and Harberd 1994). Despite of significant similarities, the plant species possess some variations in the function of concrete phytochrome type, in particular, the latter being true for the control of seedling development as a part of FR-HIR (Casal *et al*. 1997). Keeping in mind the

Received 8 August 2009, *accepted* 15 June 2010.

Abbreviations: as - antisense; FR - far-red radiation; HIR - high irradiance response; POR - NADPH:protochlorophyllide oxidoreductase; R - red radiation; W - white radiation; WT - wild-type.

Acknowledgements: The authors gratefully thank Prof. W. Rüdiger (Botanische Institute, LMU München, Germany) and Prof. K. Apel (Institute of Plant Sciences, ETH Zürich) for kindly presented antibodies, and Dr. E.V. Serduchenko (Institute of Biophysics and Cellular Engineering, NASB) for help in electron microscopy experiments.

[1] Present address: Central Botanical Garden, National Academy of Sciences of Belarus, 2b Surganova Str., 220012, Minsk, Belarus.
* Corresponding author, fax: (+375) 17 2842359, e-mail: gapeeva@ibp.org.by

necessity to enlarge the list of species with modified phytochrome content, we used *Nicotiana tabacum* as an example of polyploid species that had no phenotypically expressed phytochrome mutants to apply asRNA technique for reducing phytochrome content in plant cells. The *Nicotiana tabacum* is a convenient model for testing of *Agrobacterium* assisted transformation. Transgenic tobacco plants overexpressing heterologous oat, rice and *Arabidopsis thalliana* cDNA of PHYA, B, C were obtained to study phytochrome functions (Halliday *et al*. 1997, Robson and Smith 1997). However, the plants of this species with the biologically active phytochrome antisense RNA have not yet been reported although antisense potato and *Arabidopsis thaliana* plants with reduced phytochrome content have been obtained (Heyer *et al*. 1995, Jackson *et al*. 1998, Palecanda and Sharrock 2001).

The tobacco transgenic plants containing a genomic cassete for the synthesis of *Cucurbita pepo PHYA* asRNA were produced earlier (Luka *et al*. 1994). The aim of this study was to show the biological activity of introduced construction by detection of reduced content of PHYA and examining the transgenic plants for far-red responses assumed to be regulated by phytochrome A. The significant point which had also to be mentioned was that the far-red radiation used ($\lambda > 680$ nm) overlapped partly with the protochlorophyllide absorption spectrum that allowed to investigate a chlorophyll accumulation and expression of protochlorophillide oxidoreductase under a strong shift of the photodynamic equilibrium to the red phytochrome form.

Materials and methods

Plants and treatments: The transgenic plants with the asRNA of *Cucurbita pepo* phytochrome A (*GenBank* accession number M15265) inserted into the genome of tobacco (*Nicotiana tabacum* L. cv. Samsun) were obtained as had been described previously (Luka *et al*. 1994). The heterozygous population of the T_1 generation of the transgenic line designated as $PHYA_{antisense}$ was used for the most of the experiments.

White light (W; 350 μmol m^{-2} s^{-1}) was obtained by the combinations of an incandescent lamp (150 W) and five luminescent lamps (40 W, *SL40*, *Osram*, Smolensk, Russia). Broad band far-red radiation (FR; up to 170 μmol m^{-2} s^{-1}) was obtained by using the same lamps and a glass filter with light transmission above 680 nm (Vargin 1967). Integral irradiance was measured by radiometer *PMA2100* (*Solar Light Company*, Glenside, USA). It should be mentioned, that radiation with the wavelength of 680 - 700 nm was slightly absorbed by protochlorophyllide providing the possibility of chlorophyll formation under far-red irradiation.

For phenotype observations, the seeds of transgenic and wild-type plants were imbibed on water-soaked filter papers in Petri dishes for 2 d at 25 °C in complete darkness and then germinated under white light. After a week, the seedlings were transplanted into soil and grown from May to September under the natural irradiance to seed setting. The flowering time was determined as number of the days until to appearance of flowering shoot after that the plant height and leaf number were measured.

For hypocotyls elongation tests, measurements of chlorophyll and chlorophyll fluorescence spectra, the imbibed seeds were germinated under W, FR (40 or 170 μmol m^{-2} s^{-1}) or in darkness after preliminary induction of germination. This induction was performed by short-term exposure to W (20 μmol m^{-2} s^{-1}, about 1 min). The hypocotyl length was measured with 0.1 mm accuracy by using an enlarger.

For detection of phytochrome apoprotein, the seeds of the T_1 transgenic and wild-type (WT) plants were germinated for 10 d at 25 °C in darkness after preliminary induction of germination.

