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Plastid-nuclear complexes: permanent structures in photosynthesizing tissues of vascular plants

Turs Selga*, Maija Selga, Valters Gobiņš, Agrita Ozoliņa

Faculty of Biology, University of Latvia, Kronvalda Bulv. 4, Riga LV-1586, Latvia

*Corresponding author, E-mail: turs.selga@lu.lv

Abstract

Communication between the cytosol and organelles has been much studied in plant cells, while the nature of direct interaction of nuclei and chloroplasts, and the interaction of tubular and saccular networks derived by them, are still a challenge to research. Here we show by the use of several microscopy methods and techniques that different compact plastid-nuclear complexes (PNCs) are permanent structures of cells in gymnosperms and angiosperms like juniper (*Juniperus communis*), tobacco (*Nicotiana tabaccum*), garden onion (*Allium cepa*) and other plants. PNC acts in a continuous interrelated three-level movement: (i) by slowly tumbling the whole PNC in a fixed locality of a cell; (ii) by rocking of some chloroplasts near the nucleus; (iii) by rushing of many nuclear derivatives (endoplasmic reticulum, dictyosomes) at relatively high speed among the PNCs and other cell structures. It can be suggested that PNCs provide beneficial exchange of substances between the chloroplasts and nucleus, thus inhibiting formation of gerontoplasts during the senescence of cells.

Key words: chloroplast-nuclear complexes, endoplasmic reticulum, Golgi bodies, plastids, stromules, tubular networks, vascular plants.
Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescence protein; PNC, plastid-nuclear complexes; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

Introduction

Conventional opinion assumes random distribution of plastids within a plant cell, with light regulated movement of chloroplasts through the help of stromules and actin microfilaments (Oikawa et al. 2003). However, in several organisms from protists to plants, joining of chloroplasts to the nucleus has been described as a phenomenon (Gibbs 1962; Esau, 1965; Ehara et al. 1984). Nucleus and chloroplasts, as constituents of plant cells, are known since the 19th century. However, plastid-nuclear complexes while being among the most typical structures in plant cells, remain mostly non-described, evidently, since they are too obvious to be recognised.

Detailed study on plastid-nuclear complexes has been carried out only in the case of *Euglena gracilis*; it was demonstrated that a correlation exists between DNA synthesis and location of chloroplasts around the nucleus (Ehara et al. 1984; Ehara et al. 1985). Chloroplast nucleus contacts have been also discussed in recent years in relation to plastid stromules and their functions (Köhler, Hanson 2000).

The chloroplast-nucleus complex in bright field microscopy resembles a "daisy" like structure. We observed this structure during transmission electron microscopic analysis of the effect of ethylene producer on the ultrastructure of leaf mesophyll cells (Selga, Selga 1995). In this case, the outer membrane of chloroplasts became linked to the outer membrane of the nucleus. It was suggested that plastid-nucleus complexes (PNCs) have a specific role in the case of unicellular algae but in higher plants appear only during stress or temporarily due to growth of plastid stromules.

The aim of the present study was to investigate the appearance of PNCs as permanent structural complexes among different plant species, and to describe their changes during differentiation of epidermis and mesophyll and their behaviour *in vivo*.

Materials and methods

Plant material

Equisetum arvense L., *Athyrium filix-femina* (L.) Roth, *Juniperus communis* L., *Pinus sylvestris* L., and *Convallaria majalis* L. plants were collected in a dominant pine forest near Riga.

Cucumber (*Cucumis sativus* L.), broad beans (*Vicia faba* L.) and garden pea (*Pisum sativum* L.) plants were cultivated in aquatic culture, and winter rye (*Secale cereale* L.) and tobacco (*Nicotiana tabaccum* L.) plants in vegetation pots in soil with Helriegel's medium (Hellriegel 1898), in a greenhouse with natural light and day/night temperatures of 25/19 °C. Bulbs of garden onion (*Allium cepa* L.) cv. Golden were obtained from a seed market.

Green fluorescent protein (GFP) was fused to rat sialyltransferase located in Golgi bodies and endoplasmic reticulum (ER) of transgenic tobacco plants (Andreeva et al. 2000).

A search was made for attachment of chloroplasts to nuclei in several types of cells in leaves and needles in three stages of development: young, fully expanded green, and yellowish senescing. Samples were obtained from at least three plants for each species/stage combinations. From each plant at least five samples were prepared for microscopic analysis.

Bright field microscopy

Bulbs of onion *Allium cepa* were dissected and epidermis from different layers of leaf scales was removed. Epidermal peels from the middle of tobacco, cucumber, pea, rye, *Convallaria majalis*, needles of juniper and Scot's pine and the outer, middle and inner layer of leaf scales of onion bulbs were stripped and transferred to droplets of aceto-ethanol on microscopic slides, washed and contrasted with aceto-orcein.

