Origin of Chlorophyll *a* Biosynthetic Heterogeneity in Higher Plants

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Abstract: In this study, the origin of the monovinyl chlorophyll *a* carboxylic biosynthetic route was investigated in barley (*Hordeum vulgare* L.) and corn (*Zea mays* L.). Protoporphyrin IX accumulated *in vivo* or *in vitro* was found to be all of the divinyl form. Furthermore, the conversion of divinyl protoporphyrin IX to monovinyl protoporphyrin IX *in vitro* was not observed. In contrast, the biosynthesis and accumulation of monovinyl Mg-protoporphyrin IX and its methyl ester occurred in etiolated leaves and divinyl Mg-protoporphyrin IX was convertible to monovinyl Mg-protoporphyrin IX *in vitro*. These results suggest that the monovinyl chlorophyll *a* carboxylic biosynthetic route in plants may originate from the divinyl Mg-protoporphyrin IX pool.

Key words: chlorophyll biosynthesis, divinyl, monovinyl, Mg-protoporphyrin IX, protoporphyrin IX.

Biosynthetic heterogeneity refers to the biosynthesis of a particular metabolite by an organelle, tissue, or organism *via* multiple biosynthetic routes (Arigoni, 1994). It has well been documented in the biosyntheses of δ-aminolevulinic acid (ALA), chlorophyll (Chl) *a*, and vitamin B_{12} (Klein and Senger, 1978; Rebeiz *et al.*, 1994; Scott, 1994). In green plants Chl *a* is formed *via* two parallel biosynthetic routes; the divinyl (DV) and monovinyl (MV) carboxylic Chl *a* routes (Leeper, 1991; Richards, 1993; Rebeiz *et al.*, 1994) (Fig. 1). The intermediates of the DV carboxylic route have two vinyl groups, one at position 2 and the other at position 4 of the macrocycle (Fig. 2). In contrast, the intermediates of the MV carboxylic route have one vinyl and one ethyl group at positions 2 and 4 of the macrocycle, respectively (Fig. 2). In higher plants, the end product of the Chl *a* biosynthetic heterogeneity is invariably MV Chl *a* and *b*, with the only known exception of a lethal maize mutant (Bazzaz, 1981) which forms only DV Chl *a* and *b*. In the phytoplankton of tropical oceans, however, DV Chl *a* and *b* are the predominant Chl species (Chisholm *et al.*, 1990, 1992; Veldhuis and Kraay, 1990; Goericke and Repeta, 1992).

As shown in Fig. 1, the two routes are assumed to be linked at the several levels of the Chl *a* biosynthetic pathway. Under a dark condition, DV protochlorophyllide (Pchlide) *a* and DV chlorophyllide (Chlide) *a* in barley and cucumber are converted to MV Pchlide and MV Chlide, respectively, by [4-vinyl] reductase(s) that converts the 4-vinyl group at position 4 to ethyl (Tripathy and Rebeiz, 1988; Parham and Rebeiz, 1992). DV Mg-protoporphyrin IX methylester (MPE) could also be converted to MV MPE by an NADH-dependent enzyme (Ellsworth and Hsing, 1974). Tripathy and Rebeiz (1986) reported that in both barley and cucumber etiochloroplasts. ALA was converted to both MV and DV protoporphyrin IX (Proto) in darkness. Recently, Suzuki and Bauer (1995) reported that *Rhodobacter capsulatus* strains which are unable to reduce Pchlide *a* to Chlide *a* (such as *bchL*, *B*, or *N* mutants) accumulated a mixed pool of DV and MV Pchlide *a*.

Furthermore, the introduction of a second *bchj* mutation in these strains, which was assumed to block the conversion of DV Pchlide *a* to MV Pchlide *a*, also resulted in the accumulation of MV Pchlide *a*. The above results suggest that formation of MV tetrapyrroles is linked at earlier steps of chlorophyll biosynthesis, presumably at the level of Proto, Mg-Proto, or MPE, and one or more 4-vinyl reductases may be involved in the reactions (Suzuki and Bauer, 1995). However, the origin of Chl *a* biosynthetic heterogeneity in higher plants is still uncertain.

