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Ultrastructural Characterization of the Reversible Differentiation of Chloroplasts in Cucumber Fruit

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The changes in plastid ultrastructure in the pericarp of cucumber (*Cucumis sativus* L.) fruit were studied during fruit yellowing (which accompanied maturation) and regreening. In the course of fruit maturation, the thylakoid system was progressively reduced, and only a small number of membranes remained in the plastids of mature fruit. At the same time, the plastoglobules increased in size, often remaining in close proximity to the degrading thylakoids. In pericarp tissue which turned green again, the thylakoid network in the plastids was gradually reconstituted. Morphological similarities between the plastids in mature and regreening fruit indicated that the chloroplasts in regreened tissue were redifferentiated from the plastids of mature fruit. Reconstitution of the thylakoid system appeared to start from two morphologically distinct types of membranes: from double membranes which resembled thylakoids and from membrane-bound bodies (MBBs). The latter appeared to form thylakoids by two mechanisms: by detachment of extensions from their surfaces and by fragmentation. The plastoglobules remained in the plastids during thylakoid system reconstitution and were often observed in close proximity to developing thylakoids. In the course of chloroplast redifferentiation, several types of membraneous structures were found to be associated with the plastid envelope: (i) vesicles which appeared to separate from the envelope and to fuse subsequently with the developing thylakoids, (ii) tubules, and (iii) double-membrane sheets which appeared as *de novo* forming thylakoids.

Key words: chloroplasts, cucumber, regreening, thylakoid formation, ultrastructure

According to Schimper's (1885) classical concept, the various types of plastids are homologous cell organelles which are able to differentiate into one another and are, accordingly, also capable of reverse differentiation. In the case of chloroplasts, the latter phenomenon has been demonstrated by means of electron microscopy in various plant tissues, in a number of species. It was found that chloroplast redifferentiation can be induced in senescing soybean cotyledons by removing the epicotyls (Huber and Newman, 1976; Tuquet and Newman, 1980), in *Nicotiana rustica* by removing the shoots above a single senescent leaf (Zavaleta-Mancera et al., 1999), and in *Xerophyta scabrida* by rehydrating the desiccated leaves (Tuba et al., 1993). In *Buxus sempervirens* (Koiwa et al., 1986) and *Euonymus japonica* (Ikeda, 1979), this phenomenon occurs during regreening of yellowed leaves, while, in the spathes of *Zantedeschia aethiopica* (Pais, 1972; Tavares et al., 1998), *Z. elliottiana* (Grönegress, 1974) and *Spathiphyllum wallisii* (Palandri, 1967), and in the sepals of *Chrysosplenium alternifolium* (Sitte, 1974) and *Nuphar luteum* (Grönegress, 1974), it accompanies the fruiting process. Redifferentiation of chloroplasts has also been described in some fruits: in the rind of ripe fruits of Valencia oranges and lemon (if they are left on the tree till the following spring and summer) (Thomson et al., 1967; Ljubešić, 1984; Mayfield and Huff, 1986), and also in the subepidermis of ripe fruits of *Cucurbita pepo* (Devidé and Ljubešić, 1972, 1974) and *C. maxima* (Ljubešić, 1981). In the present work, we report the phenomenon of chloroplast redifferentiation which may occur in the subepidermal layers of the pericarp of mature cucumber (*Cucumis sativus* L.) fruit.

From a structural point of view, the main question concerning chloroplast redifferentiation is the way by which the thylakoid system is reconstituted. In spite of some initial attempts at ultrastructurally characterizing these processes (Thomson, 1967; Devidé and Ljubešić, 1974; Grönegress, 1974; Huber and Newman, 1976), they have not received much consideration, in recent years. Our studies have therefore focused on the ultrastructural changes undergone by the plastids during the maturation and subsequent regreening of the fruit, in the latter case with particular emphasis on the characterization of the processes involved in the formation of the thylakoids. Special reference was devoted to the plastid structures which may have important functions in the process of chloroplast redifferentiation: the plastoglobules and the membranous structures associated with the plastid envelope.

MATERIAL AND METHODS

Plant Material

Cucumber (*Cucumis sativus* L. cv. Vert Petit de Paris) fruit were obtained from plants grown from seeds in an experimental garden at the Ruđer Bošković Institute. The fruit were left to ripen on the plants and, at maturity, were collected, cleaned from adhering soil and stored in a dry place (room temperature, light intensity 50 $\mu mol_{photons}\ m^{-2}s^{-1}$, 16 h day/8 h night). Samples were taken from green immature fruit, maturing fruit (greenish yellow in color), mature fruit (dark yellow to orange in color), fruit at an initial stage of regreening, fruit at an advanced stage of regreening, and

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Abbreviations: MBB membrane-bound body; DAB 3,3'-diaminobenzidine

completely regreened parts of fruit.

Light Microscopy

For bright field and fluorescence microscopy, fresh hand-cut sections of pericarp tissue were used. The sections were examined using a Zeiss Axiovert 35 microscope equipped with a digital camera (Pixera Pro 150 ES).

Transmission Electron Microscopy

For ultrastructural studies, pericarp tissue (about 1 mm in thickness) was excised from the fruit surface with a razor blade, and cut into small pieces. The latter were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2), for 30 min, at 4°C, and postfixed in 1% osmium tetroxide in the same buffer, for 1 h, at 4°C. After dehydration in a graded series of ethanol, the tissue was embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate.

To localize photosynthetic activity in the plastids, a cytochemical reaction using 3,3′-diaminobenzidine (DAB) photooxidation was performed, according to a procedure described by Wrischer (1978). The tissue was fixed in 2% formaldehyde in 0.05 M phosphate buffer, in the dark, for 30 minutes, and then washed with the same buffer, in the dark, for 30 minutes. The tissue was then treated with DAB (1 mg/mL) in 0.05 M phosphate buffer (to which 5% sucrose had been added), in the dark, for 30 minutes, followed by 30 min in the light (70 $\mu mol_{photons}\ m^{-2}s^{-1}$). After washing with 0.05 M phosphate buffer, the tissue was postfixed in 1% osmium tetroxide in the same buffer, for 1 h, dehydrated in ethanol and embedded in Spurr's resin.