Mesophyll was fixed with 2% glutaraldehyde in sodium cacodylate buffer (pH 7.3), washed with water, frozen in a droplet of water, and cut by razor under 50 × magnification; cuttings were contrasted on microscopic slides with aceto-orcein.

Leaf pieces of mesophyll were excised in the middle of the leaf blade among large veins, soaked in aceto-ethanol, rinsed in distilled water, maturated in 1 M HCl at 60 °C, rinsed in distilled water, contrasted with aceto-orcein, placed on microscopic slides, covered with cover slips, and gently crushed by pressure. Palisade parenchyma cells that were largely or partly separated due to destruction of cellulose were examined.

Samples were examined under bright field illumination under a light microscope LEICA DM 2000. Photographs were taken with a digital camera Leica EC3 and images processed with LAS EZ and Paint Shop Pro 4.

Scanning electron microscopy (SEM)

Small pieces of mesophyll from the selected leaves were fixed with 2% glutaraldehyde and maturated in 1 N HCl at 60 °C. Washed samples were gently crushed by applying pressure with sample holders. The specimens were dried and coated with gold.

Small pieces of mesophyll from the selected leaves were fixed repeatedly both with 2% glutaraldehyde and 4% osmium tetroxide in 0.2 M sodium cacodylate buffer, washed with the same buffer, impregnated with 2% sucrose, frozen and cut with a razor blade, washed, dried and coated with gold. Samples were analysed with a scanning electron microscope Hitachi S-4800 in high vacuum mode at 15 kV.

Transmission electron microscopy (TEM)

Small pieces of mesophyll from the selected leaves were fixed repeatedly both with 2% glutaraldehyde and 4% osmium tetroxide in 0.2 M sodium cacodylate buffer. Samples were dehydrated and contrasted with 1% phosphowolframic acid and embedded in a mixture of epoxy resin (Epons: 812, MNA, HY 964, DYO 64). Ultrathin sections were cut with an ultramicrotome LKB 8800, contrasted with lead citrate, and examined with transmission electron microscopes Tesla BS 500 and Philips 301 under magnification 2000 to 25 000.

Laser confocal microscopy

Tobacco leaf specimens were covered with oil immersion and analysed by laser scanning confocal microscopy using a Leica DM RA-2 microscope equipped with a TCS-SL confocal scanning head (Leica Microsystems, Bannockburn, USA). Images were collected with a Leica 40 × HCX PL Fluotar objective (NA = 0.75) and 100 × HCX PlAPO oil immersion objective (NA = 1.40). GFP was excited with a 488-nm band from a four-line argon ion laser. Chlorophyll fluorescence was excited at 633 nm. GFP fluorescence was detected between 500 nm and 560 nm. Chlorophyll fluorescence was detected between 650 nm and 715 nm.

Table 1. Occurrence of plastid-nuclear complexes (PNC) in plant cells. ni, not investigated

Species	Leaf parenchyma	Epidermis	Meristem or parenchyma of a root tip
Allium cepa L.	+	+	+
Athyrium filix-femina (L.) Roth	+	+	ni
Equisetum arvense L.	+ (shoots)	+	ni
Convallaria majalis L.	+	+	+
Cucumis sativus L.	+	+	+
Juniperus communis L.	+	+	ni
Nicotiana tabaccum L.	+	+	ni
Pinus sylvestris L.	+	+	ni
Pisum sativum L.	+	+	+
Secale cereale L.	+	+	+
Vicia faba L	+	+	+

Results

Epidermal cells of all investigated vascular plants contained the specific daisy-shape PNCs (Table 1). PNCs were observed from expanding till yellowing phase. Only some cell chloroplasts were involved in PNCs, while the others were scattered through the cortical cytoplasm (Fig. 1A – D).

The PNCs were localized also inside developing and old guard cells of stomata, and also external to guard cells in the epidermal pavement cells, positioned close to the stomata (Fig. 2A, B). Frequently, in the large epidermal cells over and next to leaf veins the PNCs were located in the middle of cells; a large number of chloroplasts were associated to the nucleus via thread-like structures (Fig. 1D). The number of chloroplasts involved in the complex was highly variable.

Both transverse sections and separation of cells by maceration of tobacco leaves showed location of PNC. In the mesophyll square-shaped cells of young leaves the association of chloroplasts to nuclei was irregular, due to lack of space (Fig. 3A), but the number of chloroplasts in PNCs and closeness of chloroplast connection with nuclei increased with age of leaves, and the structure took shape of a "daisy" (Fig. 3B, C). In these cells PNC was located in the central part of the cell. During differentiation, PNC moved downwards and laterally toward the cell wall.