In this work, we demonstrate that the Chl *a* biosynthetic heterogeneity in higher plants may originate at the level of DV Mg-Proto, and would be mediated by the activity of a putative [4-vinyl] Mg-Proto reductase (4VMPR) activity.

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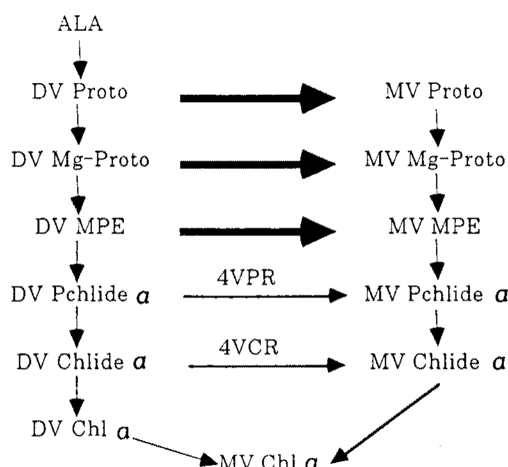
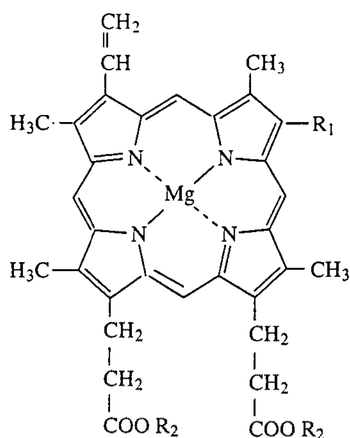


Fig. 1. The MV and DV monocarboxylic routes of Chl *a* biosynthesis in green plants. Arrows joining the DV and MV branches indicate the reactions catalyzed by [4-vinyl] reductases. Bold arrows point to putative reactions. The figure was adapted from Rebeiz *et al.* (1994).



R_1	R_2	Compound
CH_2-CH_3	H	MV Mg-Proto
$CH=CH_2$	H	DV Mg-Proto
CH_2-CH_3	CH_3	MV MPE
$CH=CH_2$	CH_3	DV MPE

Fig. 2. Chemical structures of Mg-protoporphyrins.

Materials and Methods

Plant materials

Barley (*Hordeum vulgare* L. cv. Robust) seeds were germinated in moist vermiculite, in darkness at 28℃ for 5~6 days. The etiolated leaves were used for the experiments of plastid isolation and of tetrapyrrole accumulation upon dark incubation with modulators of the Chl biosynthesis (See "*in vivo* incubation" below).

Seeds of Orobanche 1 (Oro 1) and Luteus 13 mutant corn (*Zea mays* L.), which are known to be MPE cyclase- and Mg-Proto chelatase-deficient mutant, respectively, were also germinated in moist vermiculite under a 14 h light/10 h dark photoperiod at 28℃ for 10~14 days. Light intensity was as low as about 30 $\mu W\ cm^{-2}$ to avoid any necrosis on mutant leaves. Metal halide lamps were used as a light source. Only yellow plants were allowed to grow and used as plant material for the tetrapyrrole accumulation experiments. The other plants which turned green, resulting from an incomplete mutation, were removed as soon as they had developed. The mutant seeds were obtained from the Maize Genetic Stock Center at the University of Illinois, Urbana, USA.

Chemicals

DV Proto and DV Mg-Proto were purchased from Porphyrin Products (Logan, USA) and ALA from Biosynth. (Naperville, USA). Picolinic acid (PA) was purchased from Sigma (St Louis, USA) and 1,10-phenanthroline (Oph) from Aldrich (Milwaukee, USA).

In vivo incubation

The etiolated barley and corn leaves (about 0.5 g fresh weight) were cut out and incubated in a deep Petri dish (10×8 cm) in 10 ml of solution containing various concentrations of ALA and modulators of the Chl *a* biosynthetic pathway such as PA or Oph. Unless otherwise indicated, the incubation was carried out in darkness at 28℃ for 14 h.