Sections were examined using a Zeiss EM10A electron microscope operating at 60 kV accelerating voltage. Ultrastructural analyses were performed on plastids in the pericarp subepidermis (approximately the first 5 to 7 subepidermal cell layers).

RESULTS

Macroscopical Observations and Light Microscopy

In the course of ripening the, initially green, cucumber fruit became dark yellow to orange (Fig. 1A). During this process, chloroplasts in the pericarp subepidermis turned into plastids colored by large yellowish plastoglobules, as revealed by light microscopy (Fig. 1B). In those plastids, the red autofluorescence of chlorophyll was barely detectable (Fig. 1C). When fruit were collected at maturity and stored (as described in Material and Methods), most of the fruit decayed after some time (variable for each fruit) or, occasionally, completely dehydrated. However, in fruit which did not deteriorate, the pericarp subsequently started to regreen. The quantity of such fruit depended on the season, but on the average comprised about 15% of the mature fruit (experiments were performed through three vegetational seasons monitoring about 60 mature fruit). Regreening was a slow process which usually required two to three months. During that time, parts of the fruit exposed to light partially regreened, eventually comprising about 20-40% of the total fruit surface (Fig. 1D). In the subepidermal layers of such regreened parts of the fruit pericarp, light microscopy revealed chloroplasts with strong chlorophyll autofluorescence (Fig. 1E, F).

Plastid Ultrastructure in Immature Fruit

In the pericarp of immature green fruit, the cells in all

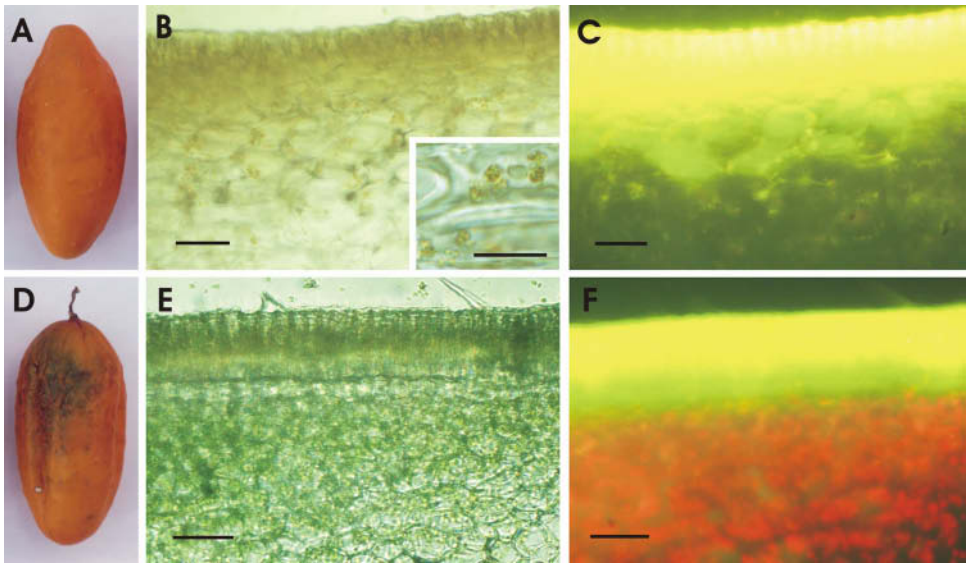

Figure 1. Features of the pericarp in mature (**A, B, C**) and partially regreened (**D, E, F**) fruit. (**A**) Mature fruit. (**B, C**) Light micrographs of a section through the pericarp of a mature fruit (bright field and fluorescence, respectively). (**B**) Plastids are visible in the subepidermal layers. Bar = 50 μm. In the inset, yellowish plastoglobules can be discerned in the plastids. Bar = 10 μm. (**C**) Red autofluorescence of chlorophyll in the plastids is barely visible. Bar = 50 μm. (**D**) Fruit with regreened areas. (**E, F**) Light micrographs of a section through the pericarp of the regreened part of a fruit (bright field and fluorescence, respectively). (**E**) Chloroplasts are visible in the subepidermal layers. Bar = 50 μm. (**F**) The chloroplasts show strong chlorophyll autofluorescence. Bar = 50 μm.