In fully-grown leaves, where the nuclear size relative to cell size is decreased, due to larger cell volume, two types of PNCs were observed: (i) those formed by the pressing of a bent nuclear protrusion towards chloroplasts (Fig. 3C), and (ii) by the direct attachment of many chloroplasts around the nucleus, forming the daisy-shaped profiles (Fig. 3D) in the medial part of the cell.

Yellowish leaves contained particularly pronounced daisy-shaped PNCs (Fig. 3D), possibly related to increased volume and vacuolisation of cells, decrease of number and size of chloroplasts and decrease of absolute size of nuclei.

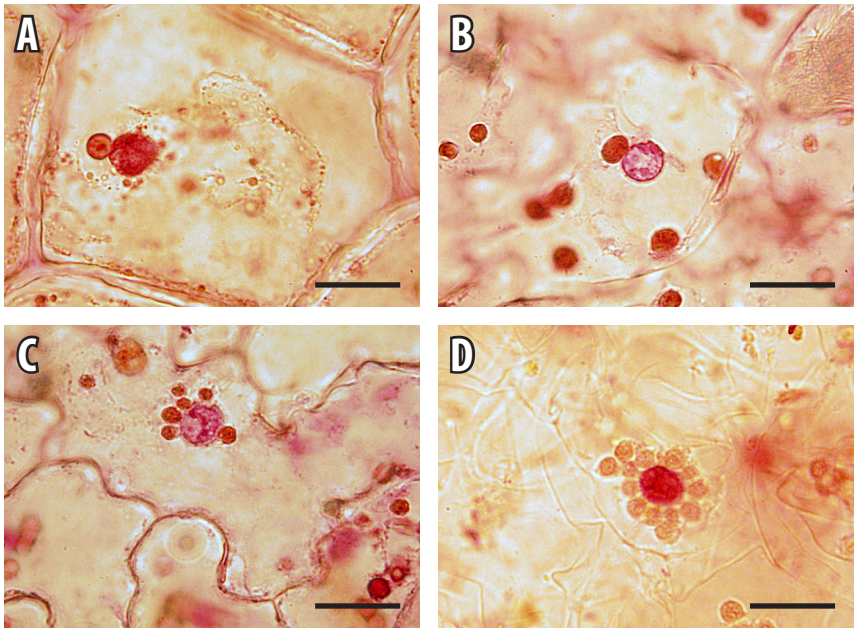

Fig. 1. Plastid-nucleus complex in tobacco (*Nicotiana tabaccum*) leaf epidermal cells. Samples were fixed with aceto-ethanol on microscopic slides, washed and contrasted with aceto-orcein. A, expanding leaf; B, mature leaf; C, yellowing leaf; D, large plastid-nucleus complex. Bars 25 μm (A - C) and 15 μm (D).

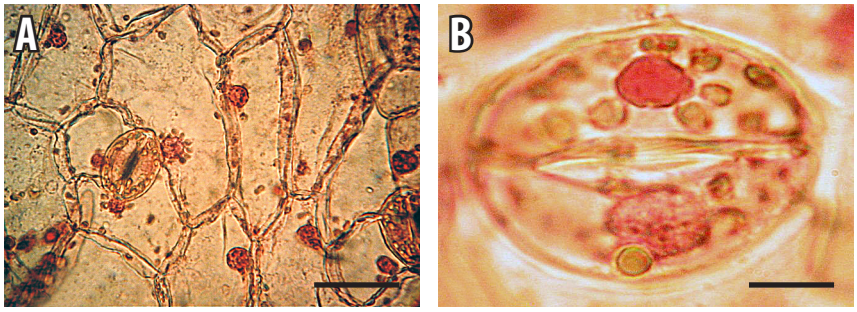

Fig. 2. Plastid-nucleus complex in tobacco (*Nicotiana tabaccum*) leaf epidermal cells. Samples were fixed with aceto-ethanol on microscopic slides, washed and contrasted with aceto-orcein. A, epidermis with PNC oriented towards stomata; B, guard cells containing PNC. Bars 30 μm (A) and 10 μm (B).

