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Chloroplast biogenesis 80. Proposal of a unified multibranched chlorophyll *a/b* biosynthetic pathway

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Abstract

A unified multibranched chlorophyll (Chl) biosynthetic pathway is proposed. The proposed pathway takes into account the following considerations: (*a*) that the earliest putative precursor of monovinyl Chl *b* that has been detected in higher plants is monovinyl protochlorophyllide *b*, (*b*) that in most cases, Chl *b* biosynthesis has its roots in the Chl *a* biosynthetic pathway, (*c*) that the Chl *a* biosynthetic pathway exhibits extensive biosynthetic heterogeneity, (*d*) that Chl biosynthesis may proceed differently at different stages of greening and in different greening groups of plants. Integration of the Chl *a* and *b* biosynthetic pathways into a unified multibranched pathway offers the functional flexibility to account for the structural and biosynthetic complexity of photosynthetic membranes. In this context, it is proposed that the unified, multibranched Chl *a/b* biosynthetic pathway represents the template of a Chl-protein biosynthesis center where photosystem (PS) 1, PS2, and light-harvesting Chl-protein complexes are assembled into functional photosynthetic units. The individual biosynthetic routes or groups of two to three adjacent biosynthetic routes may constitute Chl-protein biosynthesis subcenters, where specific Chl-protein complexes are assembled.

Additional key words: enzymes; light-harvesting pigment-proteins; photosystems 1 and 2; protochlorophyllide; protoporphyrin IX; tetrapyrroles.

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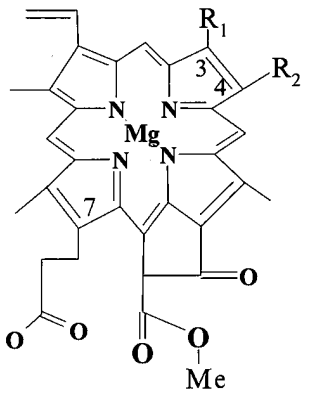
Abbreviations: ALA, 5-aminolevulinic acid; Chl, chlorophyll; Chlide, chlorophyllide; Chl(ide), Chlide and/or Chl; D, dark; DL, dark-light; DV, divinyl (vinyl groups at position 2 and 4 of the macrocycle); LHC, light-harvesting pigment-protein antenna; L, light; Mpe, Mg-protoporphyrin IX monomethyl ester; MV, monovinyl (vinyl group at position 2 and ethyl group at position 4 of the macrocycle); Pchlide, protochlorophyllide; Proto, protoporphyrin IX; PS, photosystem; 4VChlR, [4-vinyl] Chl reductase; 4VCR, [4-vinyl] Chlide *a* reductase; 4VMPR, [4-vinyl] Mg-Proto reductase; 4VpideR, [4-vinyl] Pchlide *a* reductase. Unless preceded by MV or DV, tetrapyrrole names are used generically to designate metabolic pools that may consist of MV and DV components.

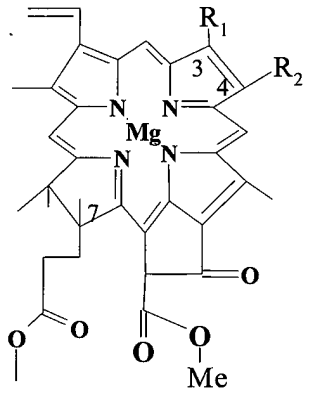
Introduction

Next to monovinyl (MV) Chl *a*, MV Chl *b* is the second most abundant Chl in green plants. Its function as an accessory pigment in photosynthesis is well established. For the past 50 years investigations aimed at elucidating the biosynthetic origin of 2-MV Chl *b* (Fig. 1) have met with limited success. After a lifetime devoted to investigating the biosynthetic origin of MV Chl *b*, Shlyk (1971) assembled a large body of circumstantial evidence in support of the hypothesis that 2-MV Chl *b* may be formed from "young", easily extractable MV Chl *a*. This hypothesis was questioned by Oelze-Karow and Mohr (1978): on the basis of kinetic analysis of the fluctuations in the MV Chlide *a* (*i.e.*, Chl *a* without esterification at position 7 of the macrocycle) and MV Chl *b* pools in etiolated mustard seedlings subjected to various irradiations, they proposed that MV Chl *b* may be formed from MV Chlide *a* instead of 2-MV Chl *a*.