Preparation of etiochloroplasts and plastid membranes

All procedures were carried out under subdued laboratory light (80 $\mu W\ cm^{-2}$). Twenty to thirty grams of etiolated barley tissue were hand-ground in a cold ceramic mortar containing 90 ml of homogenization buffer (pH 7.8). The homogenization buffer consisted of 500 mM sucrose, 15 mM Hepes, 30 mM Tes, 1 mM $MgCl_2$, 1 mM EDTA, 0.2% (w/v) BSA, and 5 mM cysteine. The homogenate was filtered through two layers of Miracloth and was centrifuged at 200 g for 5 min in a Beckman JA-20 rotor at 1℃. The supernatant was decanted and centrifuged at 1,500 g for 10 min at 1℃. The pelleted crude etiochloroplasts were gently resuspended in incubation medium using a small paintbrush. For further plastid purification, the pelleted crude etiochloroplasts were resuspended in 5 ml of homogenization buffer and were purified by Percoll density centrifugation (Lee *et al.*, 1991). The pelleted, Percoll-purified etiochloroplasts were resuspended in incubation medium. The incubation medium consisted of 500 mM sucrose, 200 mM Tris, 2 mM $MgCl_2$, 2.5 mM EDTA, 1.25 mM methanol, 0.15% (w/v) BSA, 20 mM ATP, 8 mM methionine, and where indicated 1.5 mM NA-

DPH and/or 40 mM NAD^+. The pH was adjusted to 7.8 with HCl.

For preparation of plastid membranes, the pelleted etiochloroplasts were resuspended in 8 ml of lysing buffer. The lysing buffer consisted of 25 mM Tris, 3 mM $MgCl_2$, 2.5 mM EDTA, and 0.1% (w/v) BSA at room temperature, pH 7.8. The lysed plastid suspension was centrifuged at 235,000 g for 1 h in a Beckman 80 Ti rotor at 1℃ (Lee *et al.*, 1991). The soluble stromal fraction was decanted and the pelleted membranes were resuspended in the incubation medium without sucrose.

Conversion of ALA to Proto, DV Proto to MV Proto, and DV Mg-Proto to MV Mg-Proto by etiochloroplasts or membranes

Conversion of ALA to Proto was carried out in a flat-bottomed tube (2×6.5 cm) containing 0.33 ml of the suspended plastids (about 0.9 mg protein), 0.33 ml of additional incubation medium (see above), and 0.30 ml of distilled water. Since in the absence of ATP, added ALA is converted to Proto without any significant accumulation of MP(E) and Pchlide *a* (Tripathy and Rebeiz, 1986; Lee *et al.*, 1991), ATP was omitted from the incubation medium. In order to photoreduce any Pchlide accumulated during the plastid preparation, the incubation mixture was exposed to white fluorescent light (320 $\mu W\ cm^{-2}$) at 30℃ for 3 min prior to the addition of substrate. After adding 33 μl of 30.3 mM ALA, the reaction mixture was incubated in an oscillating water bath (50 oscillations/min) at 30℃ in darkness or in the light (320 $\mu W\ cm^{-2}$) for 2 h. The reaction was terminated by adding 10 ml acetone : 0.1 N NH_4OH (9 : 1, v/v).

Conversion of DV Proto to MV Proto and DV Mg-Proto to MV Mg-Proto by etiochloroplasts or membranes were conducted in the same way as in the experiment for conversion of ALA to Proto, except that DV Proto or DV Mg-Proto was supplied as a substrate.

Extraction and separation of tetrapyrroles

All procedures were conducted under a dim, green light source. The incubated tissues were homogenized in acetone : 0.1 N NH_4OH (9 : 1, v/v) with a Brinkman Polytron at a speed setting of 8 for 40 s using a fresh weight : volume ratio of 1 : 10. The homogenate was centrifuged at 39,000×*g* at 1℃ for 12 min and the resulting supernatant stored at −85℃ until tetrapyrrole extraction. Samples from *in vitro* incubation experiments were directly subjected to the centrifugation. Chl and other fully esterified tetrapyrroles were transferred from acetone to hexane by extraction with an equal volume of hexane, followed by a second extraction with 1/3 volume of hexane. The remaining hexane-extracted acetone residue which contained monocarboxylic tetrapyrroles such as Chlide, Pchlide, and MPE, and dicarboxylic tetrapyrroles such as Proto and Mg-Proto, was used for the determination of Proto, MP(E) (a mixture of MPE and Mg-Proto), and Pchlide *a* by spectrofluorometry at room temperature (Rebeiz *et al.*, 1975; Smith and Rebeiz, 1977).

