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Acquisition of plastid movement responsiveness to light during mesophyll cell differentiation

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ABSTRACT A culture of *Nicotiana tabacum* leaf protoplasts, regenerating *in vitro*, was used to study light-induced plastid translocations. Experiments were carried out for 5 months starting with protoplasts, through single cells, microcolonies and callus to the differentiated mesophyll of regenerated plants. Although the actin cytoskeleton was fully developed at every stage of culture, blue light-mediated directional movements of chloroplasts were observed only after the full differentiation of callus into leaf tissues. These chloroplast rearrangements were similar to those observed in control plants grown from seeds. Under strong blue light, chloroplasts gathered at the cell walls parallel to the light direction (profile position); under weak blue light, they gathered at the walls perpendicular to the light direction (face position). No light-dependent plastid arrangements were found in undifferentiated cell cultures even after cell wall recovery. A characteristic pattern of plastids in the dividing cells was independent of light signals. Only trace chloroplast responses to strong blue light were detected in the first leaves regenerating from callus. We hypothesize that factors which control the developmental status of a cell, i.e. division and differentiation, take over the control of plastid redistribution from light signals.

KEY WORDS: *chloroplast movement, morphogenesis, Nicotiana tabacum, callus, protoplast regeneration*

Introduction

Blue light-induced directional movements of chloroplasts occur in many lower and higher plant species; red light is active only in a limited number of the plant genera investigated so far (Trojan and Gabrys 1996, Augustynowicz and Gabrys 1999, for review see: Wada *et al.,* 2003). Strong light induces chloroplasts to gather at the walls parallel to the light direction (profile position, avoidance response) while weak light directs them to the walls perpendicular to the light direction (face position, accumulation response). In the dark chloroplasts are distributed more or less randomly and controlled by unknown endogenous factors. It is accepted that chloroplast movements play a significant role in the optimisation of photosynthesis under changing conditions of natural light. The weak light response is thought to maximise photosynthesis, whereas the strong light response is regarded to be a strategy against photodamage of the photosynthetic apparatus (Zurzycki 1955, Zurzycki 1957, Park *et al.,* 1996, Kasahara *et al.,* 2002). The blue light signal is perceived by phototropins (Kagawa 2003), although the precise signalling pathway from the receptor to effector system remains unknown (Tlalka and Gabrys 1993, Tlalka and Fricker 1999, Grabalska and Malec 2004, Gabrys 2004). Chloroplasts redistribute in the cell using actin tracks and myosin motors (Takagi 2002, Krzeszowiec *et al.,* 2007, Krzeszowiec and Gabrys 2007). Besides, the actin cytoskeleton constitutes one component of the cell wall-plasma membrane-cytoskeleton *continuum*. This dynamic system plays a fundamental role in the control of cell expansion and cell division patterns during morphogenesis (Fowler and Quatrano 1997, Baluska *et al.,* 2003). It is suggested that the anchorage of actin filaments to the plasma membrane enables directional movements, including chloroplast responses under blue irradiation (Augustynowicz *et al.,* 2001) as well as cytoplasmic streaming (Masuda *et al.*1991, Ryu *et al.,* 1997). We have previously demonstrated that chloroplasts lose their ability to respond directionally to light signals in mesophyll protoplasts of tobacco i.e. after cell wall removal. Intriguingly, the function of the cell wall could be partly performed by unknown factors induced by low temperature. In protoplasts isolated from cold-treated plants, chloroplasts moved in the manner observed in an intact leaf mesophyll. These protoplasts

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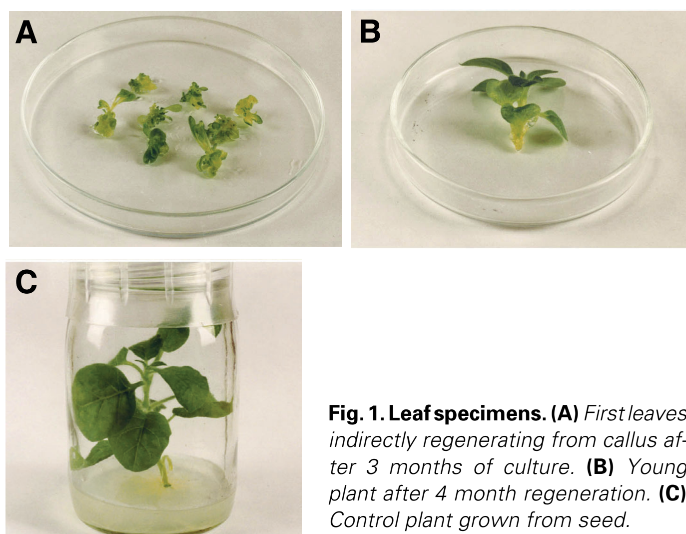