RNA-PCR analysis: Total RNA was isolated from leaves of plants grown under W with 16-h photoperiod by using *TRIzol* reagent (*Gibco/Invitrogen*, Grand Island, NY, USA). The cDNA was obtained by AMV reverse transcriptase (*Promega*, Madison, USA) and oligo dT_{18} primer. The PCR was performed by using *LC Taq* polymerase, high fidelity buffer (*Fermentas/Thermo Fisher Scientific*, Vilnius, Lithuania) and the following primers: NPTS (5'-CCTTGCTCCTGCCGAGAAAGTC TCC), NPTA (5'-CGGCAAGCAGGCATCGCCATGG GTC) for *nptII* gene (Libiakova *et al*. 2001); CUCS (5'-TGCTCAGACATCTGTTGATGCG), CUCA (5'-TGA CCTAGGCTGTTGATCTCCG) for *PHYA* of *Cucurbita pepo* (*GenBank* accession number M15265) and ActS (5'-CATGGTATTGTCAGCAATTG), Act A (5'-CCA CGCTCAGTGAGGATC) for the potato actin gene (*GenBank* accession number X55749). Annealing was performed at 58 °C for *nptII*, at 55 °C for *PHYA* of *Cucurbita pepo* and at 48 °C for the potato actin gene. The amount of cDNA template corresponded to 50 ng of total RNA. The expected sizes of RNA-PCR amplicons were 121 bp for *PHYA* gene of *Cucurbita pepo*, 241 bp for *nptII* and 373 bp for actin gene. Primers ActS, ActA designed to distinguish the actin cDNA from the potato actin gene (expected gene amplicon size is 502 bp) were used to control the quality and comparative quantity of cDNA. PCR product sizes were determined on 1.7 % agarose gel by comparison with DNA markers (*Fermentas*).

Chlorophyll content and chlorophyll fluorescence spectra: The chlorophyll *a* and *b* contents in crude clarified extracts in 80 % aqueous acetone were determined from the absorbance measured at 662 and 644 nm by spectrophotometer (*Lambda 25*, *PerkinElmer*,

(Waltham, USA) according to Schlyk (1971) .

The low-temperature fluorescence spectra (77 K) of intact tobacco leaves were recorded using a spectrofluorometer designed at the Institute of Biophysics and Cell Engineering, NASB (Domanskii *et al*. 1986). Excitation wavelength was 440 nm with the spectral slit of 2 nm; the relative emissions (E_{685} and $E_{730,}$) were recorded. The parameter E_{685}/E_{730} was used to indicate the relationship between the formation of photosystem 2 (E_{685}) and photosystem 1 (E_{730}).

Electron microscopy: The transgenic and WT plants were grown under FR for 2 and 6 d. Ultrathin sections of the cotyledons (Manankina *et al*. 1981) were fixed in buffered 2.5 % glutaraldehyde (pH 7.2) and postfixed in buffered 1 % osmium tetroxide (pH 7.2). The material was dehydrated in ethanol and acetone and embedded in the *Epon 812* resin. The thin sections were stained with uranyl acetate, contrasted with lead citrate and examined with electron microscope (*EMV-100 L*, *Selmi*, Sumy, Ukraine).

Protein electrophoresis and immunoblotting analysis: The crude extracts of total protein were obtained by adding 1 cm^3 of buffer (100 mM Tris, 28 mM 2-mercaptoethanol, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, pH 8.0) to 1 g of fresh mass of the seedlings followed by homogenization in liquid nitrogen and extract clarification by centrifugation for 5 min at 10 000 *g*.

For PHYA apoprotein detection, the crude extracts of the etiolated seedlings (50 μg of total protein) were separated by SDS-PAGE (10 % acrylamide gel), electroblotted to a nitrocellulose membrane and detected by using monoclonal antibodies (Z3-B1) against maize phytochrome (Bonenberger *et al*. 1992) kindly gifted by Prof. W. Rüdiger (Institute of Botany, LM-University of München, Germany). To detect the primary antibodies BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium) substrate was used for visualization of the mouse immunoglobulines conjugated with alkaline phosphatase. The native oat phytochrome (124 kDa) was prepared as described by Lapko (1989) and was used as a molecular mass marker.

The analysis of NADPH:protochlorophyllide oxidoreductase (POR, EC 1.3.1.33) was performed by dot immunoblotting. The seeds were placed for swelling on water-soaked filter papers in Petri dishes for 2 d, stimulated for germination as described above, transferred to the dark for 2 d, and then one part was transferred under FR (170 μmol m^{-2} s^{-1}), and another one was kept in darkness. The crude extracts were obtained after 1, 2, 3 and 4 d. POR was detected using polyclonal rabbit antiserum against barley NADH-protochlorophyllide oxido-reductase, the antibodies were kindly gifted by professor K. Apel (Institute of Plant Sciences**,** ETH Zürich). Horseradish peroxidase conjugated with mouse immuno-globulins was used for immunochemical detection.

