

Molecular basis for semidominance of missense mutations in the XANTHA-H (42-kDa) subunit of magnesium chelatase

(chlorophyll synthesis/complementation assays/protoporphyrin IX/*chlorina*/aurea)

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ABSTRACT During biosynthesis of bacteriochlorophyll or chlorophyll, three protein subunits of 140, 70, and 42 kDa interact to insert Mg^{2+} into protoporphyrin IX. The semidominant *Chlorina-125*, *-157*, and *-161* mutants in barley are deficient in this step and accumulate protoporphyrin IX after feeding on 5-aminolevulinic acid. *Chlorina-125*, *-157*, and *-161* are allelic to the recessive *xantha-h* mutants and contain G559A, G806A, and C271T mutations, respectively. These mutations cause single amino acid substitutions in residues that are conserved in all known primary structures of the 42-kDa subunit. *In vitro* complementation and reconstitution of Mg-chelatase activity show that the 42-kDa subunits are defective in the semidominant *Chlorina* mutants. A mutated protein is maintained in the *Chlorina* plastids, unlike in the *xantha-h* plastids. Heterozygous *Chlorina* seedlings have 25–50% of the Mg-chelatase activity of wild-type seedlings. Codominant expression of active and inactive 42-kDa subunits in heterozygous *Chlorina* seedlings is likely to produce two types of heterodimers between the strongly interacting 42-kDa and 70-kDa subunits. Reduced Mg-chelatase activity is explained by the capacity of heterodimers consisting of mutated 42-kDa and wild-type 70-kDa protein to bind to the 140-kDa subunit. The 42-kDa subunit is similar to chaperones that refold denatured polypeptides with respect to its ATP-to-ADP exchange activity and its ability to generate ATPase activity with the 70-kDa subunit. We hypothesize that the association of the 42-kDa subunit with the 70-kDa subunit allows them to form a specific complex with the 140-kDa subunit and that this complex inserts Mg^{2+} into protoporphyrin IX.

Chelation of Mg^{2+} into protoporphyrin IX during the biosynthesis of bacteriochlorophyll or chlorophyll requires an enzyme, Mg-chelatase, consisting of three soluble subunits (1–6). The subunits show significant conservation of their primary amino acid sequences and molecular mass from *Rhodobacter* to cyanobacteria and higher plants, including barley, thale cress, and tobacco (for reviews, see refs. 7 and 8). The three subunits have nominal molecular masses of 140 kDa (range: 123–154 kDa), 70 kDa (range: 60–87 kDa), and 42 kDa (range: 37–46 kDa), respectively. Although the functional analysis of recombinantly expressed subunits is more advanced for *Rhodobacter* than for higher-plant Mg-chelatase, genetic studies and analyses of subunits from tobacco and barley thus far have indicated congruent characteristics. The largest subunit binds the substrate protoporphyrin IX. The metal-insertion step requires Mg^{2+} and ATP, in addition to the large subunit and protoporphyrin IX. It is preceded by an activation step, consisting of ATP hydrolysis, which is carried out by the combined action of the 70- and 42-kDa subunits (2, 9).

In barley, the 140-, 70-, and 42-kDa subunits are encoded by the *Xantha-f*, *Xantha-g*, and *Xantha-h* genes, respectively (4, 5). Mutations in these loci yielded the recessive, lethal genes *xantha-f*^{f10}, *-f*^{f26}, *-f*^{f27}, *-f*^{f40}, *-f*^{f41}, *-f*^{f58}, *-f*^{f60}, and *-f*^{f68}; *xantha-g*^{g28}, *-g*^{g37}, *-g*^{g44}, *-g*^{g45}, and *-g*^{g65}; and *xantha-h*^{h30}, *-h*^{h38}, *-h*^{h56}, and *-h*^{h57} (10). These mutants lack Mg-chelatase activity and accumulate protoporphyrin IX and its monomethyl ester when fed the chlorophyll biosynthetic precursor 5-aminolevulinic acid. Some of the mutants are defective in transcription (e.g., *xantha-h*^{h56} and *-h*^{h57}); others are defective in translation (e.g., *xantha-h*^{h30} and *-h*^{h38}); and others are missense mutations (e.g., *xantha-f*^{f10}; ref. 4).