Fig. 1. Leaf specimens. (A) *First leaves indirectly regenerating from callus after 3 months of culture.* **(B)** *Young plant after 4 month regeneration.* **(C)** *Control plant grown from seed.*

exhibited also a significantly higher rigidity than the protoplasts of control plants (Augustynowicz *et al.,* 2001). In the work reported here we carried out experiments on the involvement of the cell wall - plasma membrane - cytoskeleton *continuum* in blue light-controlled chloroplast /plastid redistribution. An *in vitro* culture of regenerating protoplasts was chosen as a good model to seek a correlation between the stage of cell development and the ability of plastids to perform orientational movement. To our knowledge, no study of this type had been done before. It turned out that the restoration of the cell wall did not reinstate directional plastid rearrangements under blue irradiation. Moreover, plastids in 2-3 day old cells regenerating from protoplasts obtained from cold-treated plants lost their capacity for light-controlled directional movement even though the actin cytoskeleton and the cell wall were regenerated in these cells. No clear response in plastid arrangements could be observed under blue irradiation in undifferentiated cell cultures. Instead, a characteristic pattern of plastid distribution was detected in dividing cells. Only after a full differentiation of the callus into mesophyll tissue did the first blue light-directed movement of plastids appear.

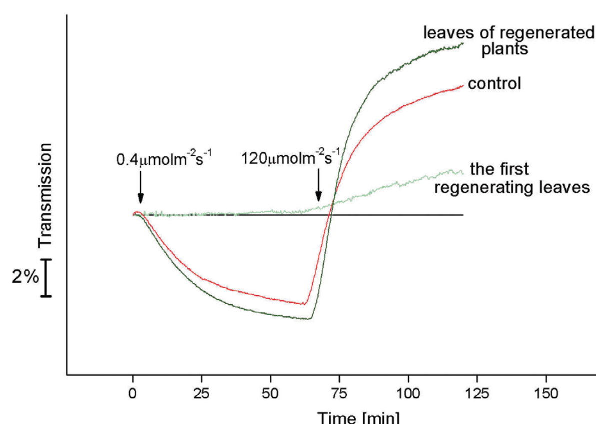
Results

Movements of chloroplasts in leaves

Chloroplast translocations were examined in the mesophyll tissue regenerating from protoplasts. Three types of specimen were used: the first leaves just regenerating from callus, small leaves after full regeneration from callus, and the leaves of plants grown from seeds as a control (Fig. 1 A,B,C respectively). Only trace chloroplast responses to continuous strong blue light were found in the first leaves regenerating from callus. The leaves were fragile and semi-transparent with an average area of 15-20 mm^2. However, they had already formed stomata and hairs. In spite of this relatively advanced level of regeneration, neither responses to weak blue light nor to blue light pulses were observed in these leaves (Fig. 2A and 3, flat traces). In the fully regenerated leaves the chloroplast rearrangements were similar to those observed in the control plants (cf. the respective traces in Fig. 2A). For these comparative experiments only leaves which had similar levels of transmission after dark adaptation (about 10%) were chosen. Amplitudes and velocities of the responses to continuous strong blue light are compared in Fig. 2B. Both parameters characterizing the avoidance response were higher in the fully regenerated leaves than in the control ones. Besides the rearrangements under continuous blue light, transient responses of chloroplasts to pulses of strong blue light were detected in the regenerated mesophyll tissue (Fig. 3). The characteristic biphasic time-courses of the corresponding transmission changes and their similarity to the changes observed in the control tissue point to a complete regeneration of the movement apparatus in the regenerated leaves.