Results

To study responses to far-red radiation that were assumed to be phytochrome A-mediated, the *Nicotiana tabacum* transgenic plants, containing a genomic cassete with 35S CaMV promoter for the synthesis of *Cucurbita pepo PHYA* asRNA (Luka 1994), were analyzed for the content of phytochrome A apoprotein. Because of significant prevalence of phytochrome A type in darkness, the content of phytochrome apoprotein was detected in ethiolated seedlings by immunoblotting using anti-maize antibodies Z-3B1 with the wide ranging cross-reactivity (Fig. 1). The marked decrease in phytochrome was observed in 10-d-old etiolated seedlings of the transgenic lines of accessions 4 and 8. The accession 8 designated as $PHYA_{as}$ was chosen for further research because of accession 4 infertility.

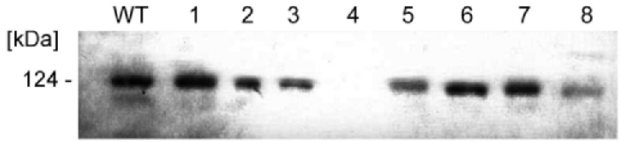

Fig. 1. Immunoblot detection of PHYA apoprotein in 10-d-old etiolated tobacco seedlings. WT - wild type, 1 to 8 - $PHY_{antisense}$ transgenic lines. Each sample is the crude extracts of etiolated seedlings contained 50 μg of total protein. The native phytochrome from etiolated oat seedlings was used as a molecular mass marker (124 kDa).

Table 1. Time to flowering and plant height and leaf number at the onset of flowering in *Nicotiana tabacum* wild-type plants and $PHYA_{as}$ transgenic plants grown under natural irradiance. Means ± SE, n = 10.

Genotype	Plant height [cm]	Leaf number	Time to flowering [d]
WT	18.9 ± 5.0	8.5 ± 1.0	62 ± 10.0
$PHYA_{as}$	41.2 ± 8.1	19.7 ± 2.0	122 ± 7.0

As it was determined by RNA-PCR, the antisense and *nptII* transgene expression for the T_1 generation of $PHYA_{as}$ plants represented the population of transgenic heterozygotes (Fig. 2). The population has shown 3:1 segregation for *nptII* gene as was determined by DNA-PCR (data are not presented).

Some phenotypic characteristics of the T_2 generation of transformed $PHYA_{as}$ plants were investigated (Table 1). The $PHYA_{as}$ plants growing under natural irradiance had much more leaves and they were higher than WT plants, while flowering was 60 d later in $PHYA_{as}$ plants than in WT plants.

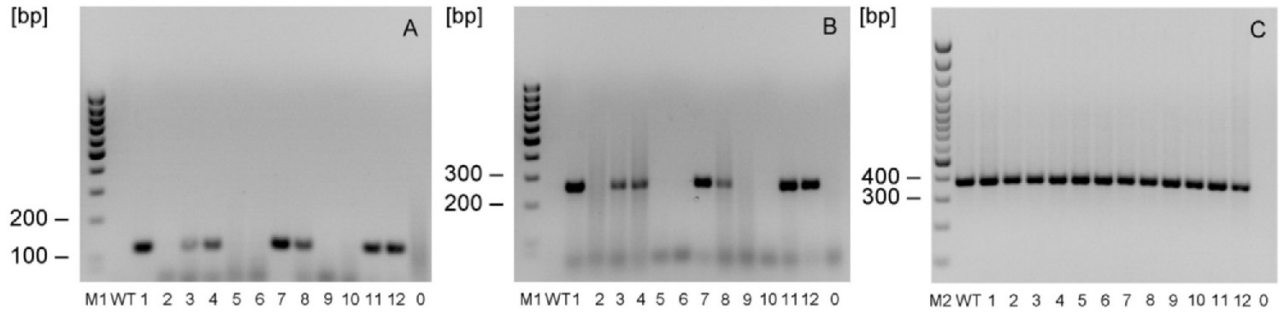

Fig. 2. PCR analysis in T_1 generation of $PHY_{antisense}$ plants (1 to 12) with primers to *Cucurbuta pepo PHYA* (*A*), npt*II* (*B*) and potato *actin* (*C*) genes. M1, M2 - 100 bp DNA ladder, WT - tobacco wild type, 0 - control PCR without template. The expected size of amplicon is 121 bp for *PHYA* of *Cucurbita pepo*, 241 bp for *nptII* and 373 bp for actin mRNA transcripts.

The transgenic seedlings grown under FR differed from those of WT tobacco in hypocotyl length (Fig. 3).

Under continuous FR the average hypocotyl length was only about 12 % of that in D in WT plants while about 20 % in $PHYA_{as}$. When the plants were grown under W, the average hypocotyl length was less than 10 % of that in D in WT plants and less than 14 % in the $PHYA_{as}$ seedlings. The hypocotyle elongation of WT seedlings was similar under FR 40 and 170 μmol m^{-2} s^{-1}, while $PHYA_{as}$ seedling hypocotyls were much longer under FR 40 than 170 μmol m^{-2} s^{-1}.