To separate MPE and Mg-Proto in the MP(E) extracted from incubated tissues, the hexane-extracted acetone residue was chromatographed on thin layers of Silica gel H developed in toluene : ethyl acetate : ethanol (8 : 2 : 2, v/v/v) in darkness at 4℃ (Belanger and Rebeiz, 1982). In this solvent the MPE migrated with an Rf of about 0.5, while Mg-Proto did so with an Rf of about 0.09. Each pool was eluted in methanol : acetone (4 : 1, v/v), was dried under nitrogen gas, and then dissolved in 80% acetone for spectrofluorometric analysis at room temperature.

DV MPE from MV MPE was separated by chromatography on thin layers of polyethylene (Belanger and Rebeiz, 1982). The plates were developed in acetone : water (9 : 1, v/v) in darkness at room temperature. MV and DV MPE migrated with respective Rfs of about 0.84 and 0.67. They were eluted separately into ether, centrifuged, and the ethereal solution was used for spectrofluorometric characteristics at 77°K (Tripathy and Rebeiz, 1985).

Quantitative determination of tetrapyrroles

The total amount of each tetrapyrrole was determined by spectrofluorometry at room temperature (Rebeiz *et al.*, 1975; Smith and Rebeiz, 1977; Belanger and Rebeiz, 1982). The amounts of MV and DV components in each monocarboxylic or dicarboxylic tetrapyrrole pool were calculated from the total amount of tetrapyrroles and from the proportions of MV and DV components determined at 77°K in diethyl ether (Tripathy and Rebeiz, 1985).

For the determination of the MV and DV proportions of tetrapyrroles, the hexane-extracted acetone fraction was further extracted with diethyl ether. Monocarboxylic tetrapyrroles were first extracted into diethyl ether by adding 1/5 volume of diethyl ether, 1/17 volume of saturated NaCl, and 1/70 volume of 0.37 M KH_2PO_4 (pH 7.0) to the hexane-extracted acetone fraction. The mixture was throughly mixed and the phases were separated by a centrifugation at 1,500×*g* for 30 s at room temperature. The ether phase was collected with a Pasteur pipette and the ether-extracted aqueous residue was re-extracted 1∼5 times with a small volume of diethyl ether. The collected ether extracts were washed by passing through 2 mM NH_4OH solution and then

used for the determination of the MV and DV proportions of MP(E) (Tripathy and Rebeiz, 1985, 1986). The remaining ether-extracted acetone phase contained mostly dicarboxylic tetrapyrroles such as Proto and/or Mg-Proto. To extract them into diethyl ether, the pH of the acetone residue was adjusted to 4.0 with 1 N HCl, and extracted with diethyl ether as described above. The ether extracts were washed by passing through a 0.5 M solution of potassium phosphate buffer (pH 4.8), and then used for the determination of the MV and DV proportions of Proto and/or Mg-Proto.

Since limited amounts of Mg-Proto were formed during *in vivo* incubation, however, the proportions of MV and DV Mg-Proto were determined by 77°K spectrofluorometry from the mixture of MV and DV Mg-Proto pool after methylation (Tripathy and Rebeiz, 1985). The methylation was achieved by adding excess of diazomethane in ether. The ethereal solution of methylated Mg-Proto was completely dried under nitrogen gas and redissolved in diethyl ether for spectrofluorometric analysis at 77°K. The MV/DV ratio was not changed during the methylation (Kim and Rebeiz, 1995).

Determination of the precise proportion of MV and DV Proto with optical electronic spectroscopy has not been successful even under the 77°K condition. This is because the fluorescence emission and excitation maxima of MV and DV Proto are not sufficiently separated, and their Soret excitation bands are rather broad (Rebeiz *et al.*, 1975b). Thus Proto was chemically converted to MPE as described previously (Kim and Rebeiz, 1995) and the proportion of MV and DV of the converted MPE was determined by using typical 77°K spectrofluorometry. The proportion of MV and DV Proto was then indirectly calculated with the equation represented by Belanger and Rebeiz (1982).