Irrespective of whether Chl *b* is formed from Chl *a* or Chlide *a*, the conversion of the Chl *a* and/or chlorophyllide (Chlide) *a* [Chl(ide) *a*] to Chl(ide) *b* involves conversion of the methyl group at position 3 to formyl (Fig. 1). Mass spectra of [3-hydroxymethyl]-Chl *b* extracted from leaves greened in the presence of either $^{18}O_2$ or $H_2{}^{18}O$ revealed that ^{18}O was incorporated only from molecular oxygen into the 3-formyl group of Chl *b* (Porra *et al.* 1993, 1994). The high enrichment using $^{18}O_2$ and the absence of labeling by $H_2{}^{18}O$ indicated that molecular oxygen is the sole precursor of the 3-formyl oxygen of Chl *b* in greening maize leaves. This in turn suggested that a mono-oxygenase and a hydroxymethyl dehydrogenase might be involved in the oxidation of the methyl group to formyl. However, these putative enzymes (which will be referred to collectively as formyl synthetase) have so far eluded detection and purification from higher or lower plants.

Recent developments in the understanding of the Chl *a* biosynthetic heterogeneity, and the discovery of putative biosynthetic intermediates and end products of the Chl *b* pathway, such as MV Pchlide *b* (Shedbalkar *et al.* 1991), MV Chlide *b* (Aronoff 1981, Duggan and Rebeiz 1982), DV Chlide *b* (Rebeiz, unpublished), and DV Chl *b* (Brereton *et al.* 1983, Wu and Rebeiz 1985), suggest that the Chl *b* pathway may also be biosynthetically heterogeneous. As a working hypothesis, the full extent of this biosynthetic heterogeneity is depicted in Fig. 2 which describes ten different biosynthetic routes capable of forming Chl *b* either from Chlide *a*, Chl *a*, or MV Pchlide *b*. Chemical structures are shown in Fig. 1. In constructing Fig. 2, the following was taken into account: (*a*) The 4-branched carboxylic Chl *a* biosynthetic pathway described earlier (Adb El Mageed *et al.* 1997) was used as a starting point of the unified Chl *a*/*b* pathway, (*b*) possible precursor-product relationships was based on structural similarities, and (*c*) results from preliminary experiments (discussed below). The possible involvement of MV Pchlide *b* phytyl ester in the biosynthesis of MV Chl *b* was not considered, on account of the demonstration that this tetrapyrrole was not photoconvertible to MV Chl *b* *in vitro* (Schoch *et al.* 1995).





1. Protochlorophyllide **2. Chlorophyllide**

	R1	R2	R3	Compound
1a	**Methyl**	**Ethyl**		**MV Pchlide *a***
1b	**Methyl**	**Vinyl**		**DV Pchlide *a***
1c	**Formyl**	**Ethyl**		**MV Pchlide *b***
1d	**Formyl**	**Vinyl**		**DV Pchlide *b***
2a	**Methyl**	**Ethyl**	**H**	**MV Chlide *a***
2b	**Methyl**	**Vinyl**	**H**	**DV Chlide *a***
2c	**Formyl**	**Ethyl**	**H**	**MV Chlide *b***
2d	**Formyl**	**Vinyl**	**H**	**DV Chlide *b***
2e	**Methyl**	**Ethyl**	**Phytol**	**MV Chl *a***
2f	**Methyl**	**Vinyl**	**Phytol**	**DV Chl *a***
2g	**Formyl**	**Ethyl**	**Phytol**	**MV Chl *b***
2h	**Formyl**	**Vinyl**	**Phytol**	**DV Chl *b***

Fig. 1. Chemical structures of various MV and DV protochlorophyllides, chlorophyllides, and chlorophylls.

