Characterization of a Family of Chlorophyll-Deficient Wheat (*Triticum*) and Barley (*Hordeum vulgare*) Mutants with Defects in the Magnesium-Insertion Step of Chlorophyll Biosynthesis

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During thylakoid membrane biogenesis, chlorophyll (Chl) biosynthesis and the accumulation of Chl-binding proteins are tightly linked, light-regulated processes. We have investigated the consequences faced by mutant plants with defects in Chl biosynthesis by studying a series of five homeologous allelic chlorina mutants in wheat (*Triticum*) and one phenotypically related barley (*Hordeum vulgare*) mutant that express the same pleiotropic mutant phenotype but to different extents. These mutants accumulate Chl at different rates, with the most severely affected plants having the slowest rate of Chl accumulation. Analysis of precursor pools in the Chl synthesis pathway indicates they have a partial block in Chl synthesis and the accumulation of Chl-binding proteins are tightly linked, light-regulated processes that are affected plants having the most severe phenotypes accumulate the most Chl. Chl mutants isolated from these mutants exhibit a lower activity of the enzyme Mg-chelatase, which catalyzes the first committed step in Chl synthesis and accumulate protoporphyrin IX (Proto), the last porphyrin compound common to both heme and Chl synthesis. The affected plants with the most severe phenotypes accumulate the most Proto. Chloroplasts isolated from these mutants exhibit a lower activity of the enzyme Mg-chelatase, which catalyzes the first committed step in Chl synthesis. The most severely affected plants exhibit the greatest reduction in Mg-chelatase activity. Heme levels and protoporphyrinogen oxidase activity were the same for mutant and wild-type plants. We suggest that a block in Mg-chelatase activity in these mutants could account for the other traits of their pleiotropic phenotype previously described in the literature.

Chl biosynthesis and the accumulation of Chl-binding proteins are two tightly linked, light-regulated processes that occur during thylakoid membrane biogenesis. Throughout this developmental process, Chl-protein complexes first appear in the membrane in a characteristic order. First, Chl a is synthesized and the Chl a-binding RCs, comprising mostly chloroplast-encoded apoproteins, are assembled. Later Chl b is synthesized and the Chl a/b-binding, nuclear-encoded LHC's accumulate. When the Chl supply is limited by a bottleneck in the Chl synthesis pathway, either by periodic light environments (Akoyunoglou et al., 1978) or through mutation (the subject of this paper), a plant preferentially makes Chl a and accumulates RCs. The mechanism by which limited Chl is targeted to the required RC complexes over the optional LHC's is not completely understood. Chl b is thought to be derived from Chl a or perhaps Chl a (Beale and Weinstein, 1990). Presumably nascent RCs being synthesized on chloroplast ribosomes (Klein et al., 1988a, 1988b) have a higher affinity for Chl a than the unidentified Chl b biosynthetic machinery. Chl b is found only in antenna complexes, and limiting Chl b affects only antenna complexes that need both Chls a and b for stability. In the Chl b-less barley mutant chlorina f2, which has nearly normal amounts of Chl a, Bellemare et al. (1982) showed that, although this mutant accumulates almost no LHCs, the LHC apoproteins are synthesized in the cytoplasm normally, imported into the chloroplast, and inserted into the thylakoid membranes, but are rapidly degraded because of the lack of Chl b. The demand for Chl a by RC polypeptides can be experimentally reduced by incubating plants with chloramphenicol, an inhibitor of chloroplast protein biosynthesis. The wheat mutant CD3 is normally Chl b and LHC deficient, but when grown in the presence of chloramphenicol it accumulates Chl b and LHC's (Duyssen et al., 1985, 1987).

The chlorina mutants discussed in this report have somewhat less Chl a and significantly less Chl b than wild-type plants but not a total loss of Chl b like the chlorina f2 mutant of barley. Although the levels of RC's remain relatively unchanged, there is a significant, but not total, loss of Chl a/b-binding LHCs and a concomitant reduction in the size and number of grana stacks, whose presence has been attributed in part to the action of LHCs (Staehelin, 1986). Many Chl b-deficient mutants express this pleiotropic mutant phenotype to different extents; this is evidenced by their temperature and light intensity dependence and the wide range of thylakoid ultrastructures observed under different growth conditions (Allen et al., 1988; Greene et al., 1988a, 1988b; Knoetzel and Simpson, 1991).