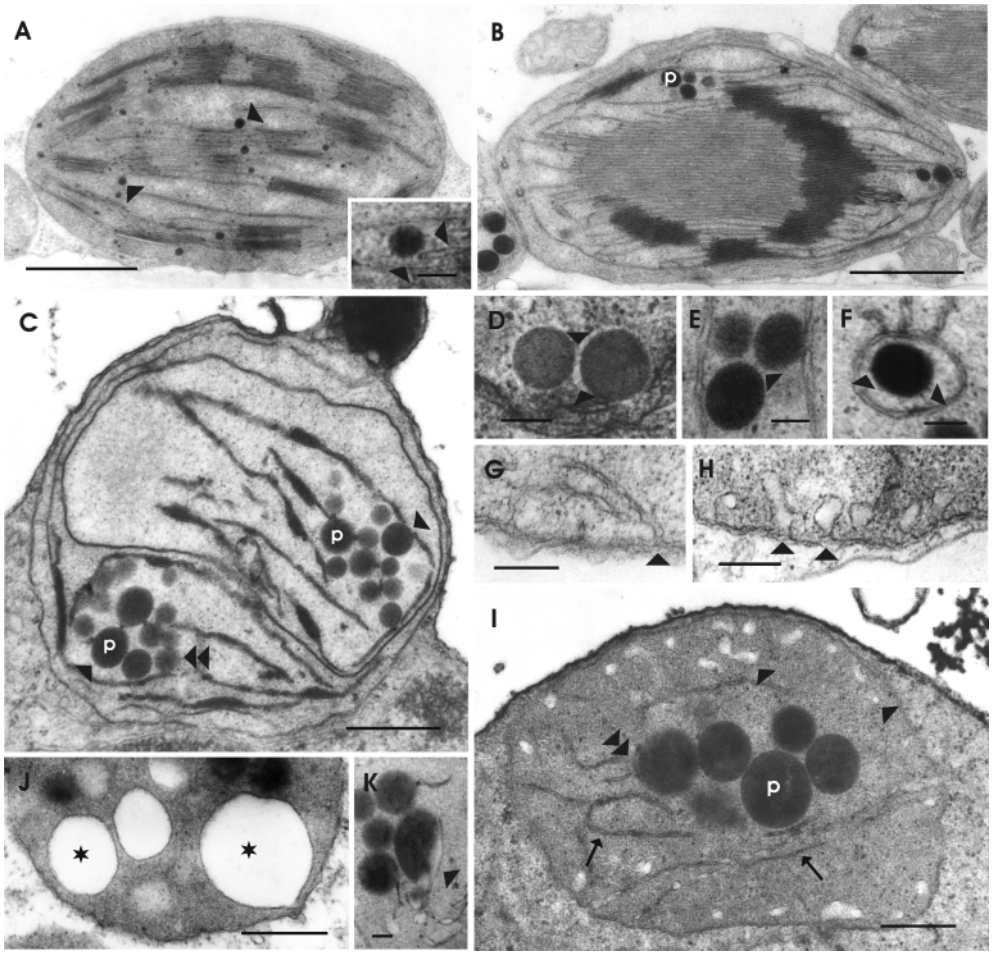

Figure 2. Ultrastructural characteristics of pericarp plastids from immature, maturing, and mature fruit. (**A**) Immature fruit. Chloroplast with well developed thylakoid system and small plastoglobules (arrowheads) scattered along the thylakoids. Bar = 1 μm. In the inset, a plastoglobule with radial protrusions (arrowheads) is seen. Bar = 0.1 μm. (**B-H**) Maturing fruit. (**B**) Degrading chloroplast with a group of parallelly arranged thylakoids. p, plastoglobules. Bar = 1 μm. (**C**) Degrading chloroplast with clustered plastoglobules (p). Plastoglobules showing a clearly bordered (arrowheads) and a "diffuse" (double arrowhead) surface are indicated. Bar = 0.5 μm. (**D-F**) Features of plastoglobules in degrading chloroplasts. (**D**) Plastoglobules encircled with a poorly stained region. Protrusions extending through these regions are indicated (arrowheads). Bar = 0.1 μm. (**E**) Plastoglobules interconnected by protrusions (arrowhead). Bar = 0.1 μm. (**F**) Plastoglobule with protrusions extending towards degrading thylakoid (arrowheads). Bar = 0.1 μm. (**G**) Part of a degrading chloroplast showing a double membrane sheet continuous with the plastid envelope (arrowhead). Bar = 0.2 μm. (**H**) Part of a degrading chloroplast showing continuity of membranes with vesicular and tubular profiles with the plastid envelope (arrowheads). Bar = 0.2 μm. (**I-K**) Mature fruit. (**I**) Plastid with remnant thylakoids (arrows) and cluster of plastoglobules (p). A close contact of a plastoglobule with a thylakoid remnant is indicated by double arrowhead. Membranes with vesicular and tubular profiles are seen in the peripheral stroma (arrowheads). Bar = 0.5 μm. (**J**) Part of a plastid with MBBs (asterisks). Bar = 0.5 μm. (**K**) Part of a plastid showing a plastoglobule in close contact with a thylakoid remnant (arrowhead). Bar = 0.1 μm.

examined layers of the subepidermis contained chloroplasts. The latter showed typical lense-shaped profiles, while their length was usually up to 4 μm (Fig. 2A). The thylakoid system exhibited normal morphology, with well-developed granal and intergranal regions. The plastoglobules were small (usually up to 120 nm in diameter) and mostly darkly stained. They were scattered throughout the plastid interior, individually placed in immediate vicinity to thylakoids. On some occasions, thin radial protrusions were observed to extend from the surfaces of the plastoglobules, some of them reaching towards the thylakoids (inset in Fig. 2A). The plastid stroma was dense with abundant ribosomes.

Plastid Ultrastructure During Fruit Maturation

During fruit maturation, the thylakoid system in the chloroplasts progressively deteriorated, often accompanied by formation of groups of parallelly arranged single thylakoids (Fig. 2B). At the same time, the plastoglobules increased in size and tended to arrange in clusters, usually remaining close to the membranes of the deteriorating thylakoid system (Fig. 2B, C). Most plastoglobules were darkly or moderately stained, while their surfaces were either rather clearly bordered or showed a more "diffuse" appearance (Fig. 2C). Some plastoglobules were encircled by a poorly stained region (Fig. 2D). In many places, thin protrusions were seen on the surfaces of the plastoglobules (Fig. 2D-F). Some of these protrusions extended between adjacent plastoglobules (Fig. 2D, E), while others appeared to connect the plastoglobules with the degrading thylakoid membranes (Fig. 2F). In the plastoglobules surrounded by a poorly stained region,

the protrusions were often found to extend through the latter (Fig. 2D).

In the course of chloroplast degradation, most commonly in its later stages, double-membrane sheets as well as mem-