In amphidiploid tobacco, the gene of the semidominant aurea mutation *Sulfur* and its wild-type allele have been cloned by transposon tagging and sequenced (11, 12). Homozygotes of aurea mutations are yellow-seedling lethals, whereas the heterozygotes have a reduced amount of chlorophyll and are yellow-green. The *sulfur* gene encodes a 42-kDa Mg-chelatase subunit of tobacco, and the *Sulfur* mutation results in a single amino acid substitution from Asn to Ile in position 269 of the subunit inherited from the *Nicotiana glauca* parent. Nguyen (11) interpreted the dominant action of the single-mutated 42-kDa subunit by the formation of inactive Mg-chelatase, which thereby limits the synthesis of chlorophyll.

The three Mg-chelatase subunits of tobacco have been investigated by Gräfe (8) for expression in yeast and for protein–protein interaction in the GAL4/two-hybrid-yeast system. A strong interaction between the 42-kDa subunit (designated CHL I) and the 70-kDa subunit (designated CHL D) was observed in the activation of transcription of the reporter gene when the two subunits were fused to the activation and binding domains, respectively, of the GAL4 transcription factor. A weak interaction was observed between 70-kDa subunits and interpreted as homodimer formation. No interaction was registered with the 140-kDa subunit (designated CHL H), although this subunit was required for Mg-chelatase activity. Dissection of the 70-kDa subunit by expression of five peptide fragments in yeast, together with the cDNAs of the 140-kDa and 42-kDa tobacco subunits, indicated that the 312 N-terminal amino acid residues of the mature protein with significant homology to the 42-kDa subunit are dispensable for maximal Mg-chelatase activity. By coexpression of the proline-rich linker region and the two flanking helical domains at the center of the primary structure of the 70-kDa subunit with the two other subunits, 30% of maximal activity was achievable.

Recently, it has been shown that the purified 42-kDa subunit of *Rhodobacter sphaeroides* (designated BchI) catalyzes a phosphate-exchange reaction from one ATP molecule to an ADP,

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generating ADP and ATP, respectively, whereas ATP hydrolysis requires the combined presence of the 42-kDa (BchI) and 70-kDa (BchD) subunits (9). The 140-kDa subunit (BchH) by itself had weak ATPase activity. Recently, the unusual ATP-to-ADP phosphate-exchange activity has been found to be an intrinsic activity in the heat-shock protein Hsp70, which is thought to act as a molecular chaperone in protein folding (13). The semidominant action of the mutated 42-kDa tobacco *Sulfur* (CHL I) subunit and the possible importance of this subunit for correct folding of Mg-chelatase subunits led us to investigate three aurea mutants in barley, designated *Chlorina-125*, *-157*, and *-161* (14, 15). They are allelic and segregate into a phenotypic ratio of one green wild-type leaf (*clo/clo*) to two viable light-green *chlorina* leaves (*clo/Clo*) to one lethal yellow *xantha* leaf (*Clo/Clo*) (Fig. 1).

MATERIALS AND METHODS

Plant Material. Grains of two barley lines were treated with the mutagen sodium azide (16) and sown in the field. The progeny grains (M_2) from these plants were germinated; 4-week-old seedlings were screened visually for light-green (*chlorina*) color, and the viable mutants were propagated for further analyses. Among the 78 viable *Chlorina* mutants selected, there were 3 allelic aurea mutants. Mutant *Chlorina-125* was induced in a wild-type line of cultivar Bonus, and *Chlorina-157* and *Chlorina-161* were induced in a wild-type line of Ca710516 (Triumph \times Goldspear). Grains of the stock collection were planted in moist vermiculite and grown in



FIG. 1. Leaves (6 days old) obtained from the seeds of a heterozygous barley *Chlorina* mutant. Because of the semidominant behavior of the *Chlorina* mutation, the leaves segregate into a phenotypic ratio of one normal green wild-type to two light-green heterozygotes to one yellow, chlorophyll-lacking homozygote. In this spike, the ratio is 3 normal green to 13 light-green to 5 yellow.

darkness at 20°C for 6 days. Homozygous yellow and heterozygous light-green seedlings were distinguishable from green wild-type seedlings after 4 h of illumination with Osram Fluora fluorescent lights (Berlin) at 2.59 W/m².