Plastid translocations in protoplasts and regenerating cells

Chloroplast translocations in protoplasts have been shown in our previous paper (Augustynowicz *et al.,* 2001). Briefly, after cell wall digestion, the chloroplasts did not effect significant directional redistributions regardless of light intensity. On the contrary,



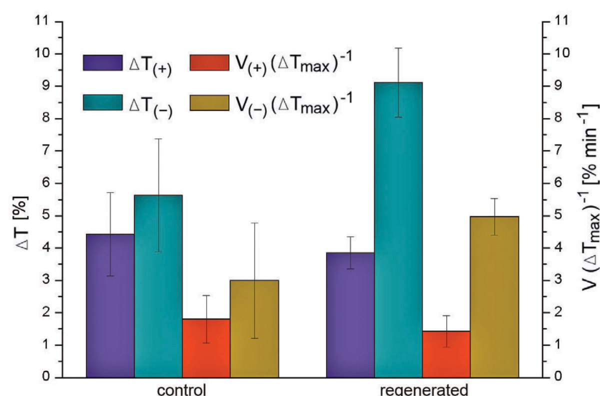


Fig. 2. Chloroplast responses in leaves of control plants and of regenerated plants. (A) *Typical time-courses of changes in transmission of light correlated with chloroplast movement to continuous blue light.* **(B)** *Parameters of chloroplast responses to continuous weak (+) (0.4 μmol·m^{-2}·s^{-1}) and strong (-) (120 μmol·m^{-2}·s^{-1}) blue light; ΔT, amplitudes; $V(\Delta T_{max})^{-1}$ velocities normalized to the maximum amplitude, each value is an average of 4-9 measurements. Error bars represent standard deviations.*

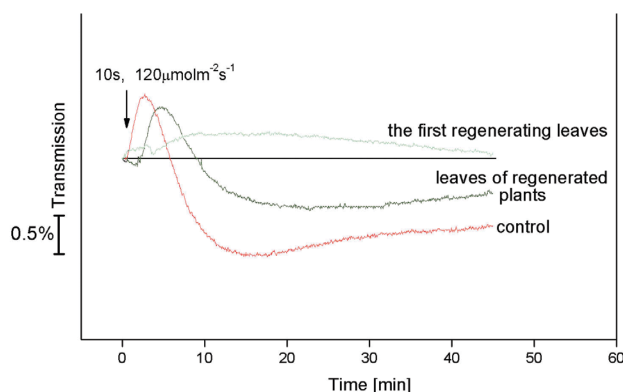


Fig. 3. Typical time-courses of changes in transmission of light *caused by chloroplast redistributions in response to a 10 s blue light pulse.*

protoplasts isolated from cold pre-treated plants showed manifest chloroplast responses under strong and weak blue irradiation - similar to those observed in the cells of the intact leaf mesophyll.

The ability of chloroplasts to effect directional movements under continuous blue light was lost 2-3 days after the isolation of protoplasts from the cold pre-treated plants. Plastid distributions in the regenerating cultures were comparable in both the control and cold-treated plants and independent of light. At the same time specific arrangements of plastids became visible: they partially redistributed towards the cell centre (Fig. 4A). These particular plastid patterns were followed by cell division, which took place after 3-5 days of culture. During cell division, a high number of plastids were clustered in the area of the division plane (Fig. 4 B,C). The plastids remaining outside the dividing plane exhibited high mobility. The velocity of plastid movements in a microcolony which was a few days old was higher than the velocity of chloroplast translocations in the protoplasts (data not shown). The accelerated motion of plastids corresponded with the higher number of transvacuolar cytoplasmic strands formed in the cells (Fig. 4A, indicated with arrows). No light-induced movements of plastids were recorded in cells regenerating from protoplasts, even after cell wall formation/cell division (compare plastid distributions in Fig. 5, left and right column). The first blue-light-controlled directional chloroplast translocations were detected in the differentiating mesophyll tissue, after ca. three months of culture (see above). A detailed analysis of the kinetic parameters of plastid translocations was not feasible because of problems with focussing: apart from movement in the XY plane, the plastids also moved in the Z direction. In consequence, they came out of focus during the collection of sequential images. Another reason was the very high optical density of the regenerating microcolonies (Fig. 6C). Added to this, chloroplasts underwent significant morphological and functional changes in the course of protoplast regeneration (as noticeable in Fig. 6).

Actin cytoskeleton in the culture of regenerating protoplasts

As shown in Fig 7 the cells from various stages of culture had a fully developed actin cytoskeleton. Yet the organization of the F-actin network looked different at various phases of regeneration. In protoplasts, many thin filaments were noticeable around and between chloroplasts (Fig. 7a). Nuclei were clearly visible (marked by arrowheads). The architecture of the cytoskeleton in 4 day-old cells regenerated from protoplasts (Fig. 7b) resembled that in the normal leaf mesophyll cell. A characteristic image of the cytoskeleton and a distinctive chloroplast arrangement were observed in the exterior cells of first leaves regenerating from callus, at the stage of development shown in Fig. 1a. In these cells the cytoskeleton consisted of long thin filaments often running parallel to each other (Fig. 7c). Nuclei were clearly visible (marked with arrowheads) with chloroplasts gathered around them. Stomata also displayed an abundant actin cytoskeleton (not shown). The fully regenerated leaf mesophyll is shown in Fig 7d. Two types of the actin filaments were distinguishable: those surrounding single chloroplasts and longer filaments connecting chloroplasts with different parts of the cell. The filaments were thicker and more curly than in protoplasts and in the regenerating callus. Nuclei were not detectable.