The wheat CD3 mutant is one such pleiotropic chlorina mutant whose thylakoid membrane composition and ultrastructure has been characterized in our laboratory (Allen et

Abbreviations: ALA, 6-aminolevulinic acid; DV, divinyl; LHC, light-harvesting antenna complex; Mg-Proto, Mg-protoporphyrin IX; MV, monovinyl; Proto, protoporphyrin IX; Protogen, protoporphyrinogen; RC, reaction center.
In the present study we used a number of other mutants similar to CD3 that are homeoalleles of CD3. These homeologous chlorina mutant loci of both hexaploid wheat <em>Triticum aestivum</em> and tetraploid wheat <em>Triticum turgidum</em> have been mapped to chromosome 7, on the A, B, or D parental diploid wheat genomes. In <em>T. aestivum</em> the loci are called cn-A1 (on the A genome) and cn-D1 (on the D genome). Two alleles are available at each locus. At cn-A1, the mutants are called Driscoll’s chlorina (Pettigrew et al., 1969) and chlorina-1 (Sears and Sears, 1968), and at cn-D1, the mutants are called CD3 (Freeman et al., 1987) and chlorina-214 (Washington and Sears, 1970). In <em>T. turgidum</em> the mutated gene is on the B genome and is called Cdd-1 (C. Cifuentes, M. Duysen, P. Otto, and N. Williams, unpublished data). All of these mutants were derived by ethyl methanesulfonate mutagenesis, except for Driscoll’s chlorina, which arose spontaneously. Included in this study for comparison was the temperature-sensitive barley mutant, chlorina-104, which has been compared previously to the CD3 wheat mutant (Knoetzel and Simpson, 1991), but it is not known whether this genetic locus in barley is related to the wheat mutants mentioned above.

Both Chl and heme are synthesized from the precursor ALA in a pathway that begins in the stroma of the chloroplast, illustrated in Figure 1. In this paper we show that, to varying extents, all of these mutants accumulate Proto, the last precursor compound shared by both the Chl and heme biosynthetic pathways. There are three possible enzymatic steps that could be affected in these mutants that would cause this compound to accumulate: ferrochelatase, protoporphyrinogen oxidase, and Mg-chelatase. We have examined these possibilities and describe the first set of mutants with reduced activity of Mg-chelatase, the first enzyme committed to Chl biosynthesis.

**MATERIALS AND METHODS**

**Plant Growth Conditions**

Wild-type hexaploid wheat (<em>Triticum aestivum</em>) strain ND496-25 (CD3 parent strain), tetraploid wheat (<em>Triticum turgidum</em>) strain Langdon-16 (CDd-1 parent strain), and all mutant wheat strains (CD3, chlorina-1, chlorina-214, Driscoll’s chlorina, Cdd-1) were obtained from Dr. Murray Duysen (North Dakota State University). <em>T. aestivum</em> strain Alex, a close relative of ND496–25, was obtained from AgriPro Seeds (Berthoud, CO). Wild-type barley (<em>Hordeum vulgare</em>) strain Svalöv’s Bonus and mutant barley chlorina-104 were obtained from Dr. David Simpson (Carlsberg Research Laboratories). Plants were grown in moist vermiculite in a growth chamber at 24°C with a 14-h light/10-h dark cycle under cool-white fluorescent light (approximately 100 μE m⁻² s⁻¹), for variable amounts of time, depending on the experiment, as described in the text. For rates of Chl determination, wild-type and mutant plants were grown in the dark for 7 d and growned under continuous illumination in the same growth chamber at approximately 100 μE m⁻² s⁻¹.

**Pigment Determination**

Leaf material from approximately three to five plants was weighed, cut into small pieces, and ground in approximately 7 mL of ice-cold acetone:0.1 mM NH₄OH (9:1) in a Tissumizer (Tekmar) homogenizer for 15 s in a Nalgene centrifuge tube. For the determination of daytime Chl precursor pools, plants were homogenized within 10 s of being removed from the growth chamber. Nighttime Chl precursor pools were treated as above but homogenized during dark incubation under a green safelight.

Potential bottlenecks in the Chl synthesis pathway were determined by incubating 14-d-old plants with ALA in darkness. This was done by adding 1 mL of a solution of ALA (10 mM ALA, 5 mM MgCl₂, 10 mM Na-P [pH 7]) to a 13- × 100-mm culture tube, immersing wheat and barley plants cut
at soil level, and incubating in darkness for 12 h. Leaf material was homogenized under a green safelight as described above, but the bottom 1 cm (which was submerged) was not used.

For all extracts, insoluble material was pelleted at 4°C for 10 min at 12,000g. The acetone extract was stored at -20°C until analysis and was stable for several weeks at this step. Chl content was determined by diluting this extract in 80% acetone and quantitating the Chl absorption using the simultaneous equations of Porra et al. (1989). After the mature Chls were removed by two hexane extractions, Chl precursors Proto, Mg-Proto, and Pchlide were quantitated by spectrofluorimetry. Spectroscopic quantitation of Chl precursors in the hexane-extracted acetone residue was done on one of three spectrofluorimeters as described in detail by Tripathy and Rebeiz (1985). Concentrations of each Chl precursor were determined for each sample by comparison to standard curves generated for each Chl precursor on each instrument. MV and DV Pchlides were determined in the time-course experiment by extracting the Pchlide pool into ether as described by Tripathy and Rebeiz (1985) and monitoring excitation spectra at 77 K.