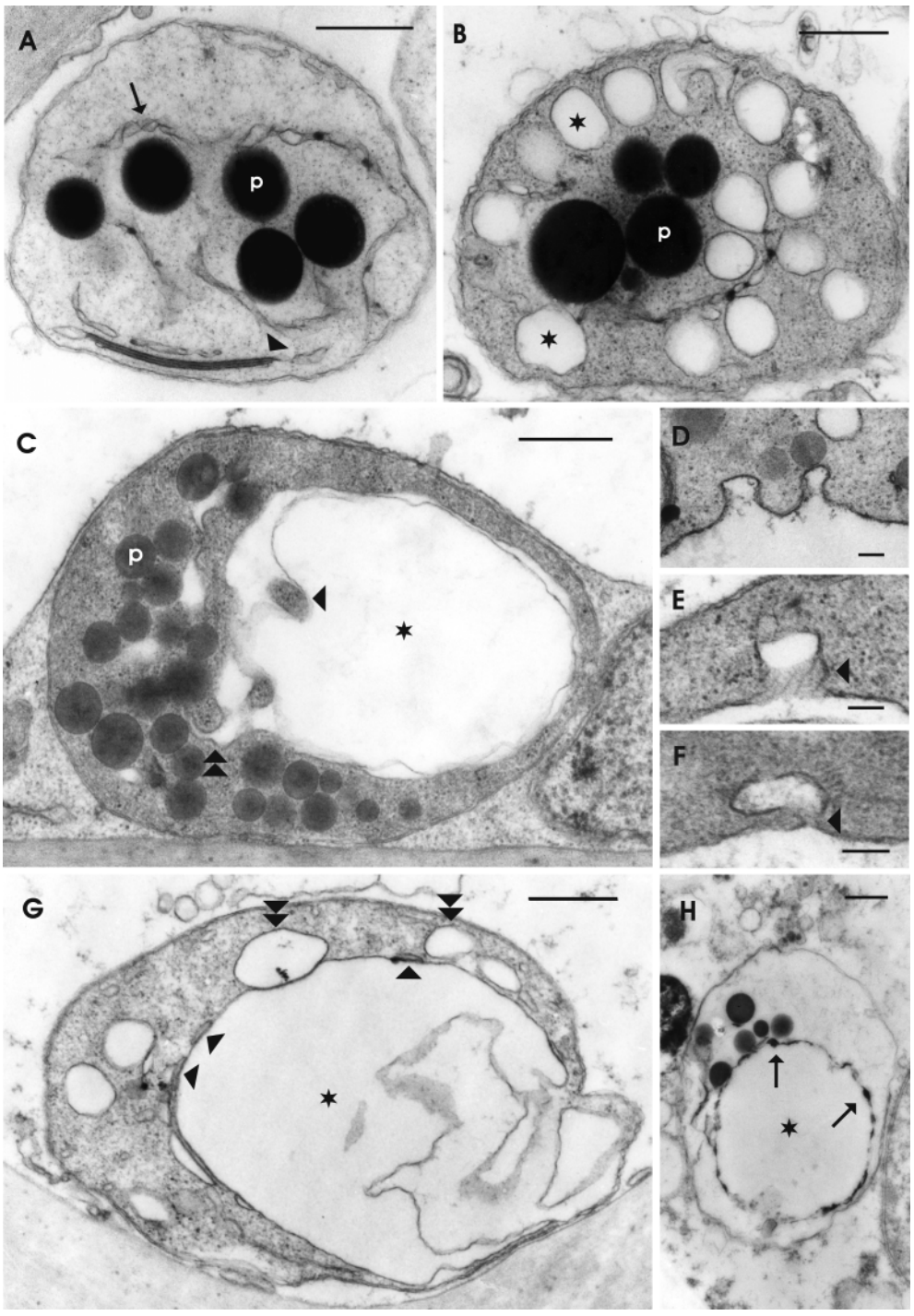

Figure 3. Ultrastructural characteristics of pericarp plastids during fruit regreening. (**A**) Plastid containing double membranes (arrow). An array of a few parallel double membranes is indicated by arrowhead. Note contact of the double membranes with the plastoglobules (p). Bar = 0.5 μm. (**B**) Plastid containing small MBBs (asterisks). Some MBBs are placed in close vicinity of plastoglobules (p). Bar = 0.5 μm. (**C**) Plastid with large MBB (asterisk) encircled by two membranes. Extensions of the MBB into its interior (arrowhead) and into the stroma (double arrowhead) are seen. p, plastoglobules. Bar = 0.5 μm. (**D**) Extensions of MBB displaying vesicular profiles. Bar = 0.1 μm. (**E**) Extension of MBB showing cross-connection between the membranes of the extension (arrowhead). Bar = 0.1 μm. (**F**) Extension of MBB displaying tubular profile. A possible cross-connection in the region of constriction is indicated (arrowhead). Bar = 0.1 μm. (**G**) Plastid containing large MBB (asterisk) along which flat (arrowheads) and swollen (double arrowheads) thylakoid-like structures are seen. Free-floating small MBBs which resemble swollen thylakoid-like structures are visible in the stroma surrounding the large MBB. Bar = 0.5 μm. (**H**) Plastid with MBB (asterisk) - DAB reaction. Precipitates of photooxidized DAB are seen along the border of the MBB (arrows). Bar = 0.5 μm.

branes with vesicular or tubular profiles were observed in the plastid periphery (Fig. 2G, H). All of these membranes were frequently found in continuity with the inner membrane of the plastid envelope.

Plastid Ultrastructure in Mature Fruit

In the pericarp of mature fruit, the plastids were rather small (the majority of them was up to 3 μm in diameter), and roundish to oval or sometimes amoeboid in shape (Fig. 2I). These plastids still contained some remnants of the thylakoid system, the latter often persisting in the form of parallelly arranged single thylakoids. We also observed, in many plastids, one or more vacuole-like, membrane-bound bodies (MBBs), which apparently formed by extensive dilatation of thylakoids (Fig. 2J). Plastoglobules were regularly present in the stroma, being rather large (mostly up to 500 nm in diameter) and usually arranged in clusters (Fig. 2I). Their morphological characteristics, such as osmiophilicity and the appearance of their surfaces, were the same as in the plastoglobules from maturing fruit. Plastoglobules frequently remained in the vicinity of remnant thylakoids and, occasionally, close contact of thylakoids with plastoglobules was observed (Fig. 2I, K).

The plastid stroma often appeared strikingly dense with abundant ribosomes and sometimes showed inclusions of electron dense particles which likely corresponded to phytoferritin accumulations. The peripheral stroma commonly contained membranes with vesicular and tubular profiles, similar to those observed during fruit maturation (Fig. 2I).

Plastid Ultrastructure During Fruit Regreening

Initial stages of chloroplast redifferentiation

In the initial stages of chloroplast redifferentiation, the major ultrastructural characteristics of the plastids were large plastoglobules and a small number of membranes, the latter appearing as precursors from which the thylakoid system started to form. These membranes can be basically classified into two morphological types: double membranes which more or less resembled thylakoids and MBBs.

The first type, *double membranes,* were either flattened in appearance, thus resembling typical thylakoid morphology, or they were partially dilated. These membranes were sometimes arranged in a parallel fashion, in the latter case appearing as remnants of single thylakoid arrays formed during fruit maturation (Fig. 3A).