Discussion

In our previous paper a common mechanism for blue-light controlled chloroplast distribution in the leaves of *Nicotiana tabacum* and other higher plant species was postulated (Augustynowicz *et al.,* 2001). In the present study we show that blue-light-directed chloroplast movements occur in the cells of plants regenerated from protoplasts. Both accumulation and avoidance responses to saturating fluence rates, as well as responses to light pulses, were comparable in the leaves of fully regenerated and of control plants. However, the amplitudes and kinetic parameters of responses to continuous strong light were higher in the regenerated leaves than in control ones. It might be hypothesized that other photoprotective mechanisms were inad-

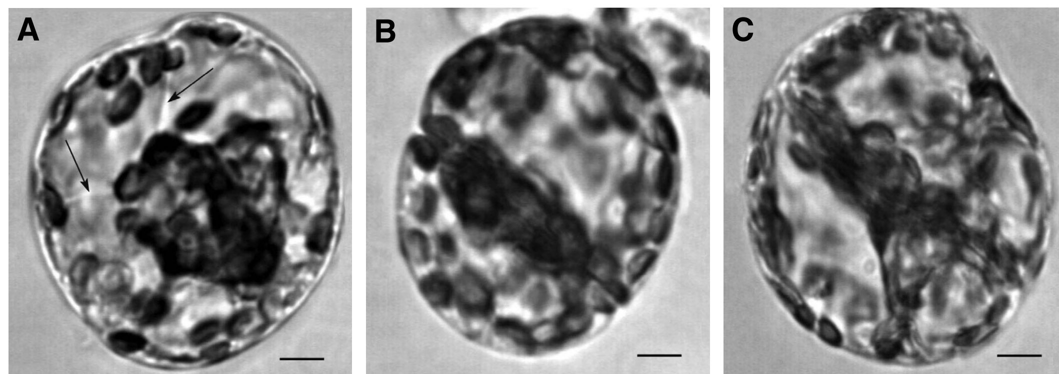


Fig. 4. Characteristic patterns of chloroplast arrangements in regenerated protoplasts independent of light conditions. (A) *2-3 day old cell before division; arrows indicate cytoplasmic strands.* **(B,C)** *4-5 day old cells during division; bars indicate 20 μm.*

equate in the regenerated mesophyll cells and that a stronger avoidance response of chloroplasts compensated for this inadequacy. A reason for the observed effect could be the influence of different developmental factors in the plants growing from seeds, as compared with plants regenerating from protoplasts. Plastid rearrangements were detected only in fully differentiated mesophyll tissue. No light-induced distribution of plastids was observed in the culture of protoplasts devoid of a cell wall, nor in non-differentiated regenerating cells up to primary leaf formation. Moreover the chloroplasts of protoplasts isolated from cold-treated plants which exhibited distinct blue-light controlled chloroplast movements (Augustynowicz *et al.,* 2001) lost the ability to respond directionally to light signals within 48-72 h. The distribution of plastids was light-independent even after cell wall recovery. The regeneration of the cell wall of protoplasts starts after the first 24 hours of culture (Tao and Verbelen 1996). Although we found that light had no effect on the direction of plastid movement in cultured protoplasts and cells, we observed particular chloroplast arrangements before and during cell division. A high number of chloroplasts (plastids) stayed immobile - clustered around the nucleus and the division plane. Only some of them resided in the peripheral part of a cell. These plastid patterns developed independently of light wavelength and intensity. Our observations are consistent with the results of Sheahan and coworkers (2004). Using cultured tobacco protoplasts they demonstrated a similar pattern of chloroplast arrangements in dividing cells. In these cells the chloroplast arrangement was actin- but not microtubule-dependent. They proposed that the clustering of chloroplasts around the nucleus enables unbiased chloroplast inheritance. In our experiments only trace plastid responses to continuous strong blue light were detected in the primary leaves differentiated from callus. Thus, the response of plastids to strong light seems to appear earlier than the response to weak light. Throughout cell ontogeny plastids differentiate into particular types, among others into chloroplasts that are present in the mesophyll of mature leaves. During leaf development a relatively low number of differentiated mesophyll cells is observed, whereas a broad merystematic zone shows high cell division activity. The proplastids which are found in the dividing merystematic cells are similar to mitochondria in shape and size, and are known for their capacity for amoeboid movement (Kutik 1998, Pyke 1999). Blue light appears to control only the distribution of chloroplasts, but not that of plastids undergoing organelle differentiation.