**Heme Determination**

Total heme was determined essentially according to the method of Weinstein and Beale (1984). Whole plants were weighed and homogenized in a solvent mixture of DMSO and acetone (1:4, v/v) to which 12.5 mM NH₄OH had been added. After the material was centrifuged insoluble material was reextracted with the same solvent mixture three times or until the supernatant was clear. The insoluble pellets were resuspended in 2 mL of DMSO and mixed with 0.5 mL of concentrated HCl plus 10 mL of cold acetone, centrifuged after a brief incubation, and the pellets were reextracted once with half volumes of DMSO, HCl, and acetone. Pooled supernatants from the DMSO-acetone-NH₄OH extractions were used for Chl determinations. Supernatants from the DMSO-acetone-HCl extractions were transferred to ether, purified and concentrated on a DEAE-Sepharose column, and eluted with ethanol:acetic acid:water (81:9:10, v/v), and hemes were quantitated in ethanol:acetic acid:water at A_{398}, using the extinction coefficient 144 mM⁻¹.

**Chloroplast Isolation**

Wheat or barley plants (7 d old, 9–13 cm tall) were cut at the soil level and cut into two 3.5-cm sections from the bottom (labeled “bottom,” “middle”), and the remaining tops of the plants were pooled as the “top” segments. For the experiment shown in Figure 8, whole leaves from wheat plants that were etiolated for 7 d and greened for 6 h were used as starting material. Pools of leaf segments (5–10 g) were sliced into very small pieces with a razor blade, added to about 80 mL of chilled, grinding buffer (50 mM Tricine-KOH [pH 7.8], 1 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.5 M sorbitol, 0.1% BSA), and homogenized for three 2-s bursts in a Polytron homogenizer with PTA 20TS generator (Brinkmann, Westbury, NY) or VirTis ®45 homogenizer with razor blades (VirTis, Gardiner, NY). The homogenate was filtered quickly first through 80-μm and then 20-μm nylon mesh, divided into two tubes, and centrifuged at 2500g in an HB-4 (DuPont-Sorvall) swinging bucket rotor for 6 min at 4°C. The pellets were combined and resuspended in 5 to 8 mL of grinding buffer and layered on a 5-mL 40% Percoll cushion in grinding buffer and centrifuged at 7700g in the HB-4 rotor for 15 min at 4°C. The pellet containing intact chloroplasts was washed by resuspending in 5 mL of grinding buffer (minus BSA) and recentrifuging at 1500g for 5 min. The pellet was resuspended in a small volume of grinding buffer (minus BSA). The yield of intact versus broken chloroplasts, approximated by the visual estimation of the proportion of the sample in the bottom of the tube versus that at the top of the Percoll cushion, was the same for wild-type and mutant plants and was much higher for the bottom leaf segments (about 50%) than for the top leaf segments (about 10%).

Soluble protein concentration of the intact chloroplast suspension was determined using the Bio-Rad protein assay system (a Bradford assay). Small aliquots of each suspension were mixed with water to release soluble chloroplast proteins. The volume of the original suspensions was adjusted with grinding buffer (minus BSA) such that soluble protein concentrations were 2 mg mL⁻¹. Chloroplast intactness was determined by assaying latent 6-phosphogluconate dehydrogenase activity (Journet and Douce, 1985). Activity of this enzyme mg⁻¹ of soluble protein in the presence of 0.025% Triton X-100 was used as a control.

**Enzyme Assays**

Mg-chelatase assays were done essentially according to the method of Walker and Weinstein (1991a, 1991b). Proto was obtained from Sigma and purified according to the method of Fuesler (1981), which improved the activity by at least 2-fold. Mg-chelatase assays were performed in 200 μL in the dark at 30°C and stopped after 20, 30, or 40 min of incubation by adding 800 μL of cold acetone. Extracts were briefly centrifuged in a clinical centrifuge, and insoluble material was reextracted by the addition of 50 μL of 0.1 M NH₄OH and 200 μL of cold acetone. Supernatants were combined and extracted twice with hexane, and the volume of each hexane-extracted acetone phase was adjusted to 1 mL with 80% acetone. Mg-Proto was determined quantitatively as detailed above, monitoring the emission intensity at 595 nm, with excitation at 420 nm. Mg-Proto used as a standard was obtained from Porphyrin Products (Logan, UT). Results were expressed as pmol of Mg-Proto produced 30 min⁻¹ mg⁻¹ of protein and normalized to percentage of the activity of the wild-type bottom segment, which was the highest. The activity of each mutant is expressed as a percentage of the wild-type activity to which it was compared on the given day. On each day, one mutant and one wild-type plant were assayed (three leaf segments each: bottom, middle, and top).