Cloning and Sequencing of the *Xantha-h* Gene Encoding the 42-kDa Subunit of Mg-Chelatase and Its Mutant Alleles. Chromosomal DNA was extracted from barley leaves with cetyltrimethylammonium bromide (17). The relevant parts of the wild-type *Xantha-h* genes and their dominant mutant alleles in the *Chlorina* mutants were cloned by PCR by using the DNA from the *Chlorina* mutants and their two wild-type segregants as templates. The PCR consisted of 30 cycles of 45 s at 95°C, 60 s at 57°C, and 120 s at 72°C, and oligonucleotides with the sequences 5'-AAC GTC ATC GAC CCC AA-3' and 5'-GCC AAA GAC TTC ATA GAA CTT-3' were used as primers. Sequencing was performed directly on the PCR fragments by using an ABI377 sequencer and the BigDye Primer Cycle Sequencing Kit supplied by the manufacturer (Applied Biosystems).

Complementation Assays of Mg-Chelatase Activity with Mutant and Wild-Type Subunits. Between 20 and 40 g (fresh weight) of leaves from the homozygous mutants were homogenized in grinding medium and processed according to the method of Kannangara *et al.* (5). Heterozygous *Chlorina* and normal green seedlings were also processed in this way. Briefly, the homogenate was squeezed through a single layer of nylon tissue, filtered through another layer, and then centrifuged at 5,000 \times g at 4°C for 6 min. After removal of the supernatant, 1 ml of the resuspended chloroplasts was purified by centrifugation through a 5-ml Percoll cushion (Amersham Pharmacia; 35% Percoll in grinding medium) at 12,800 \times g at 4°C for 10 min. The purified chloroplasts were lysed in 150 μ l of 20 mM Tricine-NaOH {*N*-[tris(hydroxymethyl)methyl]glycine-NaOH} buffer (pH 9.0) containing 1 mM EDTA, 2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. The membranes were removed by centrifugation at 21,000 \times g for 15 min, and the supernatants were used for analysis of *in vitro* complementations.

Wild-type subunits were also prepared in large scale from 3 kg (fresh weight) of leaves. This allows the 42- and 70-kDa subunits of Mg-chelatase in the stromata of purified plastids to be separated into a supernatant and pellet fraction, respectively (5). The stroma supernatant obtained by lysis of the purified plastids, as described above, was centrifuged for 90 min at 272,000 \times g, yielding a clear-as-glass pellet containing the ribosome-associated 70-kDa subunit and a supernatant containing the 42-kDa subunit. The 140-kDa subunit is present in both fractions.

Mg-chelatase activity was measured in the following way. From stock solutions of 1 mM deuteroporphyrin IX in 2% (vol/vol) ammonia and the other components, a mixture containing deuteroporphyrin (60 μ M), ATP (50 mM), creatine phosphate (250 mM), and MgCl₂ (250 mM) was prepared. The assays were started by adding 4.25 μ l of this mixture to protein solutions adjusted to 45 μ l with 20 mM Tricine-NaOH (pH 9.0) and then adding 1 μ l (2.5 units) of creatine kinase. The reaction mixtures were incubated and shaken for 20 min at 30°C, and the reaction was stopped by adding 1 ml of acetone/water/25% ammonia (80:20:1, vol/vol/vol) and 200 μ l of hexane. After mixing, the tubes were centrifuged briefly to separate the phases. The emission spectrum of the bottom acetone phase was recorded from 550 to 650 nm with an excitation wavelength of 408 nm. The amount of Mg-deuteroporphyrin formed was calculated from the fluorescence intensity at 585 nm by using a standard curve obtained with known amounts of authentic compound.

Other Methods. Total leaf protein from mutants and wild type was prepared for Western blot analysis as described (18). SDS/PAGE was performed as described (19) with the Tris-Tricine buffer system (20). Proteins were electrotrans-

ferred to Immobilon P filters (Millipore) with a semidry electroblotter (Ancos, Copenhagen, Denmark). Antigens were visualized by chemiluminescence (CLONTECH). Optimal sensitivity of detection was obtained according to published protocols (18). Protein concentrations were determined with the Bio-Rad protein assay based on the binding of Coomassie brilliant blue dye.