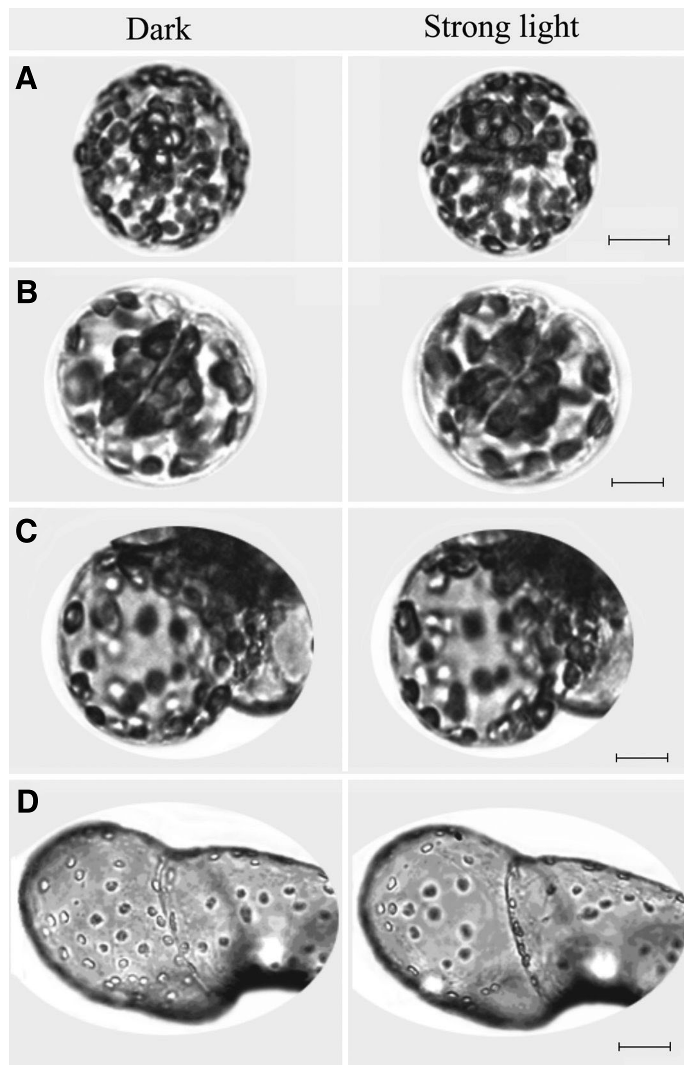


Fig. 5. Plastid redistributions in regenerating culture after dark adaptation or strong blue light irradiation (80 μmol·m^{-2}·s^{-1}). (A) *4 day old cell before division.* **(B)** *5 day old dividing cell.* **(C)** *A cell of 6 day old microcolony.* **(D)** *Cells of 1.5 month old callus; bars indicate: 15 μm (A), 20 μm (B,C) or 40 μm (D).*

Our results suggest that in a regenerating protoplast culture factors which control cell developmental status, such as division and differentiation, take over the control of plastid redistribution from light signals. The direction of chloroplast movement appears to be light regulated only in fully differentiated mesophyll tissue. Several hypothetical explanations of this phenomenon may be offered. Firstly, translocations of plastids beyond light control may be due to structural transformations of plastids, including their division accompanying cell division, as well as changes of their function (Safaris 1998). Secondly, because phototropins have been localized at the cell membrane (Harada *et al.,* 2003, Sakamoto and Briggs 2002), one may speculate that changes in membrane structure during cell ontogeny might influence their performance, and thus affect the movements.