Protopro oxidase assays (Jacobs and Jacobs, 1982, 1993) were kindly performed by Dr. Judith Jacobs (Dartmouth Medical School) in CD3 mutant, and wild-type wheat plants were etiolated for 7 d and illuminated for 4 h.
RESULTS

Alterations in Chl Content

To determine the severity of each mutation, Chl content g⁻¹ fresh weight was quantitated for each plant. From 14-d-old plants grown at 24°C, with a light intensity of approximately 100 μE m⁻² s⁻¹, first leaves were weighed and ground in ammoniacal acetone. As shown in Figure 2, there is an inverse relationship between total Chl content and Chl a/b ratio for the series of wheat and barley mutants, indicating a preferential loss of Chl b in these Chl-deficient mutants.

Next we determined whether the mutants differed in their rates of Chl accumulation (Fig. 3). One representative from the more severe mutants (chlorina-1), one from the less severe mutants (CD3), and wild-type plants were grown in the dark for 7 d and greened under continuous light. The rate of Chl accumulation in the most severe mutant, Driscoll’s chlorina, was reported by Newell and Rienits (1975). In that study, the plants were greened under different light conditions, but the wild-type accumulated Chl at the same rate as the wild-type plants shown in Figure 3, and Driscoll’s chlorina accumulated Chl at a lower rate than did chlorina-1 in this study. The results indicate that the Chl content of the mutants is related to the rate at which they accumulate Chl. After 48 h of greening all of the plants approach the Chl content values displayed in Figure 2.

Precursor Pools in the Chl Synthesis Pathway

In the 1970s, bottlenecks in the Chl synthesis pathway were determined by flooding the pathway with the precursor ALA and monitoring the accumulated Chl precursors by absorption spectroscopy (Gough, 1972). The yellow-green Driscoll’s mutant plants have been described previously (Newell and Rienits, 1975) as accumulating Proto after treatment with ALA in darkness. Figure 4 illustrates for the entire series of wheat and barley mutants the relative pools of three porphyrin precursors (Proto, Mg-Proto, and Pchlide) quantitated by fluorescence spectroscopy after similar treatments with ALA. Absolute amounts were not determined (see figure legend). In the same order of severity (with one exception, chlorina-214) as is displayed in Figures 2 and 3, the mutants accumulate more Proto and less Pchlide than wild-type plants and each other. Without ALA treatment, wild type and all mutants accumulate only Pchlide. This is shown during the
The natural levels of precursor pools can also be measured using fluorescence spectroscopy without first manipulating the pathway with ALA as shown in Figure 4. Figure 5 illustrates changes in the Chl precursor pools of two wheat mutants (Driscoll’s chlorina and CD3) and the wild type during a light/dark cycle. All of the data are represented as pmol precursor g⁻¹ fresh weight. The plants were grown for 14 d under a 14-h light, 10-h dark regime. The time-course experiment then consisted of a 14-h light phase, a 10-h dark phase, and, for some plants, an 8-h dark phase extension for reasons that are explained below. No significant difference between the time course patterns was noted for the two mutants, even though they express the Chl defect to different extents (Fig. 2).

The Proto levels for the mutants were significantly higher than that of wild type during the light cycle (Fig. 5A). Additionally, in the mutants, the Proto level seemed to decrease slightly late in the light cycle. During the dark cycle, no Proto accumulated in any of the plants. The Mg-Proto levels for the mutants during the light cycle were lower than those of the wild type (Fig. 5B). This is consistent with a bottleneck in the pathway at Proto. In Figure 5, C and D, the DV and MV components of the Pchlide pool were measured separately. During the light cycle in the wild type, the DV Pchlide levels were higher than those of the mutant (Fig. 5C), again consistent with a bottleneck earlier in the pathway. During the dark cycle, the wild-type DV Pchlide levels gradually decreased from their light cycle levels to zero. However, in the mutants immediately after the dark cycle began, the DV Pchlide pool increased significantly and then decreased, suggesting that a dark-induced apparent bottleneck in the conversion of DV Pchlide to MV Pchlide is gradually alleviated after a period in the dark. Figure 5D shows that during the light cycle the MV Pchlide levels were close to zero, both for wild type and mutants. During the dark cycle the MV Pchlide levels initially increased at the same rate for all three plants. However, the wild-type MV Pchlide pool leveled off sooner, at about 1700 pmol g⁻¹ fresh weight, a value previously reported for cucumbers (Huang and Castelfranco, 1989), whereas the mutant levels continued to increase. When the time-course experiment was extended for an additional 8 h (shaded area) to determine whether the Pchlide level in the mutants might also level off, it was found that the mutant MV Pchlide content still continued to increase. Note that in the dark the mutants produced significantly more total Pchlide (the sum of Fig. 5, C and D) than wild type.