The second type, *MBBs*, were the most conspicuous membraneous structures during early chloroplast redifferentiation (Fig. 3B, C). They were typically bordered by a single membrane, though some of them had two, or occasionally, more membranes. The MBBs showed various sizes: some of them were rather small (Fig. 3B), while others comprised up to several micrometers in length, thus occupying a large part of the plastid volume (Fig. 3C). Some plastids contained one, usually large, MBB, while in others, several were present. In their simplest form, the MBBs were roundish or oval in shape. Large MBBs, however, frequently assumed irregular shapes and their membranes showed extensions into the stroma or into their interior (Fig. 3C-F). On thin sections, the extensions into the stroma appeared either as short vesicular structures (Fig. 3D, E) or assumed longer, more tubular, profiles (Fig. 3C, F). In some of these structures, both of the vesicular and the tubular profiles, a constriction was observed (Fig. 3D-F). In the constricted region, it occasionally appeared that cross-connections between the membranes are formed (Fig. 3E, F), suggesting that these structures could eventually completely separate from the membrane of the MBB, in a process which resembles "budding".

At further stages, thylakoid-like structures appeared along the border of MBBs, suggesting formation from their membranes (Fig. 3G). Some of these structures were flattened and morphologically appeared as typical thylakoids. Others were dilated and even swollen, in the latter case resembling small MBBs. Since small MBBs were also often observed as free-floating entities in the stroma which surrounded the large MBBs, it appears plausible that, at least some of them, originated from the swollen thylakoid-like structures which primarily formed on the membranes of large MBBs and afterwards detached from them. In the tissues treated with DAB, its photooxidized form (indicating the presence of the components of Photosystem I) was often found to precipitate sporadically along the border of the MBBs (Fig. 3H). Although, in these specimens, we were unable to resolve the thylakoid-like structures appressed to the MBBs, the presence of precipitates strongly suggested that MBBs could be precursors of functional photosynthetic membranes.

Another distinctive feature observed in many MBBs was their fragmented appearance (Fig. 4A, B). Some of them were traversed by membraneous partitions and it occasionally appeared that thylakoid-like structures could be formed by such partitioning (Fig. 4A). In other cases, smaller MBBs were tightly appressed to each other or to a larger MBB, giving the impression that they were derived by fragmentation of a single larger MBB (Fig. 4B).

Reassembly of the thylakoid system

At more advanced stages of chloroplast redifferentiation, thylakoids accumulated in the stroma. These early thylakoids often appeared to be derived from the membranes of MBBs, since they were commonly observed to adhere to the latter (Fig. 4B). Sometimes even small grana composed of several thylakoids formed along the border of MBBs. Although MBBs, in many plastids, appeared to be dominant structures involved in the early formation of thylakoids, this process apparently also occurred by differentiation and multiplication of double membranes with thylakoid-like morphology, as illustrated by the plastid shown in Fig. 4C which did not contain MBBs.

As the quantity of thylakoids in the plastids increased, the MBBs progressively vanished, apparently being completely transformed into thylakoids (Fig. 4D, E). In some places, it even appeared that MBBs were fragmented into swollen grana-like structures (a constriction which could likely be involved in this process is depicted, Fig. 4D).

In the later stages of chloroplast redifferentiation, the thylakoids became linked into an organized membraneous network, the latter showing a strong DAB reaction (Fig. 4F). The thylakoid system which eventually completely reassembled exhibited well-developed grana interconnected by numer-