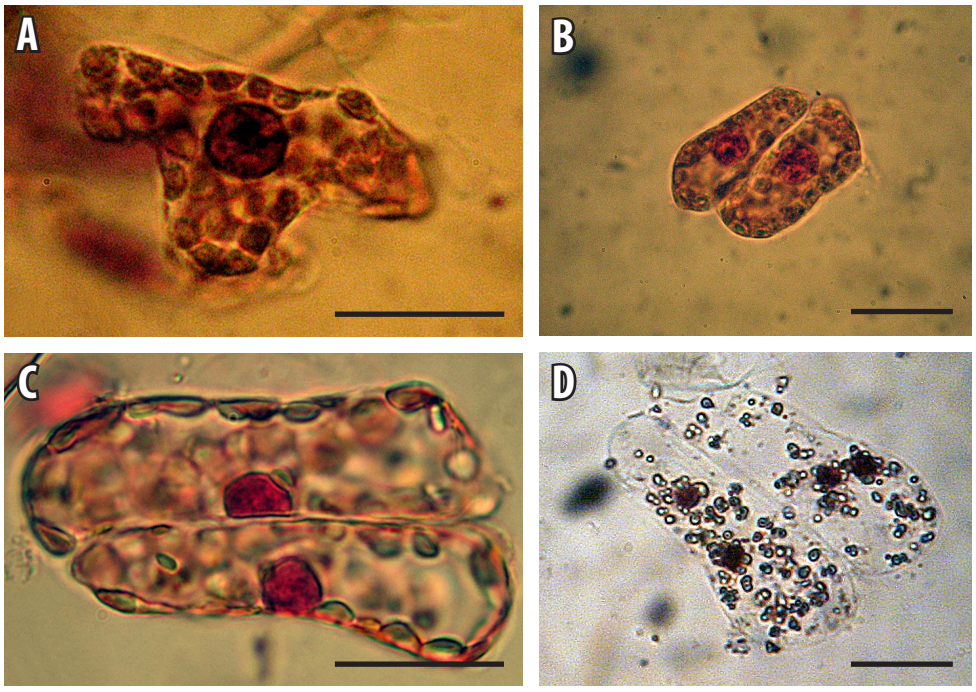

Fig. 3. Plastid-nucleus complex of leaf parenchyma cells. Mesophyll of the leaf blade among large veins were fixed with aceto-ethanol, maturated in 1 M HCl at 60 °C, contrasted with aceto-orcein, and crushed by pressure. A, cell of expanding leaf; B, cell of a mature leaf; C, cell of yellowing leaf; D, cell of a yellow leaf. Bars 20 μm (A - C) and 25 μm (D).

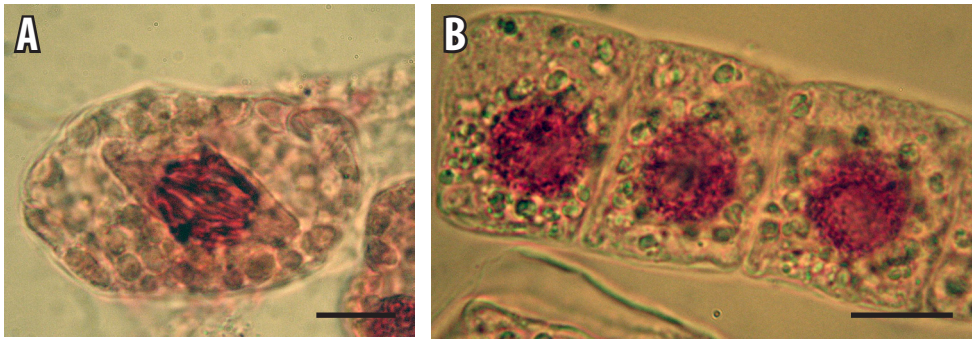

Fig. 4. Plastid-nucleus complex of root tip cells. Root tips were fixed with aceto-ethanol, maturated in 1 M HCl at 60 °C, contrasted with aceto-orcein, and crushed by pressure. A, PNC in the root meristem of broad beans (*Vicia faba*); B, PNC in the root parenchyma of cucumber (*Cucumis sativus*). Bars 10 μm.

PNCs were typical for both dividing and non-dividing root tip cells in all investigated species of flowering plants (Fig. 4A, B). During mitosis some plastids remained in close proximity of the mitotic spindle. Often, these plastids formed a more or less separated compartment from the rest of cytoplasm (Fig. 4A).

Three types of chloroplast attachment to the nucleus were observed in mechanically separated organelles: (i) direct mutual contact over a wide surface area (Fig. 5A); (ii) joining of the chloroplasts reciprocally and to the nucleus by longish connective structures (Fig. 5B); (iii) penetration of chloroplast among the surface protruberances of ragged nuclei, i.e., among wide folds of the nuclear envelope, a specialised region of the rough ER. At higher magnification, smaller ellipsoid-shaped structures were visible. Their size corresponded to that of mitochondria. In young and mature leaves they were located mainly on the lateral side of chloroplasts participating in PNC. In samples from senescent leaves, mitochondria were joined to nuclear envelope similarly to chloroplasts, or appeared in deep grooves of nuclear envelope. The precise 3D structure of location of organelles in the sections of mesophyll cells was observed. The structure of PNCs was similar as that described above (Fig. 5C).