RESULTS

The *Chlorina* Mutants Are Deficient in the Synthesis of Mg-Protoporphyrin IX. To identify the block in the biosynthetic pathway, the *Chlorina*-125, -157, and -161 mutants were analyzed after feeding 5-aminolevulinic acid to primary leaves. The primary leaves of normal green seedlings (*clo/clo*), heterozygous light-green seedlings (*Clo/clo*), and yellow seedlings (*Clo/Clo*) were detached and placed for 15 h in 20 mM 5-aminolevulinic acid and 10 mM sodium phosphate buffer (pH 7.0) in the dark. A red pigment accumulated in all homozygous (*Clo/Clo*) and heterozygous (*Clo/clo*) mutant leaves, whereas the wild type accumulated green pigment. The leaves were ground in liquid nitrogen and suspended in acetone/water/25% ammonia (80:20:1, vol/vol/vol). Carotenoids were removed with hexane, and the absorption spectra were determined. All three homozygous (*Clo/Clo*) mutants, whether grown in the dark or in the light, accumulated protoporphyrin IX. The wild-type seedlings (*clo/clo*) accumulated protochlorophyllide under these conditions, whereas the heterozygotes accumulated both protoporphyrin IX and protochlorophyllide. Accumulation of protoporphyrin IX after feeding with 5-aminolevulinic acid is also characteristic for the

xantha-f, -*g*, and -*h* mutants; however, in these cases, the mutations are recessive—the heterozygotes are normal green and accumulate only protochlorophyllide (21). We conclude that the semidominant *Chlorina* mutants are deficient in Mg-chelatase.

The Semidominant *Chlorina* Mutations Are Allelic to the Recessive *xantha-h* Mutations. Heterozygous *xantha-h*⁵⁷ plants (*Xan/xan*) were emasculated and pollinated with pollen from heterozygous *Chlorina*-161 (*Clo/clo*) plants. The F₁ progeny segregated into 25 green, 8 light-green, and 10 yellow seedlings, which is in agreement with the expected 2:1:1 ratio for allelism ($\chi^2 = 0.782$; $P \approx 0.6$). In crosses of heterozygous *xantha-f*²⁶ and *xantha-g*²⁸ with *Chlorina*-161, only green and light-green seedlings segregated in a 1:1 ratio.

The *Chlorina*-125, -157, and -161 Mutations Result in Single Amino Acid Changes in the 42-kDa Mg-Chelatase Subunit. Oligonucleotide primers for the gene encoding the 42-kDa subunit were synthesized according to the known cDNA sequence of the *Xantha-h* gene (4). From comparisons between the deduced barley *Xantha-h* gene product with the 42-kDa Mg-chelatase subunit of other organisms, it can be estimated that approximately 20 amino acid residues of the sequence of the mature XANTHA-H protein are undetermined (Fig. 2). The known part of the *Xantha-h* gene was sequenced from chromosomal DNA of the wild-type lines Bonus and Ca710516 (Triumph × Goldspear) and the homozygous *Chlorina*-125, -157, and -161 mutants. All three *Chlorina* mutations were found to be point mutations resulting in changes of single amino acid residues that are conserved strictly in the 14 sequences, 6 of which are compared in Fig. 2. When numbering the nucleotides according to the incomplete