The transgenic seedlings grown under FR radiation (170 μmol m^{-2} s^{-1}) retained the ability of chlorophyll accumulation in contrast to the wild-type tobacco (Table 2). The chlorophyll content in phytochrome A reduced plants grown under FR was comparable with

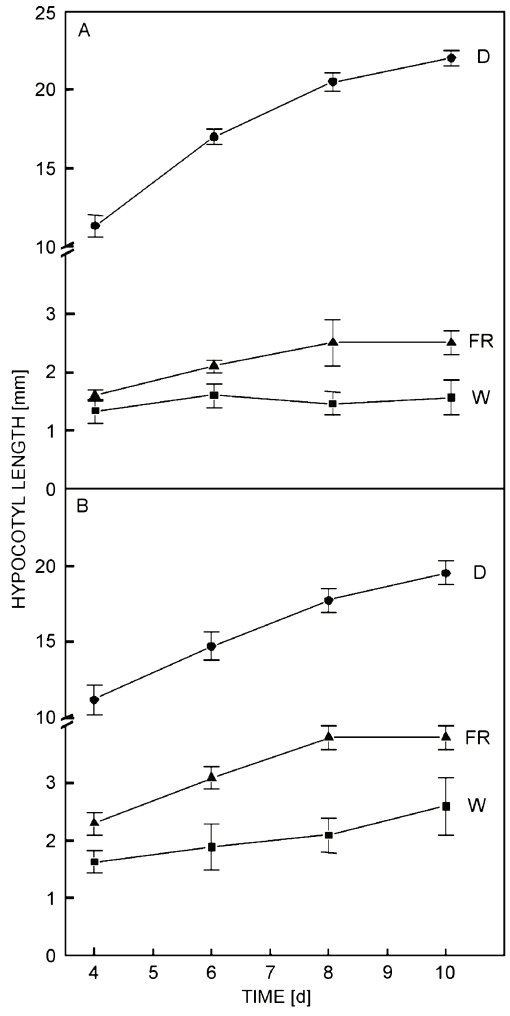

Figure 3. Hypocotyle length of wild type (*A*) and $PHYA_{antisense}$ (*B*) seedlings. D - darkness, W - white light (350 μmol m^{-2} s^{-1}), FR - far-red (170 μmol m^{-2} s^{-1}). Means ± SE of 3 replicates, each of 50 seedlings.

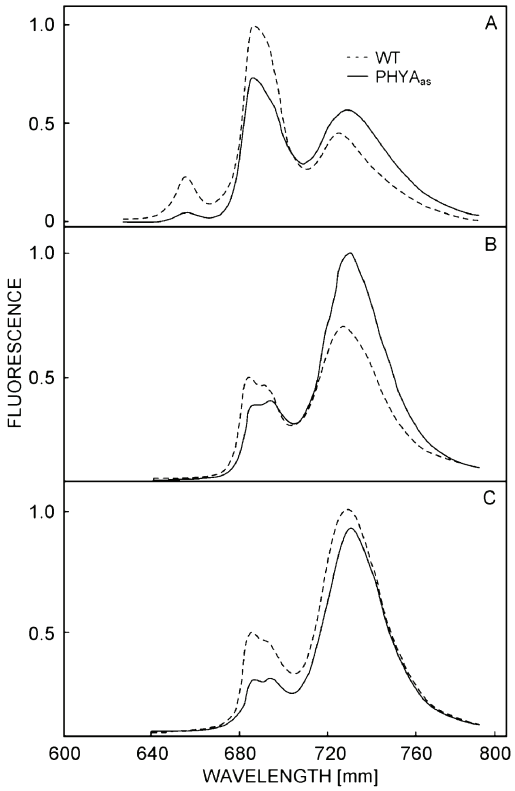

Fig. 4. Uncorrected low-temperature (77 K) fluorescence spectra (λ_{ex} = 440 nm) of cotyledons from the 1 (*A*), 4 (*B*), 7 (*C*)-d-old tobacco grown under far-red radiation.

that of plants grown under W. This value for FR-grown WT tobacco was 4.6 times smaller than for W-grown plants and 3.5 times smaller as compared with FR-grown $PHYA_{as}$ plants. However, the transgenic seedlings grown under W accumulated somewhat less chlorophyll *a+b* mainly due to less accumulation of chlorophyll *b*.

Table 2. Chlorophyll (Chl) content [mg g^{-1}(f.m.)] in 6-d-old tobacco WT and $PHYA_{as}$ seedlings grown under continuous white (W) or far-red (FR) radiation. Means ± SE of 5 replicates, each of 50 seedlings.

	Genotype	Chl *a*	Chl *b*	*a*/*b*
W	WT	0.30 ± 0.01	0.15 ± 0.08	1.98 ± 1.0
	$PHYA_{as}$	0.27 ± 0.01	0.09 ± 0.02	2.87 ± 0.6
FR	WT	0.07 ± 0.01	0.04 ± 0.01	1.86 ± 0.5
	$PHYA_{as}$	0.22 ± 0.04	0.13 ± 0.04	1.78 ± 0.6

Table 3. The ratio of emission E_{685}/E_{730} derived from low-temperature (77 K) fluorescence spectra for intact tobacco cotyledons grown under FR (170 μmol m^{-2} s^{-1}). Values are means ± SE for 5 replicates, each of 5 seedlings.