Spectrofluorometry

Fluorescence spectra were recorded on a fully corrected photon-counting, high-resolution SLM spectrofluorometer Model 8000C interfaced with an IBM Value Line microcomputer. Determination at room temperature was performed on an aliquot of the hexane-extracted acetone fraction in cylindrical microcells (3 mm in diameter) at emission and excitation bandwidths of 4 nm. Fluorescence spectra at 77°K were recorded at emission and excitation bandwidths that varied from 0.5 to 4 nm depending on signal intensity (Tripathy and Rebeiz, 1985). The photon count was integrated for 0.5 s at each 1 nm increment. For the determination of the proportions of MV and DV Mg-Proto, the photon count was integrated for 2 s at each 1 nm increment.

Protein determination

Protein was determined by the bicinchoninic acid method on an aliquot of the plastidic preparation after delipidation (Smith *et al.*, 1985).

Results

Experimental strategy

In order to investigate the biochemical origin of the MV Chl *a* biosynthetic route, we designed three step experiment. This involved (a) detection of MV Mg-Proto and MV MPE from higher plant tissues, (b) *in vivo* and *in vitro* experiments to investigate whether MV Proto is acutually formed, (c) *in vitro* demonstration of conversion of DV Mg-Proto to MV Mg-Proto. To this end, the modified tetrapyrrole extraction techniques described in Materials and Methods and the improved analysis for determining MV and DV Proto (Kim and Rebeiz, 1995) were used.

Detection of MV Mg-Proto and MV MPE accumulation in etiolated corn and barley leaves

The biosynthesis and accumulation of MV MP(E) has so far been documented mainly in lower plants such as *Euglena* and in dicotyledonous plant species such as cucumber (*Cucumis sativus* L.), red kidney bean (*Phaseolus vulgaris* L.), and a hosta mutant (*Hosta sieboldii* AUOP-15) (Belanger and Rebeiz, 1982). In this work, we investigated whether Mg-Proto and MPE in monocotyledonous plant species such as barley and corn are also heterogeneous. For this experiment, a treatment that causes MP(E) accumulation is necessary, since MP(E) is rapidly converted to Pchlide under normal conditions. For MP(E) accumulation, a mutant which lacks MPE cyclase can be used as plant material or MPE cyclase inhibitors such as Oph and 2,2′-dipyridyl (Dpy) can be applied to a normal plant. Moreover, MP(E) would significantly accumulate, if exogenous ALA is supplied as a substrate for tetrapyrrole biosynthesis. As described in Table 1, etiolated leaves incubated with ALA (corn mutant Oro 1) or with ALA+Oph (barley) accumulated detectable amounts of MV and DV MP(E). To know whether the MP(E) contained MV Mg-Proto, the MP(E) was chromatographed on thin layers of Silica gel H and polyethylene as described in Materials and Methods. This showed that 35% and 59 % of the detected Mg-Proto and MPE, respectively, was in MV from in Oro 1 leaves. Also in etiolated barley leaves incubated with ALA+Oph, 47% and 21% of the detected Mg-Proto and MPE was in MV form, respectively. These results show that Mg-Proto as well as MPE are heterogeneous in both plant species. MV Mg-

Table 1. Accumulation of MV and DV MP(E) in etiolated barley and corn mutant leaves treated with ALA or ALA+Oph

Plant	Treatment[a]	MV MP(E)[b]	DV MP(E)	MV Mg-Proto	DV Mg-Proto	MV MPE	DV MPE
				(nmol/g fresh weight)			
Barley	1 mM ALA	0.75	3.44	nd[c]	nd	nd	nd
	1 mM ALA+0.5 mM Oph	8.41	30.72	0.14	0.16	8.27	30.56
Corn (Oro 1)	none	0.96	0.54	nd	nd	nd	nd
	0.5 mM ALA	47.47	34.93	2.07	3.77	45.40	31.16

[a]Etiolated barley or Oro 1 leaf sections (about 0.5 g fresh weight) were incubated in a deep Petri dish (10×8 cm) with 10 ml of solution containing the indicated concentrations of ALA and Oph. Incubation was carried out in darkness at 28℃ for 14 h.
[b]MP(E): a mixture of Mg-Proto and MPE.
[c]nd: not determined.