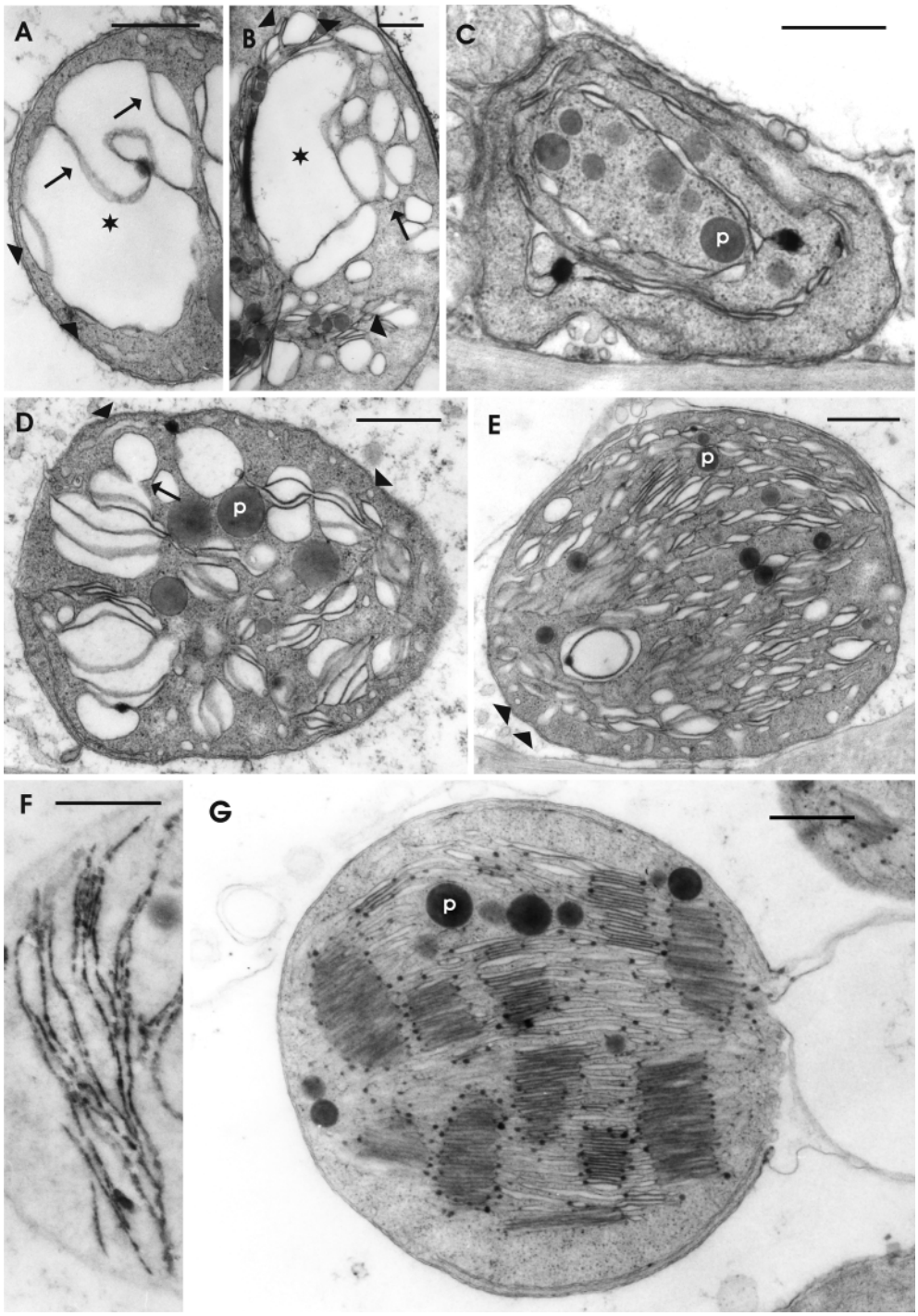

Figure 4. Ultrastructural characteristics of pericarp plastids during fruit regreening. (**A**) Part of a plastid with MBB (asterisk) traversed by partitions (arrows). Thylakoid-like structures which appear to be formed by partitioning of the MBB are indicated (arrowheads). Bar = 0.5 μm. (**B**) Part of a plastid with large MBB (asterisk) surrounded by smaller MBBs. The large MBB appears to be in the process of fragmentation. Several smaller MBBs appressed to each other which appear to be formed by such fragmentation are indicated (arrow). Note thylakoids appressed to the MBBs (arrowheads). Bar = 0.5 μm. (**C**) Plastid with developing thylakoid system, without MBBs. p, plastoglobules. Bar = 0.5 μm. (**D**) Developing chloroplast with swollen grana-like structures. A constriction which could result in fragmentation of part of the grana-like structure is indicated (arrow). Membranes with vesicular and tubular profiles are seen in the peripheral stroma (arrowheads). p, plastoglobules. Bar = 0.5 μm. (**E**) Developing chloroplast with abundant thylakoids. Note membranes with vesicular and tubular profiles in the peripheral stroma (arrowheads). p, plastoglobules. Bar = 0.5 μm. (**F**) Part of a plastid with developing thylakoid system – DAB reaction. Precipitates of photooxidized DAB are seen inside the thylakoid membranes. Bar = 0.5 μm. (**G**) Completely redifferentiated chloroplast with well-developed thylakoid system. p, plastoglobules. Bar = 0.5 μm.

ous stroma thylakoids (Fig. 4G). Both grana stacks and intergranal regions showed normal morphology. In these fully redifferentiated chloroplasts, MBBs were no longer observed.

Ultrastructural characterization of plastoglobules

In redifferentiating chloroplasts, the stroma was usually dense and contained abundant ribosomes. The electron dense particles (probably corresponding to phytoferritin accumulations), which were sporadically observed in the plastids of mature fruit, had now completely disappeared. Plastoglobules, however, remained a common motif in the plastid stroma. During early redifferentiation, the plastoglobules were mostly arranged in clusters, while, in the later stages, they often became dispersed through the plastid. They were often large (up to 900 nm in diameter) and, in most cases, moderately to strongly osmiophilic, morphologically resembling those in the plastids of mature fruit. Many plastoglobules were found to be in contact with, or in the close vicinity of, thylakoids (Fig. 5A), or membranes which appeared to be precursors of thylakoids, as can be seen on Fig. 3A-C. They were often observed to be connected to developing thylakoids by thin radial protrusions (inset in Fig. 5A), even though, occasionally, thylakoids also appeared as being pressed into plastoglobules (Fig. 5A).

Ultrastructural characterization of membranes associated with the plastid envelope

During chloroplast redifferentiation, the inner membrane of the plastid envelope was in many places found to be continuous with morphologically distinct types of membraneous structures: vesicles, tubules and double-membrane sheets.

Vesicular invaginations of the inner envelope membrane (Fig. 5B) were commonly observed in the later stages of reassembly of the thylakoid system, when the plastids already contained a substantial number of thylakoids (Fig. 4D, E). Small vesicles which appeared to be formed by those invaginations were commonly found free in the peripheral stroma and even among the developing thylakoids (Fig. 5C). Some of these vesicles were in close contact with the thylakoids and we occasionally observed places in which the vesicles appeared to be fused with thylakoid membranes (Fig. 5D). In all observed cases, those putative fusions were found in the terminal parts of the thylakoids.

The second type of membranes was tubular in shape (Fig. 5E). These tubules were often observed at the periphery of the developing thylakoid system (as can be seen in Fig. 4D, E). However, they were also sometimes found in the peripheral stroma during the initial stages of chloroplast redifferentiation.

The third type of membranes had a lamellar structure, as indicated by their uninterrupted appearance in thin sections (Fig. 5F). These double-membrane sheets, which morphologically resembled developing thylakoids, were sporadically found through the whole process of chloroplast redifferentiation. They appeared as invaginations of the inner envelope membrane, and, in some cases, were linked to the envelope in more than one place.

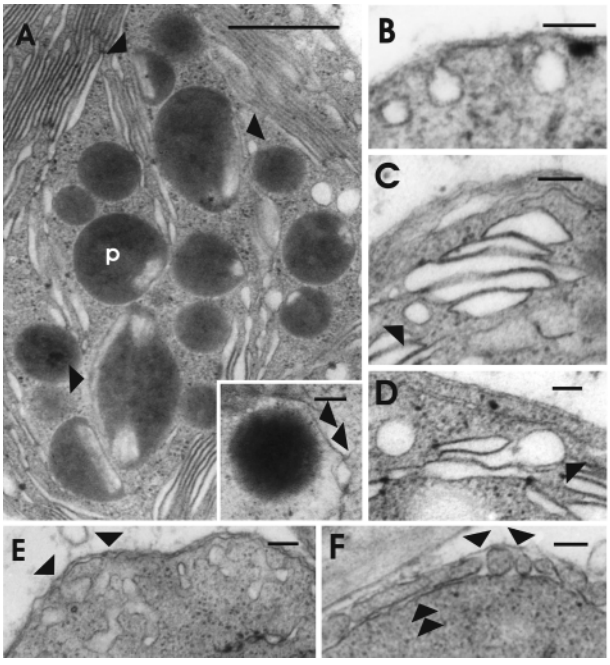

Figure 5. Ultrastructural characteristics of pericarp plastids during fruit regreening. (**A**) Part of a plastid showing plastoglobules (p) in contact with thylakoids. The arrowheads indicate places in which thylakoids appear to be pressed into plastoglobules. Bar = 0.5 μm. In the inset, a plastoglobule with radial protrusions is seen; some of the protrusions extend towards a thylakoid-like membrane (arrowheads). Bar = 0.1 μm. (**B-D**) Putative transport of vesicles from the plastid envelope to the thylakoids. (**B**) Vesicular invaginations of the plastid envelope. Bar = 0.1 μm. (**C**) Vesicle in close vicinity of thylakoids (arrowhead). Bar = 0.1 μm. (**D**) Vesicle which appears to be fused with the thylakoid membrane (arrowhead). Bar = 0.1 μm. (**E**) Tubules in the peripheral stroma. Continuity of the tubules with the plastid envelope is indicated (arrowheads). Bar = 0.1 μm. (**F**) Double-membrane sheet (double arrowhead), in several places directly connected to the plastid envelope (arrowheads). Bar = 0.1 μm.