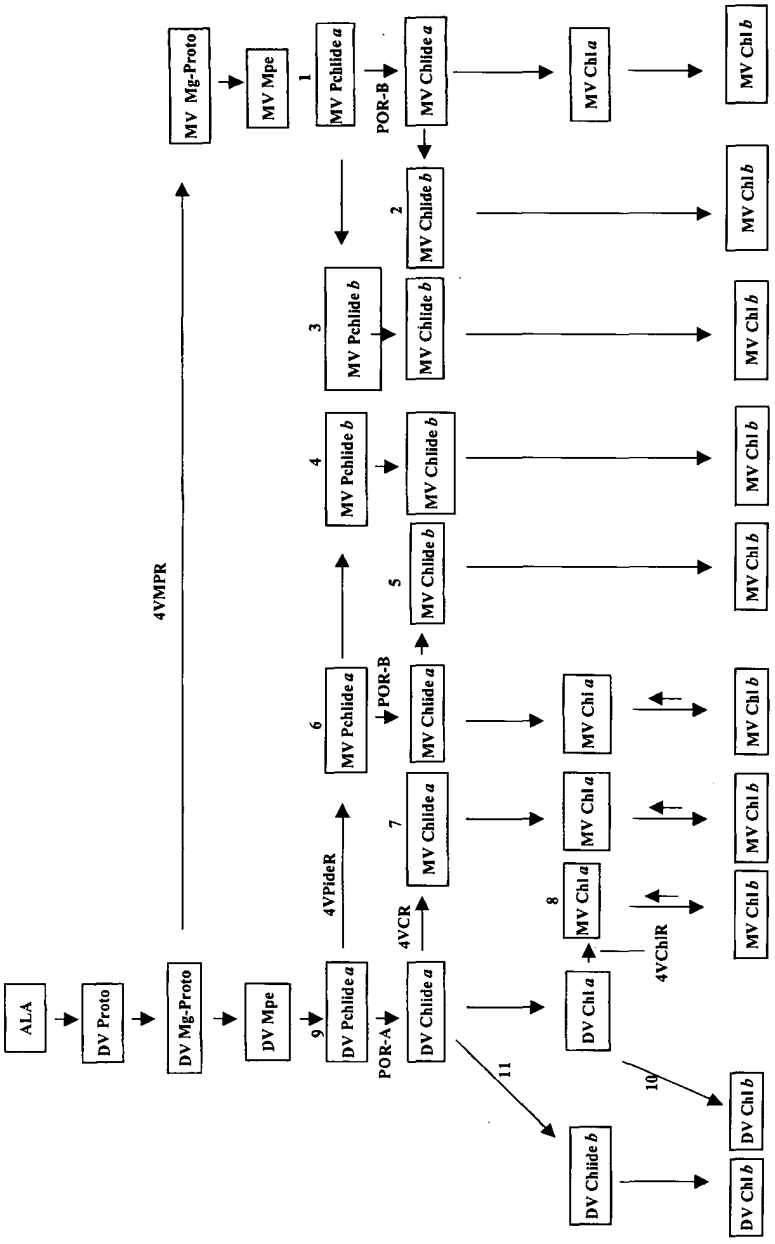


Fig. 2. Proposed unified Chl *a*/*b* biosynthetic pathway. ALA = 5-aminolevulinic acid, Chl = chlorophyll, Chlide = chlorophyllide, DV = divinyl, MV = monovinyl, Mpe = Mg-Proto monomethyl ester, Pchlide = protochlorophyllide, Proto = protoporphyrin IX, 4VCR = [4-vinyl] chlorophyllide *a* reductase, 4VChlR = [4-vinyl] Chl reductase, 4VMPR = [4-vinyl] Mg-Proto reductase, 4VPideR = [4-vinyl] protochlorophyllide *a* reductase. Arrows joining the DV and MV branches refer to reactions catalyzed by [4-vinyl] reductases. Various biosynthetic routes are designated by arabic numerals.