Table 1 demonstrates that in the middle of the light cycle the barley mutant, like the wheat mutants, accumulated dark phase of the time-course experiment illustrated in Figure 5.

Table 1. Chl precursor determinations for wild-type and mutant barley during the light cycle, in pmol precursor g⁻¹ fresh weight

<table>
<thead>
<tr>
<th></th>
<th>Proto</th>
<th>Mg-Proto</th>
<th>MV Pchlide</th>
<th>DV Pchlide</th>
</tr>
</thead>
<tbody>
<tr>
<td>c104 barley</td>
<td>361</td>
<td>142</td>
<td>110</td>
<td>210</td>
</tr>
<tr>
<td>Wild-type barley</td>
<td>234</td>
<td>234</td>
<td>90</td>
<td>640</td>
</tr>
</tbody>
</table>

Figure 5. Time-course experiment measuring Chl precursors Proto (A), Mg-Proto (B), DV-Pchlide (C), and MV-Pchlide (D) of two wheat mutants (Driscoll’s and CD3) and wild-type wheat. The 14-h light cycle (white bar) was followed by a 10-h dark cycle (dark bar) and, for some plants, an additional 8-h dark phase (shaded bar). The experiment was performed twice.
significantly more Proto and less Mg-Proto and Pchlide than did wild-type barley. Thus, the responses of the wheat mutants illustrated in Figures 4 and 5 are shared by the chlorina-104 mutant of barley.

Together, Figures 4 and 5 and Table I show that this set of Chl b-deficient wheat and barley mutants has partial blocks in Chl synthesis and that they all accumulate Proto, consistent with the presence of a bottleneck in the Chl biosynthetic pathway at the branch point between Chl and heme synthesis.

**Mg-Chelatase Activity Measurements**

Given a bottleneck at Proto, there are three possible enzymes whose activity could be affected, directly or indirectly, to cause the bottleneck. They are the ferrochelatase, Protoporphyrin oxidase, and Mg-chelatase. Total heme was determined for wild- and CD3 mutant wheat (Table II). The heme content of CD3 wheat was found to be equal to or slightly lower than that of wild type. Therefore, a defect is unlikely in the ferrochelatase, the enzyme that inserts iron into Proto to make heme. Protoporphyrin oxidase is the last enzyme shared by the heme and Chl synthesis pathways and is the target of diphenyl ether herbicides, which also result in Proto accumulation (Matringe et al., 1989; Witkowski and Hallig, 1989). The mean Protoporphyrin oxidase activity for wild-type and CD3 mutant wheat was determined and found to be essentially the same (0.79 ± 0.06 versus 0.68 ± 0.12 nmol of Proto produced mg⁻¹ of protein h⁻¹ for wild type and CD3, respectively). Thus, the lesion in the CD3 mutant does not seem to be associated with Protoporphyrin oxidase.

To measure the activity of the enzyme Mg-chelatase, we have used the assay developed by Walker and Weinstein (1991a, 1991b) for cucumbers and peas and found that this activity assay also works for monocots. The advantage of monocots for developmental studies of Mg-chelatase activity relates to the fact that monocot leaves exhibit a developmental gradient along their length with the youngest tissue at the bottom and the oldest at the tip. For our analysis, we isolated intact chloroplasts from the bottom, middle, and top segments of leaves and assayed them for Mg-chelatase activity.

Figure 6 illustrates the activities of the segments taken from wild-type plants, three from the mutant wheat plants, and the barley mutant chlorina-104. In the wild-type and mutant plants, the highest Mg-chelatase activity was present in the youngest tissue in bottom-leaf segments where the growth rate and the rate of Chl synthesis were the greatest. The lowest activities were in the oldest, top segments of the leaves. In addition, there was a major reduction in Mg-chelatase activity in the bottom-leaf segments of all of the mutants compared to wild type (Fig. 6). In fact, the difference in activity of the bottom-leaf segments of the wild-type and mutant plants parallels quantitatively the difference in the rates of Chl accumulation shown in Figure 3. For example, the rates of Chl accumulation and Mg-chelatase in CD3 were about 50% of wild type, and for chlorina-1 both rates were about 20% of wild type. The barley mutant chlorina-104 also showed a statistically significant difference from wild-type activity in the bottom-leaf segment. The error bars, which represent 95% confidence intervals, are larger for the barley samples mainly because of the small sample size (see legend).

In most of the middle- and top-leaf segments, the Mg-chelatase activity was significantly lower than that in the corresponding bottom segment. One striking exception was the chlorina-214 mutant in which the activity of the middle segment is at least as high as that of the bottom segment. Indeed, the activity of the middle segment is even greater than that of the corresponding segment in wild-type plants. On two separate occasions, the middle section of this mutant exhibited higher activity than both its own bottom section and wild type in the middle section. In general, the largest difference in activity between wild-type and mutant plants is apparent in the bottom-leaf segment.