Changes in the structure and function of actin filaments or actin associated proteins can also be put forward as factors explaining the lack of light control over plastid distribution. We have shown previously that actin is engaged in chloroplast directional movement in tobacco leaves and mesophyll protoplasts isolated from cold treated plants (Augustynowicz *et al.,* 2001). Actin cytoskeleton plays a crucial role in organelle redistribution before cell division, in plastid divisions, and in cytokinesis (e.g. in the formation of the cell plate). F-actin underlies the directional transport of Golgi and ER vesicles, including polysaccharides, to the newly forming cell wall. After the termination of cell division, filaments promote growth and differentiation events in cell development, such as cell elongation and trichome morphogenesis (Sheahan *et al.,* 2004, Smith 2003, Kost *et al.,* 1999, Volkman and Baluska 1999, Valster *et al.,* 1997). Therefore actin, an essential part of the motor apparatus of chloroplast translocations, could be engaged in other responses with greater priority to plastid avoidance or accumulation responses during cell development. The specific

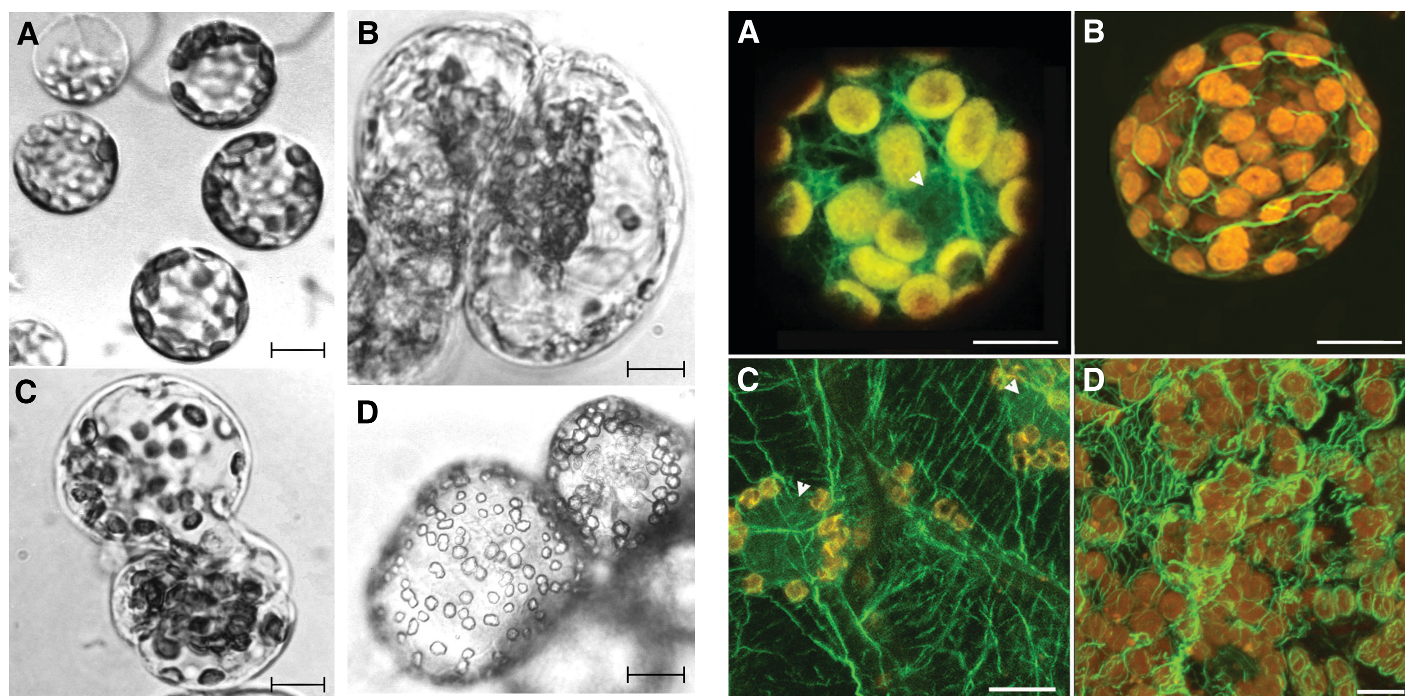


Fig. 6 (Left). Transformation of plastids in regenerating cells. (A) *Protoplasts.* **(B)** *6 day old cells.* **(C)** *2 week old microcolony.* **(D)** *2 month old callus; bars indicate 20 μm (A), 30 μm (B) or 40 μm (C,D).*