Materials and methods

Plants: Etiolated cucumber cotyledons (*Cucumis sativus* L. cv. Beit Alpha) and maize (*Zea mays* L. cv. FS 675) seeds (purchased from *FS Growmark Inc.*, Bloomington, IL, USA) were grown in moist vermiculite in glass containers (7.5 cm deep and 9 cm in diameter) for 4 and 6 d, respectively, in darkness at 28 °C.

Incorporation of ^{14}C-5-aminolevulinic acid (^{14}C-ALA) into etiolated seedlings: Three-gram batches of etiolated cucumber cotyledons excised with hypocotyl hooks, and chopped maize leaves, were incubated in 10 cm^3 of distilled water in deep Petri dishes, 9 cm diameter, under a low green irradiance (transmission maximum 503 nm, bandwidth of 40 nm, photon density *ca.* 0.01 $\mu mol\ m^{-2}\ s^{-1}$) that did not photoconvert Pchlide *a* to Chlide *a*. 185 MBq of ^{14}C-ALA (1702 GBq mol^{-1}; *Research Product International*, Elk Grove, IL, USA) were added to each Petri dish. The Petri dishes were incubated at 28 °C in darkness for 15 h.

Irradiation pretreatment of radioactively labeled etiolated seedlings: After dark-incubation, the radioactively labeled, etiolated cucumber and maize tissues were irradiated (six 1000-W metal halide lamps, 120 $\mu mol\ m^{-2}\ s^{-1}$) at 27 °C for various periods of time, prior to pigment extraction. At various time intervals, tissue samples were homogenized in acetone:0.1 M NH_4OH (9:1, v/v) at a ratio of 5 cm^3 per g of tissue. The cucumber hypocotyl hooks were removed prior to homogenization.

Pigment extraction: Partition of fully esterified (Chl *a* and *b*) and monocarboxylic tetrapyrroles (Pchlides and Chlides) between hexane and hexane-extracted acetone, and the transfer of monocarboxylic tetrapyrroles from hexane-extracted acetone to diethyl ether were described in Tripathy and Rebeiz (1986).

Separation of ^{14}C-Chl *a* from ^{14}C-Chl *b*: The hexane fraction containing Chl *a* and *b*, was dried under N_2 and the pigments were redissolved in 2 cm^3 of hexane. An aliquot (0.05 cm^3) of hexane was chromatographed on thin layers of cellulose developed in ligroin (b.p. 90-105 °C):*n*-propanol (99:1, v/v) in darkness at room temperature. The segregated Chl *a* and Chl *b* were eluted in 80 % acetone.

Measurement of ^{14}C-incorporation into Chl *a* and Chl *b*: Small aliquots (0.05 cm^3) of the ^{14}C-Chl *a* and *b* fractions were dissolved in 15 cm^3 of *BioSafe II* (*Research Products International*, Mount Prospect, IL, USA). Radioactivity was determined in a *Beckman* model *LS 3800* liquid scintillation counter operated in the automatic quench compensation mode.

Methylation of acidic side chains: Monocarboxylic tetrapyrroles such as Chlide *a* and *b* were methylated with diazomethane. Diazomethane was prepared by adding 1 g of N-nitrosomethyl urea to a flask containing 3 cm^3 of 40 % KOH and 10 cm^3 of ether, at 4 °C. The flask was swirled on ice for few minutes until the white solid disappeared and the ether turned yellow. The ether layer was decanted, washed with water and used immediately as a source of diazomethane.