Table 2. Accumulation of MV and DV Proto and MP(E) in etiolated barley and corn mutant leaf sections treated with ALA or ALA+PA

Plant	Treatment[a]	MV Proto	DV Proto	MV MP(E)[b]	DV MP(E)
		(nmol/g fresh weight)			
Barley	40 mM ALA	1.87	12.06	4.41	69.15
	40 mM ALA+ 30 mM PA	1.26	156.68	20.28	200.14
Corn[c]	5 mM ALA	0.00	92.03	0.91	3.20

[a]Five- to six-day-old etiolated barley or corn mutant leaves were incubated with the indicated chemicals for 14 h in darkness at 28℃.
[b]MP(E): a mixture of Mg-Proto and MPE.
[c]Luteus 13.

Proto was also detected in etiolated cucumber cotyledons incubated with ALA+Dpy (Belanger and Rebeiz, 1982).

MV MP(E) is not likely to have originated at the level of Proto in etiolated barley and corn leaves

If the accumulated MV MP(E) was formed from MV Proto, the latter might be detected in samples accumulating large amounts of Proto. Induction of Proto and MP(E) accumulation were achieved by treating etiolated barley leaves with ALA and PA. PA has been shown to induce the accumulation of Proto as well as MV and DV MP(E) in plants (Mayasich *et al.*, 1989; Nandihalli and Rebeiz, 1991). Although considerable amounts of MV and DV MP(E), and DV Proto were formed in etiolated barley leaves treated with ALA or ALA and PA, MV Proto accumulation was negligible (Table 2, Fig. 3). This in turn indicates either that MV Proto is not a precursor of the accumulated MV MP(E) or that MV Proto did not accumulate in the treated tissues, presumably because it was preferentially converted to MV MP(E).

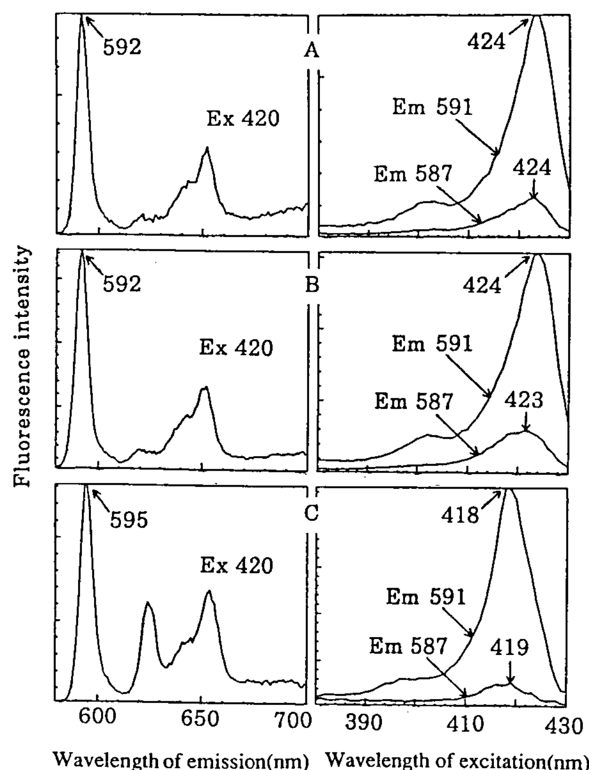


Fig. 3. Fluorescence emission and excitation spectra in ether at 77°K after Mg^{+2} insertion into standard DV Proto (A), purified (B) or unpurified Proto sample (C) extracted from barley etiolated leaves. The unpurified Proto sample was contaminated with Pchlide and/or Chlide.