Plastid Ultrastructure in Regreened Tissue

In regreened tissue, the cells in all examined subepidermal layers contained chloroplasts. The latter were roundish or lense-shaped and up to 6 μm in length. Their thylakoid system was completely restored, appearing as that shown in Fig. 4G. Many chloroplasts had high grana, which apparently resulted from fruit exposure to relatively low light intensity. The stroma was dense and contained abundant ribosomes. Plastoglobules persisted in most plastids, often appearing individually placed in the vicinity of the thylakoids. In part of the plastids, they appeared reduced in number and/or volume, although very large plastoglobules (up to 700 nm in diameter) also occasionally remained. In some plastids we observed a small starch grain.

DISCUSSION

During maturation of cucumber fruit (which was accompanied by yellowing), the chloroplasts in the pericarp subepidermis were converted to plastids with a degraded thylakoid system, large plastoglobules and dense stroma with abundant ribosomes. On some occasions, however, mature cucumber fruit were able to regreen partially. Our studies strongly suggested that the chloroplasts found in the subepidermal layers of such regreened parts of the fruit pericarp were, in fact, redifferentiated from the plastids of mature fruit. This was evident, firstly, because the plastids from regreening tissue retained some features of the plas-

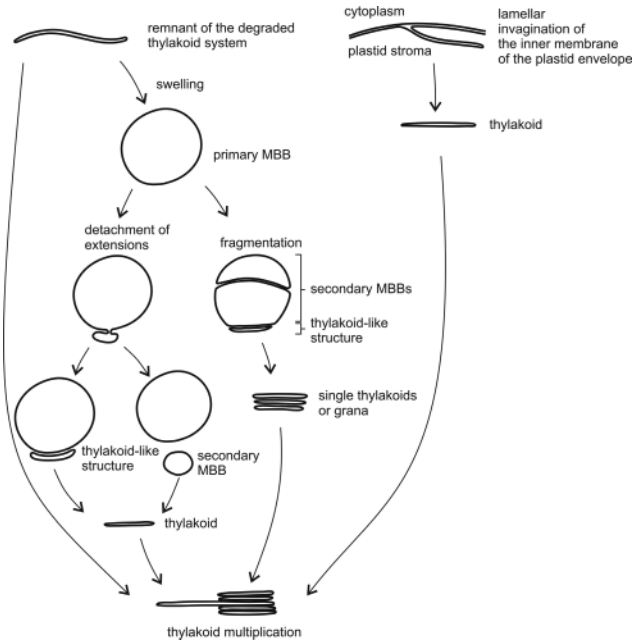

Figure 6. Overview of the main mechanisms of thylakoid formation likely to occur during chloroplast redifferentiation in cucumber fruit.

tids from mature fruit (large plastoglobules and abundant ribosomes) and, secondly, because the membranes present in the plastids undergoing early redifferentiation (MBBs and double membranes with thylakoid-like morphology) often resembled the remnants of the thylakoid system observed in mature fruit.

Ultrastructural studies further indicated that the reconstitution of the thylakoid system may start from those remnant thylakoids (the mechanisms by which this likely occurs are summarized on the left side of Fig. 6). This possibility is most convincingly suggested by the appearance and structural characteristics of the MBBs. Namely, the MBBs firstly appeared in mature fruit, apparently developing by extensive swelling of the thylakoids. Likewise, the MBBs found during early chloroplast redifferentiation, were probably, at least in part, swollen remnant thylakoids. Also, the morphological features of MBBs suggested that they are very plastic in nature and that they could even give rise to new membraneous entities which will eventually become thylakoids. Ultrastructural observations indicated that, in the latter processes, at least two mechanisms could be involved: (i) detachment of extensions of the MBB (a process which resembles "budding") and (ii) fragmentation of the MBB (Fig. 6). The possibility of the first mechanism was indicated by formation of a constriction in the extensions and, more directly, by observation of a cross-connection between the membranes in the constricted region. The formation of extensions with tubular profiles strongly suggests that the flat thylakoid-like structures, appearing along the borders of MBBs, could be formed in that way. It is, however, also possible that some of the extensions which detach from MBBs may further become secondary MBBs (Fig. 6). This was indicated by the presence of swollen thylakoid-like structures along the borders of large MBBs, the former resembling the small MBBs observed in the surrounding stroma. The second mechanism, fragmentation of MBBs, appears to be a way by which large MBBs are parceled out into smaller ones, but also a way by which thylakoid-like structures could be formed (Fig. 6). Our observations further suggest that fragmentation of the MBBs could even result in the formation of grana-like structures.

Since electron microscopy cannot provide a dynamic view of a process, the changes through which MBBs proceed during chloroplast redifferentation could only be reconstructed on the basis of static micrographs. A model which, in the light of our ultrastructural observations, appears plausible, rests on the assumption that MBBs could give rise either to thylakoid-like structures, which further become typical thylakoids, or to secondary MBBs. It is likely that these secondary MBBs subsequently flatten also becoming thylakoids (see Fig. 6). However, it should not be disregarded that secondary MBBs could also, in similar ways as the primary MBBs, give rise to new thylakoid-like structures and, possibly, to new MBBs (which subsequently both become thylakoids). The thylakoids could therefore be derived either directly from the primary MBBs or, indirectly, from secondarily formed MBBs.