Separation of ^{14}C-Chlide *a* from ^{14}C-Chlide *b*: The ether fraction containing Chlide *a* and Chlide *b* was dried under N_2 and the pigments were redissolved in 2 cm^3 of

ether. Methylation of Chlides was achieved by adding 3 cm^3 of freshly prepared diazomethane in ether to the Chlide solution (Belanger *et al.* 1982). The reaction was allowed to proceed for 6 min at 1 to 4 °C, after which the ether was evaporated completely under N_2. The residue was redissolved in ether and chromatographed on thin layers of silica gel H. The bands were eluted in 80 % acetone, and the acetone extract was collected after centrifugation.

Spectrofluorometry: Fluorescence spectra were recorded on a fully corrected photon counting spectrofluorometer *SLM 8000* interfaced with an *IBM* microcomputer model *60*. Pigment solutions were monitored at room temperature in cylindrical microcells 3 mm in diameter. The spectra were recorded at excitation and emission bandwidths of 4 nm. The photon count was integrated for 0.5 s at each nm increment.

Protein determination: The acetone-insoluble residue left after centrifugation of the tissue homogenate was resuspended in distilled water. Total proteins were determined on an aliquot of the suspension by the BCA method (Smith *et al.* 1985) after delipidation. Absorbance was determined on a spectrophotometer *Sequoia-Turner* model *340*.

Spectrophotometry: Absorption spectra were recorded on a *SLM/Aminco* dual wavelength spectrophotometer model *DW-2000*, operated in the slit beam mode, at a slit width of 2 nm.

Quantitative determination of Chl(ide) *a* and Chl(ide) *b*: The amounts of Chl *a* and Chl *b* and Chlide *a* and Chlide *b* as well as the purity of the Chl(ide) *a* and *b* bands were determined by spectrofluorometry as described in Bazzaz and Rebeiz (1979).

Investigations of precursor-product relationships *in vivo*: In 1988, equations were derived to investigate possible precursor-product relationships *in vivo*, in branched and interconnected metabolic pathways (Rebeiz *et al.* 1988). For any two compounds *A* and *B*, formed from a common precursor *P* such as ALA and having a possible direct precursor-product relationship between them, a precursor-product equation was derived. For any number of time intervals t_1 to t_2 the precursor-product equation describes the relationship between the specific radioactivity of compound *A*, possible radiolabel incorporations from compound *A* into compound *B*, and the net synthesis of compound *B* from compound *A*:

$$QB_2 = (\gamma A_1 + \gamma A_2)/2)\,(\Delta B_2) \qquad (1)$$

where QB_2 = expected (or theoretical) amount of radio-label incorporated into compound *B* during time interval $t_1 - t_2$; γA_1, γA_2 = specific radioactivity of compound *A* at the beginning and end of time interval $t_1 - t_2$, respectively; ΔB_2 = amount of compound *B* synthesized during time interval $t_1 - t_2$.

By comparing expected radiolabel incorporation into compound *B*, as calculated from Eq. (1), with experimentally determined (*i.e.*, observed) incorporations into *B*, it is possible to tell whether compound *B* was formed exclusively from compound *A* or not. The contribution of compound *A* to the formation of compound *B* can be assessed from the following equation:

$$\% \text{ conversion} = 100 - [(|\text{exp}-QB_X|/\text{exp})100] \qquad (2)$$

where % conversion = maximum possible % conversion of compound *A* to compound *B* during any time interval X; exp = observed ^{14}C-incorporation into compound *B* by the end of time interval X, as determined experimentally; QB_X = expected ^{14}C-incorporation into compound *B* by the end of time interval X; $|exp-QB_X|$ = absolute difference between the experimental and theoretical ^{14}C-incorporation of precursor *P* into compound *B* during time interval X. The above approach was successfully used in demonstrating precursor product relationships between DV and MV Pchlide *a* (Tripathy and Rebeiz 1988).

Eq. (2) shows that if the expected and observed (*i.e.*, experimental) radiolabel incorporation into compound *B* are identical or very similar, then the % conversion of compound *A* into compound *B* is 100 % or very close to 100 %. On the other hand, if the observed radiolabel incorporation into compound *B* is higher than the theoretical one, then the % conversion of compound *A* into compound *B* would fall between zero and 100 %, and the balance may be attributed to biosynthesis of compound *B* *via* other biosynthetic routes. When compound *B* is not an end product but a transient metabolite, the observed label incorporation into compound *B* is likely to be lower than the expected label incorporation. In that case Eqs. (1) and (2) cannot be used.