Electron microscopy demonstrated that plastids in expanding or mature leaf cells were anchored at the perinuclear endoplasmic reticulum (ER). Presence of all three types of interconnections of nucleus with chloroplasts was supported also by TEM studies. A complete picture of the PNC was often visible in young leaf palisade parenchyma cells (Fig. 6A), but in large cells of fully-grown leaves, PNC were evident only when in ultrathin section

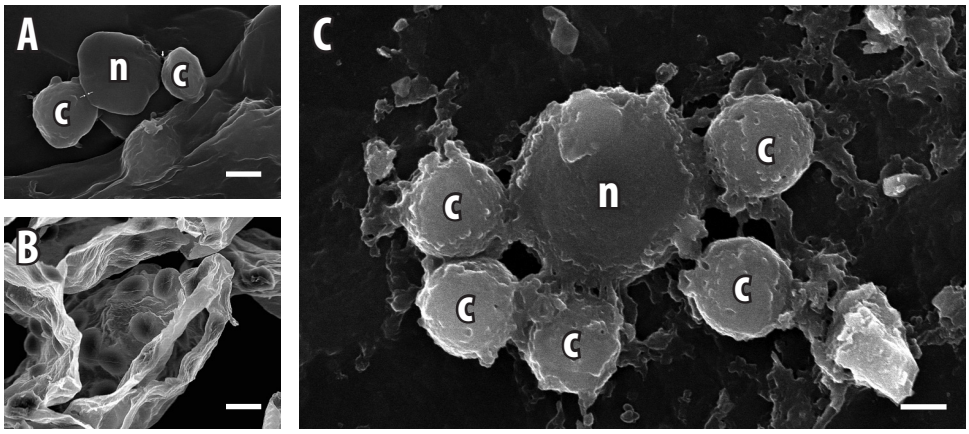

Fig. 5. Plastid-nucleus complex in mesophyll cells of tobacco (*Nicotiana tabaccum*) leaves. Mesophyll was fixed with 2% glutaraldehyde and maturated in 1 N HCl at 60 °C, crushed by pressure on sample holders, dried and coated with a gold (A, C). A, C, PNC isolated from the mesophyll cells, scanning electron microscopy; B, cross section of the leaf mesophyll cell, scanning electron microscopy. Mesophyll was fixed repeatedly both with 2% glutaraldehyde and 4% osmium tetroxide in 0.2 M sodium cacodylate buffer, cut, dried and coated with a gold. n, nucleus; c, chloroplasts. Bars 1 μm (A, B) and 4 μm (C).

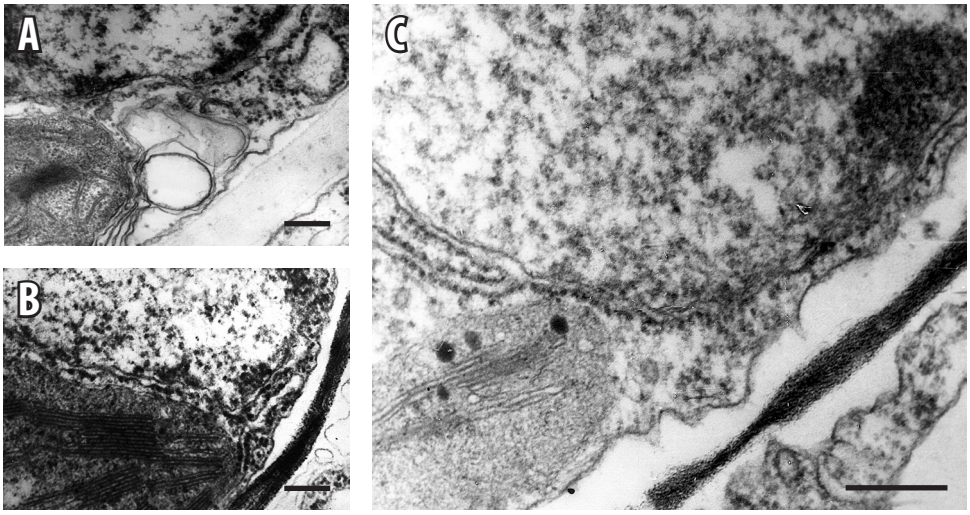

Fig. 6. Ultrastructure of the contact site between the nucleus and chloroplast. Mesophyll was fixed repeatedly both with 2% glutaraldehyde and 4% osmium tetroxide in 0.2 M sodium cacodylate buffer, dehydrated and contrasted with 1% phosphowolframic acid, embedded in the mixture of epoxy resin cut with an ultramicrotome LKB 8800, contrasted with lead citrate. A, direct flattening of the chloroplast to nucleus by contiguity of their envelopes and bridges; B, ER and multivesicular body anchors a chloroplast to the nucleus; C, outer membrane of the nucleus and tubule of ER anchors a chloroplast to the nucleus. Bars 150 nm (A, B) and 200 nm (C).