To differentiate between the two above possibilities, the biosynthesis and accumulation of Proto without significant MP(E) formation was necessary to be induced. It would be expected that if MV Proto is a precursor of MV MP(E), MV Proto as well as DV Proto will be accumulated under a certain condition. For this, both *in vivo* and *in vitro* experiments were designed. For the *in vivo* experiment, Proto accumulation in the absence of significant MP(E) was induced by incubating

Table 3. Accumulation of Proto by barley etiochloroplasts incubated with ALA in a fortified medium

Experiment	Incubation condition[a]	MV Proto	DV Proto	MP(E)[b]	Pchlide
		(nmol/100 mg protein)			
A	0 h	0.06	0.33	3.74	22.36
	2 h, dark	0.93	45.45	5.72	24.50
	2 h, light	0.18	59.52	4.88	19.36
B	2 h, dark	0.00	64.06	4.30	10.27
	2 h, dark+ 1 mM NADPH	0.00	72.96	4.46	11.51

[a] Etiochloroplasts were isolated from etiolated barley leaves as described in Methods without further purification using Percoll. ATP were omitted from the incubation medium. All assays contained 40 mM NAD^+.
[b] MP(E): a mixture of Mg-Proto and MPE.

Table 4. Lack of conversion of DV Proto to MV Proto by barley etiochloroplasts

Enzyme source	Incubation time (min)[a]	MV Proto detected (nmol/100 mg protein)
Etiochloroplasts	0	1.84
	5	1.40
	20	0.83
	60	0.46

[a] Etiochloroplasts from etiolated barley leaves were incubated with DV Proto (66.66 nmol/100 mg protein), as described in Materials and Methods. All assays contained 1 mM NADPH and 40 mM NAD^+.

Table 5. Conversion of DV Mg-Proto to MV Mg-Proto by various barley etiochloroplast preparations

Enzyme source	Incubation time (min)[c]	MV Mg-Proto detected (nmol/100 mg protein)
Etiochloroplasts[a]	0	1.66
	30	9.16
Membranes[b]	0	2.85
	60	14.11

[a] Etiochloroplasts were purified by differential and Percoll density centrifugation.
[b] Membranes were prepared from crude etiochloroplasts after lysis in lysing medium containing 1 mM NADPH.
[c] Etiochloroplasts or membranes were incubated with DV Mg-Proto of 330 and 470 nmol/100 mg protein, respectively, in darkness as described in Materials and Methods. All assays contained 1 mM NADPH.

the leaves of Luteus 13 corn, a Mg-Proto chelatase-deficient mutant, with ALA. After the incubation, only the DV form was detected (Table 2). When barley etiochloroplasts were incubated with ALA in a cofactor-fortified medium without ATP, Proto accumulated without any significant accumulation of MP(E) and Pchlide *a* (Table 3). Similar observations have previously been made (Tripathy and Rebeiz, 1986; Lee *et al.*, 1991). ATP is known to be required for the insertion of Mg^{+2} into Proto (Chastelfranco *et al.*, 1979; Lee *et al.*, 1992). As shown in Table 3, large amount of Proto accumulated with an almost exclusive DV form. Addition of a hydrogen donor such as NADPH to the incubation medium did not boost MV Proto accumulation (Table 3). These results raised further doubt about the possible involvement of MV Proto in the biosynthesis of MV MP(E).

The possibility that MV Proto is formed from DV Proto by a putative [4-vinyl] Proto reductase was also examined. The enzyme, if exists, would convert DV Proto to MV Proto. However, DV Proto was not converted to MV Proto even with the addition of NADPH (Table 4).

Since an enzyme capable of converting DV Proto to MV Proto was not detected in the *in vitro* experiment and MV Proto was not detected in the *in vivo* incubation, it is concluded that MV MP(E) seems not to be formed from MV Proto in etiolated barley and corn plants. Rather, the origin of MV MP(E) might be MV Mg-Proto.

Conversion of DV Mg-Proto to MV Mg-Proto in barley etiochloroplasts

Another possible biosynthetic source of MV Mg-Proto is DV Mg-Proto. DV Mg-Proto might be converted to MV Mg-Proto by the action of a [4-vinyl] reductase. The possibility that the enzyme could reduce the vinyl group at position 4 of the DV Mg-Proto macrocycle was examined by incubating barley etiochloroplasts preparations with exogenous DV Mg-Proto in a cofactor-fortified medium which is the medium for converting DV Mg-Proto to MV MPE and MV Pchlide *a in vitro* (Tripathy and Rebeiz, 1986). Formation of MV Mg-Proto from DV Mg-Proto in barley etiochloroplast and etiochloroplast membranes was monitored spectrofluorometrically at 77°K (Table 5). A small amount of MV Mg-Proto was formed after the dark incubation. When the boiled etiochloroplasts were used, no conversion of exogenous DV Mg-Proto to MV Mg-Proto was observed (data not shown).