Chloroplasts were tested for their intactness by measuring latent glucose-6-P dehydrogenase activity first in the absence and later in the presence of 0.025% Triton X-100. Based on this assay, 19 of the 24 chloroplast preparations were

**Table II. Chl and heme determinations for 14- and 19-d-old plants**

<table>
<thead>
<tr>
<th>Plant Age</th>
<th>Chl</th>
<th></th>
<th>Heme</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>CD3</td>
<td>Wild type</td>
<td>CD3</td>
</tr>
<tr>
<td>d</td>
<td>μmol Chl g⁻¹ fresh wt</td>
<td>nmol heme g⁻¹ fresh wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>2.1</td>
<td>0.7</td>
<td>18.6</td>
<td>14.4</td>
</tr>
<tr>
<td>19</td>
<td>2.2</td>
<td>0.9</td>
<td>15.3</td>
<td>14.9</td>
</tr>
</tbody>
</table>
shown to be composed of 100% intact chloroplasts, and the remaining 5 contained between 85 and 96% intact chloroplasts. Because a significant amount of Mg-chelatase activity is lost upon chloroplast breakage (Walker and Weinstein, 1991a, 1991b), activity values for the latter samples were adjusted to compensate for the lost activity in those samples. The activity of gluconate-6-P dehydrogenase in detergent-lysed chloroplasts was used as an additional control to demonstrate that in each of the mutants the activity of this enzyme was at least as high as that of wild type, making the possibility of a generalized chloroplast defect unlikely (data not shown).

**Kinetic Parameters of the Mutant Mg-Chelatase Enzyme**

Finally, we checked whether the reduction in the Mg-chelatase activity of the CD3 wheat mutant was due to a difference in the affinity of the enzyme for Proto or the amount of the enzyme. For these experiments, greening tissue was used instead of the bottom segments of light-dark grown plants. Greening tissue should represent a uniform developmental state and show the greatest difference in wild-type and mutant activity. Figure 7A shows that at low enzyme concentrations, at which 4 times as much protein was introduced into the assay for the mutants than for wild type (8 versus 2 µg of protein in the 200-µL assay), the mutant and wild-type kinetic behavior was nearly identical, indicating that the affinity of the enzyme for Proto is the same for wild-type and mutant enzymes. However, the total activity of the enzyme is 4-fold lower in the mutant than in the wild type (which is indeed a greater difference than was observed in Fig. 6). Our estimate for the apparent $K_m$ for Proto in this assay of about 40 nM agrees approximately with the published value for the $K_m$ of 25 nM (Beale and Weinstein, 1990). While optimizing the enzyme to substrate ratios to be used for the determination of the $K_m$ for Proto, we confirmed previous observations of substrate inhibition at Proto concentrations of 3 µM and above (Walker and Weinstein, 1991a), but we also observed possible product inhibition at low substrate concentrations and high enzyme to substrate ratios. As shown in Figure 7B, 40 µg of protein were used in the 200-µL assay, and at the lowest substrate concentrations (that were well above the apparent $K_m$ determined in part A) there is a decrease in activity. The wild-type chloroplasts appear to be subject much more to both substrate and product inhibition than are the mutants.

**DISCUSSION**

**Genetics and the Severity of the Wheat Mutants Used in This Study**

The long-term goal of our research is to understand how the biosynthesis of Chl is coupled to thylakoid membrane morphogenesis. The specific aim of this investigation was to define in biochemical terms the lesions in a series of Chl-deficient wheat mutants that exhibit a pleiotropic mutant phenotype.

The differences in severity of the phenotypes in our series of mutants could be caused either by the severity of the lesions or by different rates of expression of the allelic genes. The fact that both of the mutant alleles on the A genome (Driscoll’s and chlorina-1) are more severe than those on the D genome (CD3 and chlorina-214) could be a reflection of the fact that the products of these homeologous loci are not expressed equivalently but that the A genome’s product at this locus is more important to the whole pool than the products of other loci. Likewise, the CD-d1 mutant of tetraploid *T. turgidum* could be a reflection that the B genome’s product at this locus is relatively unimportant to the whole pool, because as a homozygote, it has a normal to mutant ratio of 2:2 and is not lethal (C. Cifuentes, M. Duysen, P. Otto, and N. Williams, unpublished results). For these reasons, it is not necessarily safe to assume that in a polyploid organism all of the gene products of homeologous genetic loci are present in equivalent pools within the plant cell. For example, in polyploid ferns, multiple copies of genes are silenced (Gastony, 1991) probably by DNA methylation (Chomet, 1991).
Implications of Dominant-Negative Mutants with Reduced Mg-Chelatase Activity