cut through the nucleus located in a thin layer of cytosol between the plasmalemma and tonoplast (Fig. 3B). The chloroplasts were located at various distance and angle around the nucleus: some were apparently tied to the nucleus, whereas the connective structures of others were not visible, probably, since they were out of the plane and angle of this ultrathin section (20 to 30 nm).

Nuclear activity in development of PNCs was evidently involved in several structural ways. Direct flattening of chloroplasts to nuclei by continuity of their envelopes in a vast area was the most common type (Fig. 6A). The nucleus frequently embraced a chloroplast in an inward bend, or by an outward bend joined to chloroplast, creating direct multimembrane contact sites (Fig. 6B). There were also some 15 to 30 nm thick grey bridges, which might be the surface of ER without ribosomes, surface of polysome with newly synthesised proteins or cytoskeleton with unidentified cross-linking proteins.

The ribosome-mediated junctions developed bit by bit between the touching chloroplast and nucleus, between a number of small curves of the nuclear external membrane richly covered by ribosomes contacting the chloroplast (Fig. 6C), and between rough ER tubes touching both near- and distant-situated chloroplasts. Two types of rough ER cisterns connected the nuclei with chloroplasts. Firstly, long tubes of rough ER retaining an open continuity between the perinuclear space and the ER lumen in one end, stretched far from the nucleus, branching and penetrating closely between the chloroplast and plasmalemma. An open connection between the ER lumen and the space between

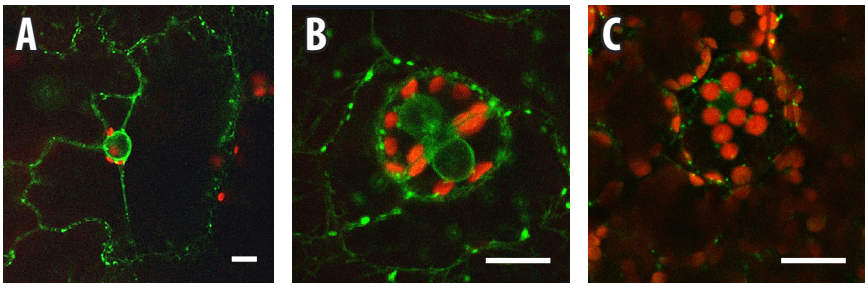

Fig. 7. Plastid-nucleus complex in leaf cells of tobacco (*Nicotiana tabaccum*). Golgi bodies and ER of transgenic tobacco plants show sialyltransferase, green due to the presence of green fluorescent protein. Chloroplasts are red due to chlorophyll autofluorescence. A, PNCs in leaf upper epidermis; B, PNC in stomata guard cells; C, PNC of leaf parenchyma cell. Bars 10 μm..

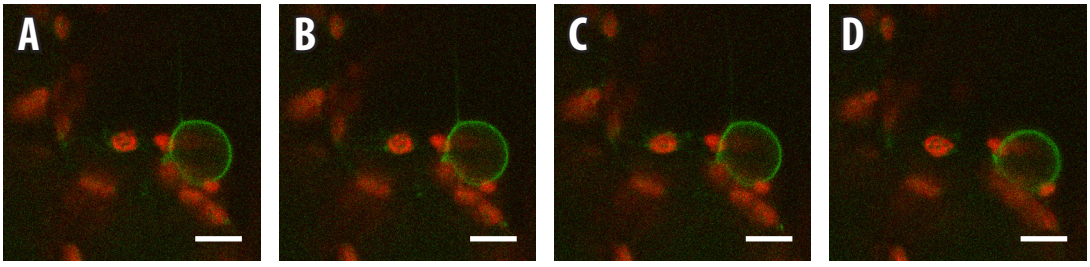

Fig. 8. Movement of plastids apart from nucleus in the PNC. Golgi bodies and ER of transgenic tobacco plants show sialyltransferase, green due to the presence of green fluorescent protein. Chloroplasts are red due to chlorophyll autofluorescence. A, 2 s; B, 15 s; C, 30 s; D, 45 s. Bars 10 μm.

chloroplast envelope membranes was not observed. Secondly, separate short cisternae of the rough ER also were common components of the leaf mesophyll cells. Some of them were attached perpendicular to the tips of chloroplasts, between the chloroplast and plasmalemma, as short trident tubes connected by a short "petiole" with the nucleus and by two "branches" touching both chloroplast and plasmalemma, or probably moving as mobile long-distance carriers between the nucleus, chloroplasts and other cell compartments.