Genotype	1-d-old plants	4-d-old plants	7-d-old plants
WT	2.61 ± 0.50	0.63 ± 0.10	0.45 ± 0.05
$PHYA_{as}$	1.10 ± 0.20	0.37 ± 0.04	0.29 ± 0.10

The 1-d-old seedlings of both WT and $PHYA_{as}$ plants grown under FR (170 μmol m^{-2} s^{-1}) contained proto-chlorophyllide that retained the ability for photoreduction accompanied by the fluorescent emission at 655 nm (Fig. 4*A*). However, the parameter E_{685}/E_{730} (see Materials and methods) was lower for the transgenic plants (Table 3). The fluorescence at 655 nm corresponding to the active form of protochlorophyllide was not recorded after 4 and 7 d under continuous FR (Fig. 4*B,C*). The spectra region at shorter wavelengths resembled that of photosystem 2 with two weakly resolved bands appearing at 685 and 692 nm. In this case the E_{685}/E_{730} ratio was 0.37 for the $PHYA_{as}$ and 0.63 for WT plants. The difference in E_{685}/E_{730} ratio was also observed for the 7-d-old plants (Table 3).

The WT seedlings grown under FR (170 μmol m^{-2} s^{-1}) lagged behind transgenic plants significantly in plastid development. The high developed prolamellar bodies that are typical for etioplasts were revealed in plastids of the 2-d-old WT seedlings (Fig. 5*A*), and on the contrary, they were not found in $PHYA_{as}$ plants. The plastids of 2-d-old cotyledons in the $PHYA_{as}$ seedlings contained the chloroplasts with typical grana and small number of thylakoids (Fig. 6*A*). Besides, 2-d-old cotyledons of $PHYA_{as}$ contained large starch granules while only small starch granules were observed in WT plants. The 6-d-old WT seedlings still retained prolamellar bodies (Fig. 5*B*) while in chloroplasts of $PHYA_{as}$ plants grana were observed (Fig. 6*B*).

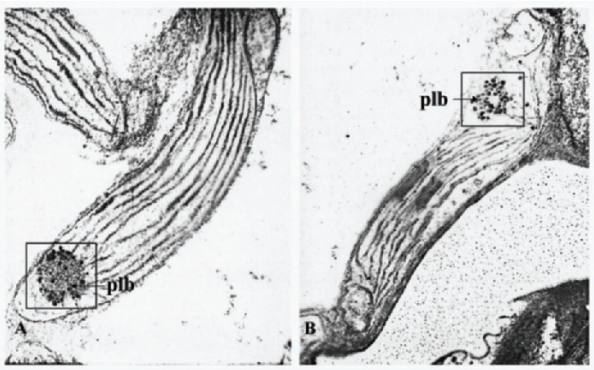

Fig. 5. The plastids of cotyledons from 2 (*A*) and 6 (*B*)-d-old wild type tobacco seedlings grown under far-red radiation: plb - prolamellar body; 10 000×.

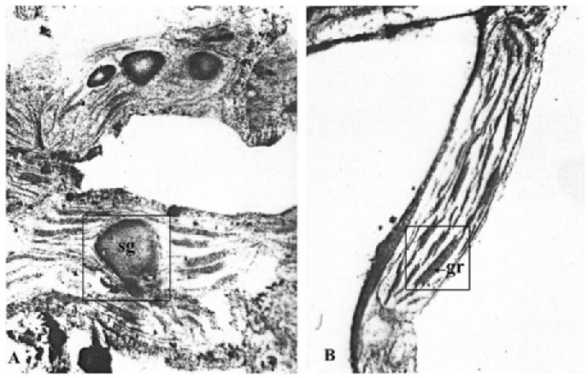

Fig. 6. The plastids of cotyledons from 2 (*A*) and 6 (*B*)-d-old $PHYA_{antisense}$ tobacco seedlings grown under far-red radiation: sg - starch granule, gr - grana; 10 000×.

For dark-grown WT and $PHYA_{as}$ plants the POR protein accumulation was similar. POR protein content in WT plants grown under FR was not changed significantly until the fourth day of irradiation. On the contrary, the FR-germinated transgenic seedlings accumulated POR protein gradually with the age.