Hvu	---	MASALGT	SSIAVLPSRY	FSSSSSKPSI	HTLSLTSQGN	YGRKFYGGIG	IHGKIGRAQ.	LSVTNVATE	VNSVDAQSI	A.SKESQRPV	YPFSAIVGQD	94
Gma	---	MASALGT	SSIAVLPSRY	FSSSSSKPSI	HTLSLTSQGN	YGRKFYGGIG	IHGKIGRAQ.	LSVTNVATE	VNSVDAQSI	A.SKESQRPV	YPFSAIVGQD	94
Nta	---	MASLLTSSS	AAAAILASTP	LSSRSCKPAV	FSLFPSSGQS	QGRKFYGGIR	VPVKKGRSQF	HVAISNVATE	INLLKNVRVN	L.LEESQRPV	YPFSAIVGQD	99
Ath	---	MASLLGT	SSSAIWAASP	LSSPSSKPS	SPICFRPGKL	FGSKLNAGIQ	IRPKNRSRY	HVSMNVATE	INSTEQVVGK	FDSKKSARPV	YPFSAIVGQD	97
Rca	---	---	---	---	---	---	---	---	---	---	---	30
Rsp	---	---	---	---	---	---	---	---	---	---	---	14
↓ 161												
Hvu	ELHLVLLLN	IDPKIGGVI	MGDRGTGKST	TVRSLVDLLP	DISVVVDPF	NSDPYDPEVM	GPEVR.DRLL	KGESLPVTTT	KITMVDPLPG	ATEDRVCGTI	102	
Gma	EMKLCLELN	IDPKIGGVI	MGDRGTGKST	TVRSLVDLLP	EIKVVAGDPY	NSDPDQPEFM	GVEVR.ERVL	QGEELSVLIT	KINMVDPLPG	ATEDRVCGTI	193	
Nta	EMKLCLELN	IDPKIGGVI	MGDRGTGKST	TVRSLVDLLP	EIKVISGDPF	NSDPDQPEVM	SAEVR.DKLR	SGQQLPIRSR	KINMVDPLPG	ATEDRVCGTI	198	
Ath	EMKLCLELN	IDPKIGGVI	MGDRGTGKST	TVRSLVDLLP	EINVVAGDPY	NSDPIDPEFM	GVEVR.ERVE	KGEQVPVIAT	KINMVDPLPG	ATEDRVCGTI	196	
Rca	DMKLALLTA	VDPGIGGVLV	FGDRGTGKST	AVRALAALLP	EIEAVEGCPV	SSPNVEM...IPDWA	TVLSTNVIRK	PTPVVDPLPG	VSEDRVVGAL	122	
Rsp	QMKQAMVLT	IDPKIGGVLV	FGDRGTGKST	AVRALAALLP	LKAVEGCPV	NSARPED...CPEWA	HVSSTTMIER	PTPVVDPLPG	VTEDRVVGAL	106	
* * * * *												
↓ Sulfur ↓ 125												
Hvu	DIEKALTEGV	KAPEPGLLAK	ANRGILYVDE	VNLLDDHLVD	VLLDSAASGW	NTVEREGISI	SHPARFILIG	SGNPEEGELR	PQLLDRFGMH	AQVGTVRDAE	202	
Gma	DIEKALTEGV	KAPEPGLLAK	ANRGILYVDE	VNLLDDHLVD	VLLDSAASGW	NTVEREGISI	SHPARFILIG	SGNPEEGELR	PQLLDRFGMH	AQVGTVRDAE	293	
Nta	DIEKALTEGV	KAPEPGLLAK	ANRGILYVDE	VNLLDDHLVD	VLLDSAASGW	NTVEREGISI	SHPARFILIG	SGNPEEGELR	PQLLDRFGMH	AQVGTVRDAE	298	
Ath	DIEKALTEGV	KAPEPGLLAK	ANRGILYVDE	VNLLDDHLVD	VLLDSAASGW	NTVEREGISI	SHPARFILIG	SGNPEEGELR	PQLLDRFGMH	AQVGTVRDAE	296	
Rca	DIERAISKGE	KAPEPGLLAR	ANRGYLYIDE	CNLEDDHIVD	LLLDVAQSGE	NVVERDGLSI	RHPARFVLVG	SGNPEEGDLR	PQLLDRFGLS	VEVLSPRDVE	222	
Rsp	DIERALTRGE	KAPEPGLLAR	ANRGYLYIDE	VNLEDDHIVD	LLLDVAQSGE	NVVEREGLSI	RHPARFVLVG	SGNPEEGELR	PQLLDRFGLS	VEVRSPRDVE	206	
* * * * *												
↓ 157												
Hvu	LRVKIVEERA	RFDKPKTEFR	QSYLEEQDKL	QEQTISARSN	LGSVQLDHLV	RVKISQVCSE	LNVDGRLRGI	VNTRAYAKAL	AALKGKDVVT	VEDISTVIPT	302	
Gma	LRVKIVEERG	RFDKPKTEFR	DSYKAEQEKL	QQQITSARSV	LSSVQIDQDL	KVKISKVCAE	LNVDGRLRGI	VNTR.AKAL	AALKGRDNVS	AEDIATVIPN	392	
Nta	LRVKIVEERS	RFDKPKTEFR	ESYKAEQEKL	QNQIDSARNA	LSAVTIDHLD	RVKISKVCAE	LNVDGRLRGI	VNTR.ARAL	AALKGRDKVT	PEDIATVIPN	397	
Ath	LRVKIVEERA	RFDKPKTEFR	DTYKTEQDKL	QDQISTARAN	LSSVQIDREL	KVKISRVCS	LNVDGRLRGI	VNTR.AKAL	AALKGKDRVT	PDDVATVIPN	395	
Rca	TRVEVIRRRD	TYDADPKAFL	EEWRPKMDMI	RNQILEARER	LPKVEAPNTA	LYDCAALCIA	LGSDGLRGEL	TLLRS.ARAL	AALLEGATAVG	RDHLKRVATM	321	
Rsp	TRVEVITRRD	AYDADHDAFM	EKWAEDMQL	RGRILGARAA	LPQLKTPNTV	LHDCALCIA	LGSDGLRGEL	TLLRA.ARAQ	AAFEGEAAVG	RSHLRSVATM	305	
* * * * *												
Hvu	VLRHRLRKDP	LESIDSGLLV	VEKFYEVFG	331								
Gma	CLRHRLRKDP	LESIDSGLLV	TEKFYEVFS	421								
Nta	CLRHRLRKDP	LESIDSGVLV	VEKFYEVFA	426								
Ath	CLRHRLRKDP	LESIDSGVLV	SEKFAEIFS	424								
Rca	ALSHRLRRDP	LDEAGS TARV	ARTVEETLP	350								
Rsp	ALSHRLRRDP	LDEAGS VSRV	ERCVCVEVLP	334								
* * * * *												