Discussion

In green plants two parallel biosynthetic routes of Chl *a* are assumed to be linked at the several levels of the Chl *a* biosynthetic pathway (Parham and Rebeiz, 1992; Richards, 1993; Rebeiz *et al.*, 1994). However,

the origin of the MV and DV Chl *a* biosynthetic routes still remains to be determined unequivocally. On the basis of the detection of putative MV Proto, the origin of the MV and DV Chl *a* biosynthetic carboxylic routes was at first assigned to the level of coproporphyrinogen III or protoporphyrinogen IX (Rebeiz *et al.*, 1981, 1983). Because of the failure to demonstrate the conversion of coproporphyrinogen III or DV protoporphyrinogen IX to MV Proto *in vitro* (C. A. Rebeiz, unpublished) and the demonstrated conversion of exogenous MV Proto to MV MP(E) and MV Pchlide *a in organello* (Tripathy and Rebeiz, 1986), recent assignments were at the level of DV Proto (Rebeiz *et al.*, 1994). However, our results do not support any of those assignments. It was not possible to induce the biosynthesis of MV Proto either *in vivo* or *in vitro* at rates that could account for the formation of MV MP(E) and MV Pchlide *a* (Table 2, 3). Furthermore, it was not possible to demonstrate the conversion of DV Proto to MV Proto by putative [4-vinyl] Proto reductase (Table 3). In contrast to our result, Tripathy and Rebeiz (1986) reported that MV Proto was formed in etiolated barley and cucumber etiochloroplasts incubated with exogenous ALA. However, they might check for an artifact at that time, which could be partially induced during Mg-insertion reaction in Proto pools contaminated with Pchlide and/or Chlide. When purified Proto pools were used for Mg-insertion reaction, the spectral characteristics of the reacted mixture were identical to those of standard DV MPE (Figs. 3A, 3B). This indicates that only DV Proto was present in the mixture. With unpurified Proto pools, however, a quite different spectrum from that with purified Proto pools was observed (Fig. 3C). It was rather similar to that of MV MPE, except that the emission maximum of unpurified Proto pools was found at 595 nm. Emission and exitation maxima of MV MPE have been reported to be 591 ± 1 nm and 418 ± 1 nm, respectively (Belanger and Rebeiz, 1982). These similar spectral characteristics could lead us to interpret the results incorrectly, expecially when the proportion of MV and DV is determined on the basis of the excitation spectrum only. The artifacts may be formed through the opening of ring E of the macrocycle by Grignard's reagent used. However, the precise mechanism of the artifact formation during the reaction is not known.

Mg-Proto and MPE were heterogeneous in both barley and corn leaves (Table 1). Exogenous DV Mg-Proto was found to be converted to MV Mg-Proto in barley etiochloroplast and etiochloroplast membranes (Table 5). Therefore, it is proposed that the MV Chl *a* carboxylic biosynthetic route may originate not at the level of DV Proto but in DV Mg-Proto. In addition, Suzuki and Bauer (1995) reported that genetic manipulations of *R. capsulatus* strains, which blocks both the conversion of Pchlide *a* to Chlide *a* and the conversion of DV Pchlide *a* to MV Pchlide *a*, resulted in the accumulation of DV Pchlide *a*, MV Pchlide *a* and MV Mg-protopophyrins. This suggests that a putative 4VMPR and the [4-vinyl] protochlorophyllide *a* reductase (4VPR) may be two different enzymes.

The characterization of 4VMPR was not successful in our assay system, since the amount of MV Mg-Proto present after incubation was not high enough for a quantitative determination of the enzyme activity. Therefore, more detailed studies using an improved assay system are needed urgently. A high degradation rate of tetrapyrroles under *in vitro* condition (Martinoia *et al.*, 1982; Brown *et al.*, 1991), the conversion of Mg-Proto and MPE to Pchlide, Mg^{+2} deletion from Mg-tetrapyrroles (Lee *et al.*, 1992), loss of Mg-tetrapyrroles during analysis process, and slow reaction of the enzyme (Chereskin *et al.*, 1983) should also be considered for development of an improved 4VMPR assay system.

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