Discussion

The antisense RNA approach was used to obtain the tobacco plants with decreased PHYA content. Because of the absence of strict regularities connecting the structure of antisense sequence used and the effect of the inhibition of transcription, the question of functioning of a *Cucurbita pepo* antisense *PHYA* RNA in tobacco cells was of particular interest. The PHYA detection by immunoblotting showed the remarkable decrease in apoprotein amount in some transformed lines and suggested that heterologous *Cucurbita pepo* antisense

PHYA RNA can be used for the inhibition of the PHYA expression in tobacco (Fig. 1). However, the presence of antisense construction in the genome (T_0 generation) did not induce the PHYA decrease in all the lines that might be due to the peculiarities of T-DNA insertion in individual transgenic lines. The phytochrome A-deficient line denoted as $PHYA_{as}$ was chosen for the further research. The heterozygous population of the T_1 generation of $PHYA_{as}$ plants that showed expression of antisense RNA (Fig. 2) was used in the majority of experiments.

Three types of phytochrome responses are discussed: responses to very low irradiance (VLFR, 0.1 to 1 μmol m^{-2} s^{-1}), responses to low irradiance (LFR, 1 to 1 000 μmol·m^{-2} s^{-1}) and responses to high-irradiance (HIR, above 1 000 μmol m^{-2} s^{-1}). The published results and the studies on the mutant and transgenic plants are in accordance with the point of view that phytochrome B and sometimes other light-stabile phytochromes mediate LFR, while phytochrome A mediates VLFR and HIR (Casal *et al*. 1998). In the present study the continuous broad band far-red radiation (170 μmol m^{-2} s^{-1}) with $\lambda > 680$ nm was applied to study photoregulatory functions of phytochrome A. The PHYA-controlled processes develop in such a case under the strong shift of the photodynamic equilibrium to the red form and correspond to high irradiance response so called FR-HIR (Mohr 1962). As stated above, the radiation with wavelength from 680 to 700 nm allows also the greening.

The $PHYA_{as}$ plants had enlarged height, leaf number and time to flowering under natural photoperiod (Table 1). The same effects have been detected for the null PHYA mutant of *Arabidopsis thaliana* (Whitelam *et al*. 1993) under growth conditions approximated to natural ones. In our study, the PHYA-deficient transgenic plants of tobacco differed from the WT plants when grown under natural irradiance. It may be assumed that change in phytochrome content in tobacco is very critical for the processes determining the period before flowering so that causes above mentioned morphological peculiarities of plants grown under natural irradiance.

Morphogenesis of the white light-grown seedlings in comparison with dark control was characterized by quick inhibition of hypocotyl elongation, cotyledon opening and extension and chlorophyll accumulation. The seedling development under continuous FR was characterized by partial morphogenesis without chlorophyll accumulation, at least in the case of *Arabidopsis thaliana*. The *Arabidopsis* seedlings were yellow with shortened hypocotyls and opened cotyledons that corresponded to the classic HIR. This response was assumed to be PHYA-mediated (Whitelam *et al*. 1993).

In this study the tobacco seedlings grown under continuous FR also showed HIR expressed in inhibition of hypocotyl elongation (Fig. 3) but the WT plants were observed to react more significantly. and the degree of inhibition being independent on irradiance. The effects observed for $PHYA_{as}$ plants depended strongly on irradiance. The same dependence was noted in the *phyA-105 missens Arabidopsis* mutant (Xu *et al*. 1995). The weakening of inhibition of hypocotyl elongation under continuous FR may be explained by PHYA decrease in $PHYA_{as}$ cells like in the case of PHYA-antisense potato (Heyer *et al*. 1995) and PHYA-deficient *Arabidopsis* mutants *phyA-1, phyA-2, hy8* (Parks 1993). The decrease in sensitivity to inhibition of hypocotyl elongation of the $PHYA_{as}$ seedlings grown under W was greatly less than that of FR-grown plants. It is in accordance with the conception that PHYA plays only a negligible role in the regulation of germination under W (Whitelam *et al*. 1993, Casal *et al*. 1997).

As mentioned above, using of FR with $\lambda > 680$ nm, which partially overlapped with the protochlorophyllide absorption spectra, allowed the deetiolation and of expression of protochlorophillide oxidoreductase (*por*) gene. Therefore, WT and $PHYA_{as}$ tobacco seedlings were investigated for the chlorophyll accumulation under FR (Table 2). The WT seedlings were characterized by low chlorophyll content under continuous FR, while the $PHYA_{as}$ plants retained the ability to accumulate chlorophyll (*a*+*b*) at the same level as W-grown plants. Dynamics of the changes in low temperature (77 K) chlorophyll fluorescence spectra (Fig. 4) and in plastid ultramicroscopic structures (Figs. 5,6) were consistent with the more rapid formation of photosynthetic apparatus in the FR-grown seedlings with reduced PHYA than in WT plants. Besides, the ratio E_{685}/E_{730} of the $PHYA_{as}$ was significantly lower than that of WT seedlings (Table 3). Considering the E_{685} to be associated with photosystem 2 (PS 2) and E_{730} with PS 1, it may be assumed higher PS 1 formation in the $PHYA_{as}$ plants than in WT. Thus, the tobacco cells with a decreased content of PHYA are less sensitive to inhibitory effect of FR on chlorophyll formation, and their photosynthetic apparatus is formed quicker under FR.