Results

Experimental strategy: If MV Chl *b* was formed from MV Chl *a* or MV Chlide *a* *via* a simple linear biosynthetic route, then this should be readily confirmed by precursor-product relationship investigations *in vivo* between MV Chl *a* and MV Chl *b*, and MV Chlide *a* and MV Chl *b*, in the various greening groups of plants. Therefore *in vivo* precursor-product investigations were carried out using cucumber cotyledons, a dark (D) DV-light (L) DV-DLDV plant tissue, and maize, a DMV-LDV-DLMV plant tissue (Adb El Mageed *et al.* 1997).

Precursor-product relationships between MV Chl *a* and MV Chl *b* in greening maize and cucumber seedlings: If MV Chl *b* is formed exclusively from MV Chl *a*, then the expected and observed ^{14}C-incorporations into MV Chl *b* should be identical or very similar. On the other hand, if the observed ^{14}C-incorporations into MV Chl *b* are much higher than the expected ones, this would strongly suggest that MV Chl *b* is not formed from MV Chl *a*, but is formed *via* other biosynthetic routes. According to Fig. 2, MV Chl *b* may be formed *via* routes 1-6 in greening DMV-LDV-LDMV plant species such as maize, which possess [4-vinyl] Mg-protoporphyrin IX reductase (4VMPR), and [4-vinyl] Pchlide *a* reductase (4VpideR) activities, but lack [4-vinyl] Chlide *a* reductase (4VCR) activity (Tripathy and Rebeiz 1988, Kim and Rebeiz 1996, Adb El Mageed *et al.* 1997), and [4-vinyl] Chl *a* reductase (4VChlR) activity (Rebeiz, unpublished). In DDV-LDV-LDDV plant species such as cucumber, which do not possess 4VMPR activity but have instead 4PideR and 4VCR and 4ChlR activities (Tripathy and Rebeiz 1988, Kim and Rebeiz 1996, Adb El Mageed *et al.*

1997), MV Chl *a* can be converted to MV Chl *b* *via* routes 6, 7, and 8. Monovinyl Chl *b* may also be formed from MV Chlide *a* *via* routes 4 and 5.

Table 1. Precursor-product relationships between MV Chl *a* and MV Chl *b* in greening maize seedlings. Three-grams batches of cut etiolated maize leaves, about 1 cm in length, were incubated in 10 cm^3 of distilled water in deep Petri dishes, 9 cm diameter, under the low green irradiance. 185 MBq of ^{14}C-ALA were added to each Petri dish. The Petri dishes were incubated at 28 °C in darkness for 15 h. After dark-incubation, the radioactively labeled etiolated cucumber and maize tissues were irradiated (six 1000-W metal halide lamps, irradiance 120 μmol m^{-2} s^{-1}, 27 °C). At the indicated incubation time, tissue samples were homogenized prior to pigment extraction and determination of expected and observed ^{14}C-incorporations into MV Chl *b* [disintegrations per s per g of tissue].

Experiment	Incubation with ^{14}C-ALA [h]	^{14}C-incorporation into MV Chl *b* expected	observed	Maximum possible % conversion of MV Chl *a* to Chl *b*
A	0	0	0	0
	5	99	6078	1.6
	7	430	9499	3.1
B	0	0	0	0
	5	82	4905	1.7
	7	289	5448	5.3

The expected and observed ^{14}C-ALA incorporations into MV Chl *b* in maize seedlings was extremely dissimilar, during the 7-h irradiation (Table 1). The extremely high ^{14}C-ALA incorporation into MV Chl *b* suggested that MV Chl *b* was not formed from MV Chl *a* *via* routes 1 and 6, but was probably formed *via* other biosynthetic routes such as routes 2-5 (Fig. 2).