Recent biochemical studies of Walker et al. (1992) have shown that the Mg-chelatase activity can be fractionated into two chloroplast components, a "light membrane" (probably envelope) fraction and soluble (stromal) fraction. This finding is consistent with the idea of Mg-chelatase being part of a membrane-bound enzyme complex consisting of at least two subunits. Mg-chelatase activity could be affected by mutations in any of its subunits. Because the number and identity of the subunits are not yet known and the genetic loci used in this study have not yet been studied on a molecular level, we can only suggest that the reduced Mg-chelatase activity of the mutants in this study is most likely caused by the inactivation of enzyme complexes by defective subunits. Two observations lend support to this theory. First, the reduction in Mg-chelatase activity for the mutants together with the fact that the apparent $K_m$ for Proto (approximately 40 nM in this assay) is the same for the wild-type and the CD3 mutant suggests that the mutant enzymes are similar to those of wild type, but the amount of functional enzyme is reduced in the mutants. Second, the Proto-accumulating wheat mutants in this study are dominant-negative mutants. If the Mg-chelatase is a hetero-oligomeric multisubunit complex, dominant mutations can be expected to be found (Herskowitz, 1987) and, conversely, damage to any number of loci might have a dominant effect in a multienzyme complex. Dominant-negative mutations encode poison products, or "defective monomers," which have the effect of reducing the number of functional enzyme complexes in the chloroplast, which in turn would reduce the total enzyme activity expressed per mg of protein. Additionally, if each enzyme complex uses several molecules of this gene product as subunits, by combinatorics "defective monomers" (Sears, 1969, 1972) would have a greater effect on the population than if only one gene product were included per enzyme complex. This hypothetical model could explain the 50 to 75% reductions in Chl content and Mg-chelatase activity in these mutants rather than 33% reductions, which might be expected for mutants having two mutant copies and four normal copies of the allele in question. We have found that a large number of other dominant-Chl-deficient multisubunit also accumulate Proto (T.G. Falbel and L.A. Staehelin, unpublished data). The Mg-chelatase may be one of the few multisubunit complexes in Chl synthesis, which might be why there are so many mutants, both dominant and recessive, that accumulate Proto.

The CD3 mutant chloroplasts are less susceptible to both substrate and possible product inhibition than are wild-type chloroplasts. This puzzling observation could either indicate that the active enzymes in CD3 are different from those in the wild type or that the defective enzymes in CD3 are able to compete for substrate with the active ones, which could change the apparent extent of substrate and product inhibition. As an alternative to product inhibition, we cannot rule out the possibility that only a small portion of the Proto introduced into the assay is available to be converted by the enzyme to Mg-Proto. At concentrations below 1 $\mu$M, the supply of substrate could be rapidly exhausted by wild-type chloroplasts.

6-Phosphogluconate dehydrogenase activity, heme content, and Protogen oxidase activity did not differ for mutant and wild-type plants. These control results make the possibility of a generalized chloroplast defect unlikely and rule out defects in two of the other enzymes that would lead to Proto accumulation if defective. However, we cannot rule out the possibility that there may be other reduced activities in these mutants that are undetected at present. Proto could accumulate indirectly as a consequence of a block further along in the Chl synthesis pathway: Mg$^{2+}$ ions could be lost from accumulated Mg-Proto, but this process probably does not occur at an appreciable rate because the barley mutant viridis k23, shown previously to accumulate Mg-Proto (Simpson and vonWettstein, 1980), accumulates much more Mg-Proto than Proto (T.G. Falbel and L.A. Staehelin, unpublished data).

Thus, the most likely explanation of our data is that the reduction in Mg-chelatase activity causes the Proto accumulation in vivo. Our kinetic observations and the fact that the mutants at this locus are dominant negatives, taken together with the fact that the Mg-chelatase has at least two components (Walker and Weinstein, 1991b), strongly suggest that the mutations directly affect the Mg-chelatase complex. Of course, this conjecture cannot be proven until some basic characterization of the enzyme's structure has been accomplished and the genetic loci have been characterized at the molecular level.

Differences in the Extent of the Mg-Chelatase Defect in Development

Although Proto-accumulating mutants occur in other species (Gough, 1972; Mascia, 1978; T.G. Falbel and L.A. Staehelin, unpublished data), we chose to use wheat and barley mutants because these cereals form relatively large plants when they are young, as opposed to most dicots. We found that the youngest tissue at the bottom of the leaves displayed the greatest difference in Mg-chelatase activity between wild-type and mutant plants. In the older tissue closer to the top of the leaves, Mg-chelatase activity for most of the mutants was still lower than wild type, but relatively less so. These observations suggest that the mutations exert their greatest effects on the forming thylakoids in the basal cells where Chl synthesis is the most active and have the least impact on the thylakoid maintenance activities in the apical cells. The lesion could affect a more active isoform of the enzyme or could indirectly affect the proper timing or expression levels of the Mg-chelatase. There could be several Mg-chelatase isoforms present in the developing chloroplast, especially in a hexaploid species. The chlorina-214 mutant, unique in having higher Mg-chelatase activity in its middle segment than its bottom segment, could be exploited in future study of developmental differences in the regulation or stability of the Mg-chelatase.