It is known that *Arabidopsis* and tomato seedlings grown under continuous FR are able neither to synthesize chlorophyll, nor become green upon subsequent transfer into W (Van Tuinen *et al*. 1995, Barnes *et al*. 1996). At the same time, PHYA-deficient mutants of *Arabidopsis thaliana* (*phyA-201*) and also its mutant deficient in PHYA signal transduction pathway (*fhy-1*) do not lose the ability of greening after transfer to W following the 3-d development under FR. In our case, when FR-grown tobacco plants were able to accumulate chlorophyll partially, the greening of WT plants was inhibited but deficiency in PHYA in transgenic plants caused the loss of sensitivity to inhibitory action of continuous FR.

The greening inhibition in *Arabidopsis* seedlings was shown to be PHYA-mediated due to the inhibition of *por* gene (Barnes *et al*. 1996). To explain similar effect detected here for *Nicotiana tabacum*, the POR accumulation has been examined. POR catalyzes the single light-dependent stage in chlorophyll biosynthesis pathway, namely photoreduction of protochlorophillide to chlorophillide. The enzyme and its mRNA decreased strongly after irradiation with W (Apel 1981, Batschauer and Apel 1984). The phytochrome control of *por*

transcription has been described for the barley. The level of *por* mRNA in barley fell strongly after R irradiation and it was reversed by FR (Mosinger *et al*. 1985). The data obtained in this work conform to the fact of *por* repression by phytochrome. This repression is absent or markedly weakened in $PHYA_{as}$ tobacco plants showing that FR-induced inhibition of *por* expression may be PHYA-mediated. In contrast to the WT the $PHYA_{as}$ plants accumulated POR both in darkness and under FR. Data obtained do not contradict the fact of back-regulation of *por* expression by phytochrome and confirm the possibility of this regulation by light-labile form of phytochrome.

Thus, it may be concluded that transgenic plants obtained are characterized by decreased content of phytochrome A and reveal the properties of *phyA* mutants. We do not take into account possible decrease in phytochrome B (PHYB) content because of the absence of visible changes in PHYB mRNA transcript level as it has been detected by RNA-PCR (data are not presented) and because of using continuous FR corresponding to PHYA-regulated FR-HIR. It should be mentioned, that despite of being undetectable by RNA-PCR, some antisense RNA-inhibition of PHYB expression can not entirely be excluded, and, so, it may influence the results obtained for natural and W irradiance (Table 1, Fig. 3). In any case, the data described here give evidence that the cassette for the expression of a *Cucurbita pepo* antisense *PHYA* RNA is biologically active in tobacco cells. The $PHYA_{as}$ plants displayed the reduced sensitivity to inhibitory action of FR as follows from hypocotyl elongation response, photosynthetic apparatus formation and accumulation of light-dependent NADPH:protochlorophyllide oxido-reductase. So, the plants obtained can be used for investigation of phytochrome regulation, for example, for study the phytochrome control of gene expression in tobacco.

References

Adam, E., Kozma-Bognar, L., Schafer, E., Nagy F.: Tobacco phytochromes: genes, structure and expression. - Plant Cell Environ. **20**: 678-684, 1997.

Apel, K.: The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.): pPhytochrome-induced decrease of translatable mRNA coding for the NADH:protochloro-phyllide oxidoreductase. - Eur. J. Biochem. **120**: 89-93, 1981.

Bae, G., Choi, G.: Decoding of light signals by plant phytochromes and their interacting proteins. - Annu. Rev. Plant Biol. **59**: 281-311, 2005.

Barnes, S.A., Nishizawa, N.K., Quaggio, R.B. Whitelam, G.C., Chua, N.-H.: Far-red light blocks greening of *Arabidopsis* seedlings via a phytochrome A-mediated change in plastid development. - Plant Cell **8**: 601-615, 1996.

Batschauer, A., Apel, K.: The inverse control by phytochrome of the expression of two nuclear genes in barley (*Hordeum vulgare* L.). - Eur. J. Biochem. **143**: 593-597, 1984.

Bonenberger, J., Schendel, R., Schneider-Poetsch, H.A., Rüdiger, W.: Structural studies on the photoreceptor phytochrome: reevaluation of the epitope for monoclonal antibody Z-3B1. - Photochem. Photobiol. **56**: 717-723, 1992.

Casal, J.J., Sanchez, R.A., Botto J.F.: Modes of action of phytochromes. - J. exp. Bot. **49**: 127-138, 1998.

Casal, J.J., Sanchez, R.A., Yanovsky, M.J.: The function of phytochrome A. - Plant Cell Environ. **20**: 813-819, 1997.

Domanskii, V., Samoylenko, A.G.**,** Lis, P.I.: [Automatic spectrofluorimeter controlled by microcomputer.] - Izv. Acad. Nauk BSSR, Ser. Biol. **5**: 100-103, 1986. [In Russ].