Fig. 7 (Right). Actin cytoskeleton in *Nicotiana tabacum*. (A) *A protoplast just after digestion of the cell wall.* **(B)** *A 4 day old cell regenerated from protoplast.* **(C)** *First, yet undeveloped leaf directly regenerating from callus.* **(D)** *Mature mesophyll tissue, fully regenerated leaf. All samples were dark-adapted (12 hours) before preparation and then fixed in a paraformaldehyde solution. F-actin, stained with Alexa Fluor 488-phalloidin is yellow/green. The red/orange color originates from chloroplast autofluorescence. Arrowheads mark the nuclei. Images (A, C, D) are maximum projections of 5-10 single confocal scans. The thickness of optical sections was 1 μm. The image (B) is a maximum projection of 25 scans with and optical section of 0.5 μm. Scale bars, 10 μm. Image (B) published with permission from Molecular Probes, Inc. (www.probes.invitrogen.com).*

organization of the cytoskeleton observed at early stages of leaf regeneration from callus seems to speak in favour of this interpretation.

Protoplasts and cells had a well developed actin cytoskeleton at every tested stage of culture. An interesting implication of our results is that two factors, namely a developed actin cytoskeleton and regenerated cell walls, are not sufficient to restore chloroplast responses to light in the regenerating tissue. Two hypothetical explanations are possible. The first one is that the responses may require polarity of the cell, achievable only in fully regenerated leaves. Secondly, the proteins that in the mature cells of mesophyll tissue anchor the cytoskeleton to the cell wall may be absent in the regenerating cell wall-plasma membrane-cytoskeleton continuum. Further investigations are planned to test these hypotheses.

A certain ambiguity might have been introduced by the high concentration of sucrose used in the culture medium, particularly at the initial stages of protoplast regeneration (Spangenberg and Potrykus 1994). Sucrose has recently been shown to inhibit blue light-induced chloroplast movements in *Arabidopsis thaliana* (Banas and Gabrys 2007). Notably, the inhibition occurred only with the direct contact of sugar with the cell. Sucrose delivered via roots either did not affect or even intensified chloroplast responses, depending on light conditions. Thus, while the lack of chloroplast movements in cultured protoplasts/early regenerated cells may be ascribed to the presence of sucrose in the medium, the effects observed in the tobacco callus and in the first leaves regenerating from it must have a different explanation.

Materials and Methods

Plant material

The seeds of *Nicotiana tabacum* cv. Samsun were kindly provided by the Laboratory of Botany, University of Agriculture (Kraków, Poland). Plants were grown on a sterile MS medium (Duchefa, Biochemie BV, Haarlem, Holland) at half concentration of macro- and microelements and full concentration of vitamins, pH 5.7. The medium was supplemented with 1% sucrose and 0.8% agar. The leaves of plants which were approximately two months old were used for experiments. The isolation of protoplasts was based on the protocols of Potrykus and Schillito (1986) and Tao and Verbelen (1996) with some modifications. Leaves cut into pieces were digested in an enzymatic solution (pH 5.5) for 12 h, in darkness, at 25°C. The digesting solution contained 2% celulase Onozuka R10, 0.2% macerozyme R10 (Yakult Honsha Co., Ltd), 0.35 M mannitol dissolved in a K3A medium (Potrykus and Schillito 1986). Protoplasts were isolated from debris by filtering through a 100 μm pore size filter (Partec) and centrifuged three times at 60 g for 5 min in K3A. A band of good quality protoplasts was formed on top of the centrifuge tube. Following isolation, the protoplasts were cultured and regenerated on a solid medium according to the protocols of Spangenberg and Potrykus (1994) with some modifications by Augustynowicz *et al.* (2001). Chemically pure water was used for all media. The osmotic potential of media

was measured by a vapour pressure osmometer (Wescor, Logan, Utah, USA). An appropriate osmotic potential was especially critical when working with protoplasts. During the first twenty four hours, protoplasts were cultured in the dark at 22-25°C. Then the culture was transferred into a light regime of 12 hours 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (fluorescence lamps TLM 115/ 33RS, Philips) at the same temperature. The cells started to divide after three days. Microcolonies formed after a one-month culture were placed into a MS-morpho medium solidified with 0.6 % agarose and kept in a continuous light of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (the source of light as before) at 25°C. The microcalli which appeared after the next month of culture were moved to the shoot-inducing medium MS k/p and cultured in the conditions described above. Multiple shoots regenerating from the three month old callus were cut and placed into a modified MS medium. The same composition of medium and conditions of culture were applied as for plants growing from seeds. Five months after protoplast isolation the regenerated plants looked indistinguishable from those used as the source material.