In transgenic tobacco, Golgi bodies, endoplasmic reticulum and nuclear envelope were marked by GFP. Nuclear envelope seemed thick, reflecting many layers of perinuclear endoplasmic reticulum (Fig. 7A). The thickest nuclear envelope (0.5 μm) was found in expanding leaves. Diameter of envelope decreased with senescence, and in yellowing leaves the fluorescence signal disappeared.

Laser confocal microscopy confirmed the regular appearance of compact PNCs in the mesophyll of all tobacco leaf cells (Fig. 7A, B, C). The method demonstrated that, during senescence, nuclei move towards the border of the cell and sometimes only two or three chloroplasts remained in the center of the cell to form the "daisy"-like structure around the nucleus. Analysis of fluorescence in the tobacco cells proved that chloroplasts and nuclear envelope were co-localised. In mesophyll cells and epidermis of yellowing leaves, chloroplasts involved in PNC were larger and showed brighter chlorophyll fluorescence. Free chloroplasts began to lose chlorophyll faster and their fluorescence became dark red or yellow-green.

Time-lapse study showed that interaction of organelles in the PNCs occurred by interrelated movement of diverse amplitude and frequency. The PNCs themselves tumbled slowly in a fixed locality of the cell. Three populations of chloroplasts formed the PNC. The first group was fixed to the nuclear envelope and oscillated together with nucleus.

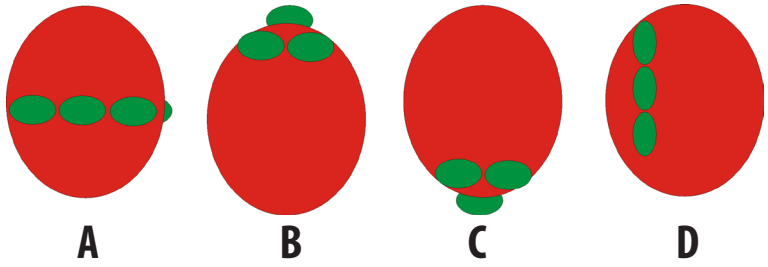

Fig. 9. Location of plastids around the nucleus analysed along the "z"axis. The upper surface of the leaf was considered as apical part of the cell. A, plastids connected to medial part of the nucleus; B, plastids at the apical part of the nucleus; C, plastids at the basal part of the nucleus; D, plastids connected to the nucleus from the top to bottom. .

The second group was attached to nucleus by short (approximately 0.5 μm long) joints and changed distance to nucleus during oscillation. (Fig. 8A – D). The red coloured chloroplasts were placed between the green folds of endoplasmic reticulum and nuclear envelope, showing wide yellow-coloured areas of co-localisation (Fig. 8A). The third group were localized relatively far from the nucleus, at a distance of at least few micrometers. These chloroplasts actively moved towards or apart from the nucleus at a velocity 1 μm s^{-1}. Green fluorescence in joints showed that strands connecting nucleus with chloroplasts contained endoplasmic reticulum, visible in the second and the third group of chloroplasts. Contraction towards the nucleus indicated activity of actin cytoskeleton. PNC reflected also an active secretory activity. Many Golgi bodies were connected to the nuclear envelope and were released after a short period of time.

Many green-coloured dictyosomes rapidly encircled the PNCs from all sides, mediating the association of red-coloured chloroplasts to the greenish nuclear envelope and scurried in various directions and distances from the PNCs. The green-coloured nuclei clung by red-coloured chloroplasts, i.e. PNCs, making long green intercellular connections, intersecting vacuoles (Fig. 8A).

Discussion

The present study supported the view that members of the main groups of vascular plants contain PNCs. The presence of PNCs in distinct vascular plants can be considered as an evolutionary ancient structure that is typical for photosynthesizing tissues. PNCs were not visible in all investigated cells. Epidermal and mesophyll cells were analysed along the "z" axis from the upper leaf surface. The majority of cells contained PNC but the frequency of occurrence was unclear, since, plastids located on the top or bottom of nucleus were not visible as a part of a complex (Fig. 9). This feature of "z" axis may explain why PNCs have not been characterized previously. To elucidate the frequency of occurrence further observations with 3D reconstruction of nuclei are required. The typical appearance of chloroplast clusters around the nucleus suggested that this was not a sign of mutation, as reported in the case of mitochondrial clusters in plants and *Dictyostelium* cells (Possingham 1980; Van Gestel 2002).