Halliday, K.J., Thomas, B., Whitelam, G.C.: Expression of heterologous phytochromes A, B or C in transgenic tobacco plants alters vegetative development and flowering time. - Plant J. **12**: 1079-1090, 1997.

Heyer, A.G., Mozley, D., Landschutze, V., Thomas, B., Gatz, C.: Function of phytochrome A in potato plants as revealed through the study of transgenic plants. - Plant Physiol. **109**: 53-61, 1995.

Intrieri, M.C., Buiatti, R.M.: Phytochrome A as a functional marker of phyletic relationships in *Nicotiana* genus - Biol. Plant. 52: 36-41, 2008.

Jackson, S.D., James, P., Prat, S., Thomas, B.: Phytochrome B affects the levels of a graft-transmissible signal involved in tuberization. - Plant Physiol. **17**: 29-32, 1998.

Jiao, Y., Lau, O.S., Deng, X.W.: Light-regulated transcriptional networks in higher plants. - Nat. Rev. Genet. **8**: 217-230, 2007.

Lapko, V.N.: [The quick method for oat phytochrome preparation.] - Izv. Acad. Nauk BSSR, Ser. Biol. **2**: 104-107, 1989. [In Russ.]

Libiakova, G., Jurgensen, B., Palmgren, G., Ulskov, P., Johansen, E.: Efficacy of an intron-containing kanamycin resistance gene as a selectable marker in plant transformation. - Plant Cell Rep. **20**: 610-615, 2001.

Luka, Z.A., Pogidaeva, L.I., Volotovski, I.D.: [The obtaining of transgenic tobacco with the genetic construction for the phytochrome antisense RNA.] - Proc. Acad. Sci. Belaruss. **1**: 45-49, 1994. [In Belaruss.]

Manankina, E.E., Serduchenko, E.V., Radyk, M.S., Shlyk, A.A.: [Reversible changes of ultrastructural organization of *Chlorella* cells under influence of levulinic acid.] - Izv. Acad. Nauk USSR **6**: 917-925. 1981. [In Russ.]

Mohr, H.: Primary effects of light on growth. - Annu. Rev. Plant Physiol. **13**: 465-488, 1962.

Mosinger, E., Batschauer, A., Schafer, E., Apel, K.: Phytochrome control of *in vitro* transcription of specific genes in isolated nuclei from barley (*Hordeum vulgare* L.). - Eur. J. Biochem. **147**: 137-142, 1985.

Palecanda, L., Sharrock, R.A.: Molecular and phenotypic specificity of an antisense *PHYB* gene in *Arabidopsis*. - Plant mol. Biol. **46**: 89-97, 2001.

Parks, B.M., Quail, P.H.: *hy 8*, a new class of *Arabidopsis* long hypocotyl mutants deficient in functional phytochrome A. - Plant Cell **5**: 39-48, 1993.

Robson, P.R., Smith, H.: Fundamental and biotechnological applications of phytochrome transgenes. - Plant Cell Environ. **20**: 831-839, 1997.

Sharrock, R.A., Quail, P.H.: Novel phytochrome sequences in *Arabidopsis thaliana*: structure, evolution, and differential expression of a plant regulatory photoreceptor family. - Genes **3**: 1745-1757, 1989.

Shinomura, T., Uchida, K., Furuya, M.: Elementary processes of photoperception by phytochrome A for high-irradiance response of hypocotyls elongation in *Arabidopsis*. - Plant Physiol. **122**: 147-156, 2000.

Shlyk, A.A.: Detection of chlorophyll and carotenoides in extracts of green leaves. - In: Pavlinova, O.A. (ed.): Biokhimicheskie metody v Fiziologii rastenii. [Biochemical Methods in Plant Physiology.] Pp. 154-170. Nauka, Moskva 1971.

Van Tuinen, A., Kerckhoffs, L.H.J., Nagatani, A., Kendrick, R.E., Koornneef, M.: Far-red light-insensitive, phytochrome A-deficient mutants of tomato. - Mol. gen. Genet. **246**: 133-141, 1995.

Vargin, V.V. (ed.): Katalog Tsvetnogo Stekla. [Catalogue of colored glass.] - Mashinostroenie, Moskva 1967.

Whitelam, G.C., Johnson, E., Peng, J., Carol, P., Anderson, M.L., Cowl, J.S., Harberd, N.P.: Phytochrome A null mutants of *Arabidopsis* display a wild-type phenotype in white light. - Plant Cell **5**: 757-768, 1993.

Whitelam, G.C., Harberd, N.P.: Action and function of phytochrome family members revealed through the study of mutant and transgenic plants. - Plant Cell Environ. **17**: 615-662, 1994.

Xu, Y., Parks, B.M., Short, T.W., Quail, P.H.: Missense mutations define a restricted segment in the C-terminal domain of phytochrome A critical to its regulatory activity. - Plant Cell **7**: 1433-1443, 1995.