In cotyledons of cucumber, a DDV-LDV-LDDV plant species, conversion of MV Chl *a* to MV Chl *b* proceeded briskly, as evidenced by the extent of expected and observed ^{14}C-ALA incorporations into MV Chl *b* (Table 2). The *in vivo* precursor-product technique used in this work cannot differentiate between multiple routes such as routes 6, 7, and 8. In other words, the observed conversion of MV Chl *a* to MV Chl *b* in cucumber may have proceeded *via* one, two, or the three routes depicted in Fig. 2. The possible reverse reaction, *i.e.*, conversion of MV Chl *b* to MV Chl *a* (Ohtsuka *et al.* 1997) was probably not strong enough to obscure the conversion of MV Chl *a* to MV Chl *b*. However, since the expected and observed ^{14}C-incorporations into MV Chl *b* were not identical or nearly identical, it can be concluded that MV Chl *b* was also formed *via* other biosynthetic routes which did not involve MV Chl *a*, such as routes 4 and 5. These results reinforce the notion that MV Chl *b* formation is not a simple linear process and proceeds differently in different greening groups of plants.

Precursor-product relationships between MV Chlide *a* and MV Chl *b* in greening maize seedlings: Since green maize leaves contain abundant amounts of MV Chl *b*, the latter must be formed *via* alternate routes not involving MV Chl *a* such as routes 2, 3, and 4 (Fig. 2). The possible operation of route 2 was probed by investigating

possible precursor-product relationships between MV Chlide *a* and MV Chl *b*. Although MV Chlide *b* is a transient intermediate, MV Chl *b* is an end product. If the conversion of MV Chlide *a* to MV Chlide *b* were very rapid, then precursor-product investigations *in vivo* should show significant conversion of MV Chlide *a*

Table 2. Precursor-product relationships between MV Chl *a* and MV Chl *b* in greening cucumber seedlings [disintegrations per s per g of tissue]. Etiolated cucumber cotyledons were excised and treated as described in Table 1 for maize leaves.

Experiment	Incubation with ^{14}C-ALA [h]	^{14}C-incorporation into MV Chl *b* expected	observed	Maximum possible % conversion of MV Chl *a* to Chl *b*
A	0	0	0	0
	5	21	80	27
	7	57	123	72
B	0	0	0	0
	5	38	57	67
	7	42	53	80
C	0	0	0	0
	4	1822	3903	47
	6	3618	2206	36
	8	1030	1515	68
D	0	0	0	0
	4	529	1701	31
	6	1004	800	75
	8	272	466	58
E	0	0	0	0
	4	710	1813	39
	6	1279	724	23
	8	246	437	56

Table 3. Precursor-product relationships between MV Chlide *a* and MV Chl *b* in greening maize seedlings [disintegrations per s per g of tissue]. Etiolated maize leaves were excised and treated as described in Table 1.

Experiment	Incubation with ^{14}C-ALA [h]	^{14}C-incorporation into MV Chl *b* expected	observed	Maximum possible % conversion of MV Chl *a* to Chl *b*
A	0	0	0	0
	5	2160	6078	36
	7	5729	13727	60
B	0	0	0	0
	5	1331	4905	27
	7	2807	8446	52

into MV Chl *b*; since the esterification of MV Chlide *b* to MV Chl *b* proceeds very rapidly *in vivo*. The *in vivo* precursor-product results (Table 3) supported a partial (27-60 %) conversion of MV Chlide *a* to MV Chl *b*. The magnitude of this conversion leaves enough room for the possible involvement of routes 3 and 4 in MV Chl *b* formation in greening maize seedlings (Fig. 2).