The presence of PNCs in cells of expanding, mature and yellowing leaves suggest that they represent permanent structures in cells rather than short time interaction between organelles.

The relative autonomy of such cellular structures raises a question about the location of PNC in individual cells according to their place in the spatial system of cellular organization. Polar spatial location of organelles is important, as shown by the existence of polarity of auxin synthesis in *Arabidopsis* leaves (Aloni et al. 2002). Polarity appears also in tobacco leaves during senescence, when the number of Golgi bodies is higher at the basal part of mesophyll cells; during stress Golgi bodies concentrate at the apical part of the cell to export substances towards the upper epidermis (Selga, Selga 2003).

One of the models used in the present study, transgenic tobacco leaf cells, exhibited auto-fluorescence of chlorophyll and fluorescence of GFP-marked chloroplasts, nuclear envelope and endoplasmic reticulum. Scanning along the "z" axis demonstrated that frequency of appearance of PNCs in leaf cells is much higher than observed with a light microscope. Thus, it can be concluded that all cells of photosynthesizing tissues, from expanding to yellowing leaves, contain PNCs. In addition, observation of cells *in vivo* and *in situ* proved that PNC is not an artefact due to fixation or staining of samples. Attachment of chloroplasts to nucleus *in vivo* has been reported also in other investigations (Arimura et al. 2001; Holzinger et al. 2007).

Since the thickness of nuclear envelope with perinuclear ER in cells of expanding leaves is up to 0.5 micrometers we can conclude that chloroplasts are joined to the perinuclear endoplasmic reticulum and that the outer membranes of nucleus and chloroplasts are apart.

Time-lapse movies showed oscillation of PNC. This fact supports the idea that chloroplasts and nuclei are closely located and cross-linked.

Successful attempts were made to isolate and cultivate PNC from living cells. Both nuclear envelope and chloroplasts maintained fluorescence for several weeks, if cultivated in Murishage and Skoog basal medium at room temperature. This system can serve as a cell-free system to study transport mechanisms among organelles (Selga, unpublished data).

SEM studies proved that the PNCs can be isolated from the mesophyll cells of tobacco leaves, retaining durable association of chloroplasts with nuclei. Similar attachment of chloroplasts to nucleus has been demonstrated relatively long ago (Pssingham 1980). This reflects close location among cross-linking elements, chloroplasts and nucleus, because glutaraldehyde fixation strongly anchored this system permitting mechanical separation. SEM showed that PNC is a permanent complex and not an accidental group of organelles that appeared due to lack of space in the cytoplasm. In addition, the organelles are physically connected, not only co-localised as in investigations with confocal laser scanning microscopy. Detailed structure of elements linking chloroplasts to the nucleus is visible only with transmission electron microscopy.

The recently described plastid stromules, which join chloroplasts mutually, encircle mitochondria contacting with plasmalemma, and move mitochondria and peroxisomes to the nucleus, are acknowledged as tubular pathways for trafficking of proteins among organelles under control of the actin cytoskeleton (Tulett et al. 1969; Gray et al. 2001; Kwok, Hanson 2004).

Continuity of outer membranes of chloroplasts and nucleus or perinuclear endoplasmic reticulum and outer membrane of chloroplast was also reported by several researchers (Ehara et al. 1984; Osafune 1994). It was shown that ER is a heterogeneous organelle, developing different types of ER junction with each organelle that partners the ER, and this network has a central role in the trafficking of lipids among organelles (Levine 2004; Hu et al. 2008; Sakamoto et al. 2008). The striking presence of bound and free ribosomes in the narrow border zone between the nucleus and chloroplasts might represent a structure that enables protein metabolism between organelles, probably facilitating plant organellar protein targeting (Mackenizie 2005).

Plastid to nucleus signalling has been studied for a long time and two directions of signals, antrograde and retrograde, are recognized (Pogson et al. 2008). Nuclear gene expression is controlled by redox signals from photosynthesis (Bräutigam et al. 2009). In addition, the chloroplast envelope plays an important role in transfer of redox signals (Jäger-Vottero et al. 1997). Thus, not all signals from plastids to nucleus pass cytosol and existence of redox signalling becomes more intresting because it permits signal transduction without passing the cytosol. Our observations suggests that only a few permanently attached chloroplasts could be involved in such direct regulation of nuclear gene expression and the others use signalling through the cytosol.

In general, it seems that the plastid-nucleus complex can be considered as a semi cell, e.g. a structure capable of metabolism, photosynthesis, protein and RNA synthesis but not covered by a plasmalemma.

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