Fig. 2. Polypeptide sequence alignment of 42-kDa subunits of Mg-chelatase. The barley sequence and 5 of the 14 sequences available in the GenBank Database: Hvu (*Hordeum vulgare*; barley; GenBank accession no. U26545), Gma (*Glycine max*; soybean; D45857), Nta (*N. tabacum*; tobacco; AF014053), Ath (*Arabidopsis thaliana*; thale cress; X91411), Rca (*Rhodobacter capsulatus*; Z11165), and Rsp [*R. sphaeroides*; AF017642 (AJ001690)]. The N-terminal sequence of the soybean, tobacco, and thale cress polypeptides is the same as that of the unprocessed proteins. Conserved amino acid residues in all 14 sequences are marked by asterisks. The species of origin of the 42-kDa subunits (not displayed) are *Porphyra purpurea* (U38804), *Odontella sinensis* (Z67753), *Cryptomonas phi* (Z21976), *Anabaena variabilis* (D49426), *Synechocystis* PCC6803 (U35144), *Olisthodiscus luteus* (Z21959), *Chlorobium vibrioforme* (Z83933), and *Heliobacillus mobilis* (AF080002). Arrows indicate the amino acid residues changed in mutants: L91F, D187N, and R269K. The tobacco *Sulfur* mutation is N269I.

barley *Xantha-h* sequence, the *Chlorina-125*, *-157*, and *-161* mutations are G559A (resulting in amino acid exchange D187N), G806A (R269K), and C271T (L91F), respectively (indicated by arrows in Fig. 2). The original *Xantha-h* sequence (4) very likely contains a sequencing mistake, which appears as a CTA insertion at positions 831 to 833. The extra codon adds a tyrosine residue to the polypeptide sequence at position 278 (barley numbering). This tyrosyl has no homology in the amino acid sequences of the 42-kDa subunits of other species and causes a gap in polypeptide alignments (Fig. 2). The tobacco *Sulfur* mutation is an amino acid substitution (N269I; ref. 11). This asparagine residue is also a completely conserved amino acid residue and corresponds to N175 in the barley sequence (indicated by an arrow in Fig. 2). As the N-terminal half of the 70-kDa subunit shows substantial similarity to the complete 42-kDa subunit, it is interesting to note that the four mutations change amino acid residues that are conserved in an alignment between the two subunits.