Discussion

It is unlikely that all the proposed reactions depicted in Fig. 2 may be found in a single plant species at all stages of greening. Although MV Chl *a* biosynthesis can proceed *via* routes 1-9 in etiolated plant species of all greening groups (Adb El Mageed *et al.* 1997), MV Chl *b* biosynthesis needs potentiation by light before taking off (Rebeiz 1967). On the basis of the *in vivo* precursor-product relationships reported in this work, MV Chl *b* is unlikely to be formed from MV Chl *a* in greening DMV-LDV-LDMV plant species such as maize and barley (Table 1). Also routes 2 and 3 (Fig. 2), are likely to be most active in greening DMV-LDV-LDMV plant species, which possess the 4VMPR activity. Indeed, MV Pchlide *b* biosynthesis does not take place in etiolated tissues (Ioannides *et al.* 1997), and 4VMPR activity has not yet been detected in DDV-LDV-LDDV plant species, such as cucumber (Rebeiz, unpublished). On the other hand, routes 4, 5, and 6 are likely to be active in DDV-LDV-LDDV plant species, following prolonged exposure to darkness as at daybreak, since in these plant species 4VpideR activity is only expressed after prolonged exposure to darkness (Tripathy and Rebeiz 1988). Also route 7 is likely to be active in DDV-LDV-LDDV plant species, but not in greening DMV-LDV-LDMV plant species, which lack 4VCR activity (Adb El Mageed *et al.* 1997). Finally, DV Chl *b* formation from DV Chlide *b* (route 11) and from DV Chl *a* (route 10) can only take place in the Nec2 mutant and the picoplankton of the euphotic zone of the world tropical and temperate oceans, and the Mediterranean Sea, where DV Chl *a* and *b* are the predominant Chl (Brereton *et al.* 1983, Wu and Rebeiz 1985, Chisholm *et al.* 1988, 1992, Veldhuis and Kraay 1990, Goericke and Repeta 1992).

The possible operation of multiple Chl *a*/*b* biosynthetic routes has far reaching implications for the understanding of the development of structural heterogeneity of photosynthetic membranes. It is presently acknowledged that thylakoid membranes consist of many apoproteins some of which bind MV Chl *a*, while others bind MV Chl *a* and MV Chl *b*. A description of the various thylakoid apoproteins can be found in Sundqvist and Ryberg (1993), as well as on our menu-driven web site devoted to the chemistry and biochemistry of greening (http://w3.aces.uiuc.edu/nres/lppbp).

At this stage, it is extremely difficult to reconcile the operation of a single-branched Chl *a*/*b* biosynthetic pathway with the structural and biosynthetic complexity of photosynthetic membranes. On the other hand, the proposed unified multibranched Chl *a*/*b* biosynthetic pathway possesses the functional flexibility to account for the assembly of highly complex thylakoid membranes. The unified multibranched Chl *a*/*b* biosynthetic pathway (Fig. 2) may be visualized as the template of a Chl-protein biosynthesis center, as visualized by Shlyk *et al.* (1978),

where the assembly of PS1, PS2, and LHC may take place. The multiple Chl biosynthetic routes may be considered, individually or in-group of two or three adjacent routes, to constitute Chl-apoprotein biosynthesis subcenters earmarked for the coordinated assembly of individual pigment-protein complexes. Apoproteins destined to some of the biosynthesis subcenters may possess specific signals for specific Chl biosynthetic enzymes peculiar to that subcenter, such as 4-vinyl reductases, formyl synthetases, or Chl *a* and Chl *b* synthetases (Rüdiger 1993).

Once an apoprotein formed in the cytoplasm or in the plastid reaches its biosynthesis subcenter destination, and its signal is split off, it may bind nascent Chl formed *via* one or more biosynthetic routes. During Chl binding, it may fold properly and act, at that location, as a template for other thylakoid apoproteins having appropriate signals. It is even feasible to visualize how certain biosynthesis subcenters are capable of Chl *a* and *b* formation to meet the demand of Chl *a/b* binding apoproteins, while other centers are only capable of Chl *a* formation as in routes 1 and 6, in greening DMV-LDV-DLMV plant species such as maize (Fig. 2).

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