Time-lapse microscopy

Time-lapse microscopy was used to study chloroplast and plastid translocations in the regenerating culture, protoplasts, single cells, microcolonies and callus, up to the formation of leaf mesophyll. Chloroplast /plastid movements were recorded using a Nikon Eclipse TE200 inverted microscope (Tokyo, Japan) with a long – distance objective (Nikon, Plan Fluor ELWD 40H, NA 0.60). The microscope was equipped with a CCD camera (Bielski & Co., Poland) and connected to a computer through a frame grabber card (Meteor, Matrox). The frames were collected at 2-4 min intervals by a custom-written programme. In the course of measurements samples, immobilised on agar (agarose), were closed in Petri dishes under sterile conditions at room temperature. They were irradiated from above with actinic blue light of 0.8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a measuring red light of 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The actinic light was obtained from a 150 W, 15 V halogen lamp supplemented with a light-guide. A dichroic mirror D52, 535 (Edmund Scientific Co., Barrington, NY, USA) directed the beam perpendicular to the surface of the sample. The measuring red light came from a microscope halogen lamp. The following sets of filters were used: BG12 + heat absorbing C805 + neutral density (blue light), a high pass 620 nm (red). All the filters except the dichroic mirror were purchased from Schott. Specimens were incubated in the dark for 12 h at room temperature before each experiment.

Photometric measurements

A photometric method was used to examine chloroplast movement in the leaf tissue (Walczak and Gabrys, 1980). The method - described in detail by Augustynowicz and Gabrys (1999) - is based on changes in the transmission of the measuring red light (0.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$) coming through a leaf sample. The transmission changes are caused by chloroplast redistribution under blue light. A decrease in light transmission corresponds with the response to weak blue light; an increase in transmission goes with the response to strong blue light. Before each experiment the leaf samples were adapted in the dark for 12 h at room temperature. Translocations of chloroplasts were activated by continuous weak 0.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ or strong 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ blue light as well as a 10 s blue light pulse of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. To analyse the time-courses of transmission changes two parameters, amplitude and velocity, were used.

Cold pre-treatment

For the experiments on cold effects, whole plants were cooled at 4°C for 8 h. Following this treatment the leaves were immediately digested for protoplast liberation. Studies on protoplasts were always performed about 20 h after the cold treatment.

Visualization of the actin cytoskeleton: fixation and staining

The leaves were cut into small pieces and infiltrated in a syringe with a fixing solution. The fixing solution comprised ASB (actin-stabilization buffer; 50 mM Pipes, 10 mM EGTA and 5 mM $MgSO_4$) with 2% paraformaldehyde (Sigma), 1% (w/v) DMSO (Sigma), 1 tablet (in 50 ml) of proteinase inhibitor cocktail (Roche) and Na_2-ATP (Sigma). The samples were incubated for 2 h in the fixing solution and then washed in ASB. After rinsing, the specimens were incubated for 1 h in staining solution (0.02 μM Alexa Fluor® 488–phalloidin (Invitrogen/Molecular Probes) and 1% DMSO, prepared in ASB).

Confocal microscopy

Confocal microscopy was performed using a BioRad MRC1024 system (Zeiss, formerly Cell Science Division of Bio-Rad Laboratories, Hemel Hempstead, U.K.) with an inverted Nikon Diaphot 300 microscope (Nikon). The microscope was equipped with a ◊60 PlanApo 1.4 NA oil-immersion lens and a 100 mW argon ion air-cooled laser (ITL). Two fluorescence detection channels were used: one for autofluorescence of chlorophyll (with a 585 low-pass filter) and the second for Alexa Fluor® 488 (with a 540 DF30 filter). Both fluorescence emissions were excited with 488 nm. All images shown are 5-10 single confocal scans with Kalman filtering to eliminate noise.

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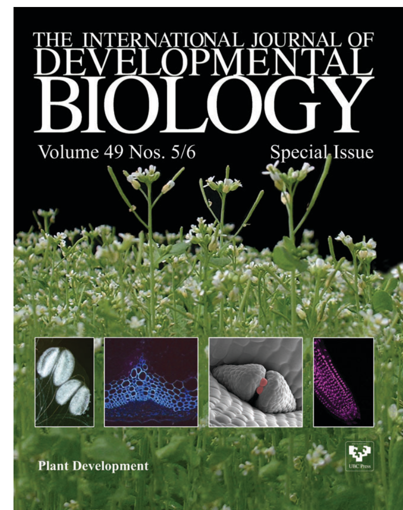

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