Pchlide Accumulation in the Mutants

The time-course experiment clearly demonstrated a block at Proto for the mutants during the times of the light cycle, yet the results from the dark cycle were puzzling. The ALA
experiment (Fig. 4) demonstrates that there is a clear block in the Chl synthesis pathway at night in the mutants, but in the time-course experiment (Fig. 5), the rate of MV Pchlide accumulation during the first 6 h of darkness is not distinguishable between wild type and even the most severe mutant, Driscoll's chlorina.

There is also a brief burst in the level of DV Pchlide in the mutants just after dark (Fig. 5), which indicates that the DV Pchlide precursors are being made faster than the 4-vinyl-reductase enzyme can convert them to MV Pchlide under conditions in which the conversion of MV Pchlide to Chlide is inhibited (Fig. 1). Therefore, in fact, the total Pchlide pool (MV plus DV) is accumulating slightly faster in the mutants than in wild type. The final level of Pchlide for the mutants is also higher than that of the wild type. We have also observed Pchlide overproduction in the barley mutant *Triturus* *tigris* k23, which has a block later in Chl synthesis (data not shown).

These two findings are puzzling but could be related to the fact that as a result of their mutations the mutants build up a much larger pool of Proto than do the wild-type plants, and this pool works its way through the pathway in the dark. Alternatively, although the pathway is clearly blocked during both day and night, the block could be more extensive during the daytime. Thus, the mutations could be affecting the regulation of the enzyme’s activity, or possibly the mutations could indirectly perturb the phytochrome-dependent mechanism (Huang and Castelfranco, 1989) that shuts off ALA synthesis at night, which involves heme and the products of the *tigrina* loci (in barley) (reviewed by Somerville, 1986). Clearly, these results will be more easily addressed in the future when both the Mg-chelatase and the nature of these mutants are defined at the molecular level.

Consequences of the Block in Chl Biosynthesis in the Mutants

In this study we have demonstrated that a subset of the chlorina mutants, the allelic wheat mutants chlorina-1, chlorina-214, and CD3, and the barley mutant chlorina-104, all have a deficiency in Chl synthesis at the Mg-insertion step, which causes a buildup of Proto with a concomitant decrease in Mg-Proto. Our results suggest further that the mutations actually affect the Mg-chelatase complex. This being the case, the question arises as to how the alteration in activity of one enzyme in the Chl synthesis pathway can give rise to the light and temperature sensitivity of these mutants as well as their pleiotropic phenotypes.

The high-light sensitivity of the Chl b-deficient mutants has two potential origins: (a) the bottleneck in the Chl synthesis pathway could prevent the mutant plants from recovering efficiently from high light or UV photodamage, and/or (b) the plants could be poisoned by the higher than normal pool of Chl precursors due to their photosensitization properties. After ruling out UV as a major source of photodamage by growing the Driscoll’s mutant under Plexiglas and noting no change in Chl content (data not shown), we now favor hypothesis b for the following reason. Plants exposed to diphenyl ether herbicides accumulate Proto. These herbicides specifically target the enzyme Protopogen oxidase, which converts Protopogen to Proto. Recently, two groups (Jacobs and Smith et al., 1993) have concurred in their proposed mechanisms to explain the surprising accumulation of Proto after herbicide treatment: large amounts of Protopogen are exported from the plastid to the cytoplasm where non-enzymic oxidation to Proto takes place. After high-light excitation, these Proto molecules dissipate their energy through free radical formation, resulting in oxidative photodamage. In the chlorina mutants that accumulate Proto in the chloroplasts, an analogous mechanism could lead to increased photobleaching at high-light intensities, thereby increasing the rate of Chl degradation and the strain on the Chl synthesis pathway.

Temperature sensitivity has been postulated to be a general phenomenon in Chl-deficient mutants of sweetclover (Yang et al., 1990) and *Arabidopsis* (Markwell and Osterman, 1992). We postulate that the bottleneck in the Chl synthesis pathway in these mutants, seen especially early in development, could simply exaggerate the low-temperature slowdown in de novo protein synthesis (Greer et al., 1991) required for the processes of development and repair and thereby enhance the mutational effects. This and the other "downstream effects" of the mutations that give rise to this pleiotropic phenotype will be discussed in a second report that will focus on the morphological features and the biochemical properties of this series of Chl b-deficient mutants.

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