The *Chlorina-125*, *-151*, and *-161* Mutants Contain the 42-kDa Subunit Protein. It has been shown that the recessive *xantha-h* mutants devoid of Mg-chelatase activity lack the 42-kDa but not the 140-kDa subunit (4). Therefore, it was of interest to determine whether three semidominant missense mutations in the same gene result in stable Mg-chelatase subunits. Western blot analyses with antibodies specific for the 42-, 70-, and 140-kDa subunits were performed according to the method of Hansson *et al.* (18). The homozygous *Chlorina* mutants contained all three subunits (Fig. 3). The recessive *xantha-f¹⁰* mutant also contains all three subunits and has been diagnosed as having a missense mutation in the 140-kDa subunit. In contrast, the *xantha-g²⁸* mutant lacked the 70-kDa subunit, and the *xantha-h⁵⁶* mutant lacked both the 42- and 70-kDa subunits.

In Vitro Complementation and Reconstitution of Mg-Chelatase Activity with Mutant Extracts. *In vitro* complementation of barley Mg-chelatase activity takes place when stroma fractions of the *xantha-f*, *-g*, and *-h* mutants are combined pairwise (5). Analogous complementation experiments were performed with the stroma proteins of *Chlorina-161* (Table 1). The wild-type stroma had a Mg-chelatase activity that yielded 188 pmol of Mg-deuteroporphyrin IX in 20 min. The homozygous mutant had no activity, whereas the stroma proteins of the heterozygous seedlings had 21.6% of wild-type activity (per mg of protein). Soluble stroma of homozygous *Chlorina-161* was mixed with soluble stroma proteins from homozygous *xantha-f¹⁰*, *-g²⁸*, or *-h⁵⁶* mutants and assayed for Mg-chelatase activity. Stroma of *Chlorina-161* mixed with stroma from

Table 1. *In vitro* complementation of Mg-chelatase activity with barley chloroplast extracts of wild-type and *Chlorina* and *xantha* mutants.

Plastid stroma assayed	Activity, pmol/20 min	Subunits present
Wild-type	188	F, G, H
<i>Chlorina-161</i> (<i>Clo/Clo</i>)	0.0	F, G, h
<i>Chlorina-161</i> (<i>clo/clo</i>)	3.0	F, G, H, h
<i>xantha-h⁵⁶</i> (<i>xan/xan</i>)	0.0	F
<i>xantha-f¹⁰</i> (<i>xan/xan</i>)	0.0	f, G, H
<i>xantha-g²⁸</i> (<i>xan/xan</i>)	0.0	F, G
<i>Chlorina-161</i> (<i>Clo/Clo</i>) + <i>xantha-h⁵⁶</i>	0.0	F, G, h
<i>Chlorina-161</i> (<i>Clo/Clo</i>) + <i>xantha-f¹⁰</i>	4.1	F, f, G, H, h
<i>Chlorina-161</i> (<i>Clo/Clo</i>) + <i>xantha-g²⁸</i>	0.0	F, G, H, h
<i>Chlorina-161</i> + wild-type	181	F, G, H, h
<i>xantha-h⁵⁶</i> + wild-type	185	F, G, H
<i>xantha-f¹⁰</i> + wild-type	176	F, f, G, H
<i>xantha-g²⁸</i> + wild-type	146	F, G, H

Subunits: F = 140 kDa, G = 70 kDa, H = 42 kDa, h = inactive 42 kDa, f = inactive 140 kDa. The amounts of protein used in 50- μ l assays were 725 μ g of wild-type plastid stroma, 138 μ g of *Chlorina-161* (*Clo/Clo*), 250 μ g of *Chlorina-161* (*clo/clo*), 275 μ g of *xantha-h⁵⁶*, 200 μ g of *xantha-f¹⁰*, and 150 μ g of *xantha-g²⁸*.

xantha-f¹⁰ showed slight activity. As expected, the addition of the *xantha-h⁵⁶* extract did not reconstitute Mg-chelatase activity of stroma from *Chlorina-161*, a result which is in agreement with the lack of the 42-kDa XANTHA-H subunit in the *xantha-h⁵⁶* cell extract (Fig. 3). The extract of *xantha-g²⁸* did not restore activity, probably because the protein concentrations attained were not high enough. The combinations of the mutant with the wild-type preparations in assays indicate the absence of inhibitors of Mg-chelatase in the mutant extracts.