Membrane-bound structures which resemble the MBBs were also described during chloroplast differentiation in some other plant species, such as in developing leaves of tobacco (Stetler and Laetsch, 1969) and *Coleus blumei* (Marty, 1973), in leaves of deetiolating *Hedera helix*, in aerial roots of some *Orchidaceae* (Salema et al., 1972), and in aspen tissue culture (Blackwell et al., 1969). Similar structures have also been reported to form in chloroplast redifferentiation in pumpkin fruit (Devidé and Ljubešić, 1974) and in the spathe of *Zantedeschia elliottiana* (Grönegress, 1974). The ultrastructural characteristics of these structures often suggested their involvement in the formation of thylakoids, in ways which, in some cases, appear to be similar to those observed in cucumber fruit. Thus, in developing leaves of tobacco and *Coleus blumei*, the thylakoids were found to be appressed to membrane-bound structures (Stetler and Laetsch, 1969; Marty, 1973), as was also observed for the MBBs. Also, a process which strikingly resembles the formation of secondary MBBs by detachment of extensions from primary MBBs was described for the reversion of chromoplasts to chloroplasts in pumpkin fruit (Devidé and Ljubešić, 1974). In the latter, membrane-bound structures termed plastid "vacuoles" were found to give rise to small vesicles, which further, by extending into one direction, formed structures similar to thylakoids.

As discussed above, the reconstitution of the thylakoid system during regreening of cucumber fruit could occur, at least to a certain extent, by redifferentiation and multiplication of the membranes which remain after degradation of the thylakoid system during fruit maturation. It is, however, also highly probable that reconstitution may simultaneously begin from thylakoids formed *de novo* (as schematically represented on the right side of Fig. 6). This was indicated by the observation of double-membrane sheets which were sporadically found to be in continuity with the inner envelope membrane, during the whole course of chloroplast redifferentiation. These double membranes strongly resembled structures which are commonly found during chloroplast

development from proplastids, and are considered as incipient thylakoids formed by invagination of the inner envelope membrane (Frey-Wyssling and Mühlethaler, 1965; Kirk and Tilney-Bassett, 1978). The possibility that, during chloroplast redifferentiation, the inner membrane of the plastid envelope may be involved in the formation of thylakoids has also been suggested for regreening Valencia oranges (Thomson, 1967), pumpkin fruit (Devidé and Ljubešić, 1974), *Zantedeschia elliottiana* spathes (Grönegress, 1974) and soybean cotyledons (Huber and Newman, 1976).

Our studies indicated that thylakoid formation during regreening of cucumber fruit is not a uniform process but can proceed by different mechanisms. Such multiple ways of thylakoid formation have also been reported for chloroplast redifferentiation in pumpkin fruit and soybean cotyledons. In the first case, thylakoids appeared to form in at least three different ways: from the inner membrane of the plastids envelope, from small thylakoid fragments and from the membranes of the plastid "vacuoles" (Devidé and Ljubešić, 1974), while, in the latter case, membrane fragments and vesicles were suggested to be involved in thylakoid formation (Huber and Newman, 1976).

In the majority of examples of reversible chloroplast differentiation, the plastoglobules were reported to accumulate and/or enlarge during disassembly of the thylakoid system and to reduce in number and/or size, during its reconstitution (Devidé and Ljubešić, 1974; Grönegress, 1974; Sitte, 1974; Huber and Newman, 1976; Ikeda, 1979; Koiwa et al., 1986; Zavaleta-Mancera et al., 1999). This led to the proposal that plastoglobules deposit at least part of the constituents of the degraded thylakoid membranes, which could, in turn, be used in rebuilding the thylakoid system. Biochemical profiling of plastoglobules indicated that they may indeed serve as storage subcompartments for thylakoid membranes, but could also be involved in lipid biosynthesis and metabolism (Steinmüller and Tevini, 1985; Kessler et al., 1999; Austin et al., 2006; Vidi et al., 2006; Ytterberg et al., 2006). In addition, freeze-fracture electron microscopy and electron tomography of samples prepared by high-pressure freezing/freeze-substitution methods demonstrated that plastoglobules are permanently attached to thylakoids through a half-bilayer membrane that encloses the plastoglobule and is in direct continuity with the thylakoid outer leaflet (Austin et al., 2006). Based on these recent discoveries, it was proposed that one of the functions of plastoglobules could be active exchange of lipid metabolites with the thylakoid membranes (Austin et al., 2006; Bréhélin et al., 2007). During chloroplast degradation which accompanied the maturation of cucumber fruit, plastoglobules considerably increased in size, remaining present in the stroma during the whole course of chloroplast redifferentiation. Their involvement in thylakoid system disassembly as well as in its reconstitution was strongly indicated by the fact that they were frequently found in close proximity to both degrading and developing thylakoids. Moreover, plastoglobules were often observed to be connected to thylakoids *via* thin radial protrusions. Although our samples were fixed by a standard chemical procedure, such protrusions could correspond to the connections of plastoglobules to thylakoids recently characterized by Austin et al. (2006).

The plastid envelope is considered to have a crucial role in the formation of thylakoid membranes, not only because thylakoids are believed to form initially by invagination of the inner envelope membrane, but also for the reason that the envelope continues to supply the lipid material required for their further growth and maintenance (Vothknecht and Westhoff, 2001; Westphal et al., 2001). The assumption that, in the latter case, lipids could be carried from the envelope to the thylakoids *via* some kind of vesicle-mediated transfer was inspired by ultrastructural observations of stromal vesicles and has been further corroborated by a number of biochemical studies (Thomson et al., 1967; Salema and Abreu, 1972; Morré et al., 1991; Andersson et al., 2001; Kroll et al., 2001; Westphal et al., 2001; Wang et al., 2004). Evidence has been presented that vesicles derived from the inner envelope membrane migrate towards the growing thylakoids, eventually fusing with them (Thomson et al., 1967; Salema and Abreu, 1972; Morré et al., 1991; Westphal et al., 2001). Ultrastructural observations of the redifferentiation of chloroplasts in cucumber fruit also strongly corroborated the involvement of such vesicles in the formation of the thylakoid system, as particularly evident in the later stages of this process. The vesicles, which apparently formed by invagination of the inner envelope membrane, were in some places in close contact with the surface of the thylakoids and we even found places in which the membrane of a vesicle had, apparently, just fused with a thylakoid membrane.

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