The Mg-chelatase subunits of barley chloroplasts can be separated by high-speed centrifugation into a clear supernatant containing the 42-kDa XANTHA-H subunit and a pellet containing the 70-kDa XANTHA-G subunit (5). Both fractions contain the 140-kDa XANTHA-F subunit. The supernatant and pellet fractions individually showed little and no activity, respectively (Table 2, lines 4 and 5). Reconstitution of the pellet with the supernatant restored high activity (Table 2, lines 1–3). The stromata of the three *chlorina* wild types (*clo/clo*) had high Mg-chelatase activity, whether tested alone or with fractions containing XANTHA-H or XANTHA-G (Table 2). Activity expressed on a protein basis could indicate some reduction in activity when the pellet fraction was added. Mg-chelatase activity in extracts of the homozygous mutants *Chlorina-125*, *-157*, and *-161* (*Clo/Clo*) was reconstituted only when the 42-kDa XANTHA-H subunit in the supernatant fraction of wild type was supplied (Table 2). This result shows that the 42-kDa subunits present in the *Chlorina* mutants are functionally defective. The assays of the stroma preparations of the heterozygous *Chlorina-125* and *-161* genotypes (*Clo/clo*) showed a reduced Mg-chelatase activity, ranging from 25 to 50% of the activity present in the wild type, which could be normalized by supplying wild-type 42-kDa subunits but not by providing the 70-kDa subunits. The stroma preparation of the *Chlorina-157* heterozygotes had an unexpectedly high Mg-chelatase activity, which might be caused by misclassification of the seedlings extracted (i.e., an inadvertent admixture of some wild-type leaves).

DISCUSSION

In prokaryotic and eukaryotic photosynthetic organisms, the insertion of Mg²⁺ into the natural substrate protoporphyrin IX or the more water-soluble *in vitro* substrate deuteroporphyrin IX requires the interaction of three protein subunits with nominal molecular masses of 140, 70, and 42 kDa. The reaction

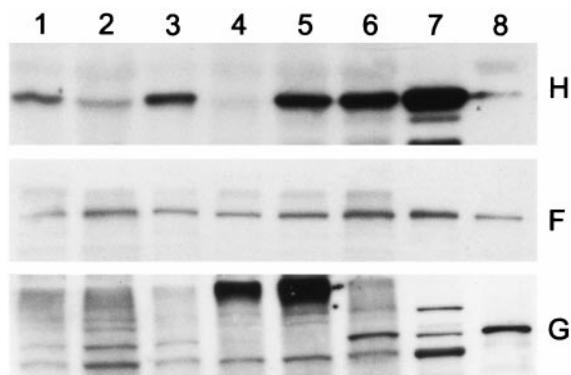


FIG. 3. Western blot analysis of the barley Mg-chelatase subunits XANTHA-H (Top), XANTHA-F (Middle), and XANTHA-G (Bottom). Lanes: 1, *Chlorina-125*; 2, *Chlorina-157*; 3, *Chlorina-161*; 4, *xantha-h⁵⁶*; 5, *xantha-g²⁸*; 6, *xantha-f¹⁰*; 7, wild-type chloroplast high-speed supernatant; and 8, wild-type chloroplast high-speed pellet. The molecular masses of the standard bands labeled in lanes 7 and 8 are 42 kDa for H, 150 kDa for F, and 76 kDa for G.

Table 2. Reconstitution assays of Mg-chelatase activity in soluble chloroplast extracts from barley *Chlorina* mutants

Plastid stroma	Wild-type subunits supplied	Mg-chelatase activity	
		pmol/30 min	pmol/min/mg protein
Wild-type	None	164 ± 64	17.6 ± 3.0
	F, H	318 ± 11	19.9 ± 2.7
	F, G	148 ± 66	4.78 ± 3.43
None	F, H	18.6	2.38
	F, G	0.0	0.0
	F, G, H	307	10.9
	None	0.0	0.0
<i>Chlorina-125 (Clo/Clo)</i>	F, H	49.7	4.04
	F, G	0.0	0.0
	None	0.0	0.0
<i>Chlorina-157 (Clo/Clo)</i>	F, H	64.4	6.71
	F, G	0.0	0.0
	None	0.0	0.0
<i>Chlorina-161 (Clo/Clo)</i>	F, H	141	9.15
	F, G	0.0	0.0
	None	0.0	0.0
<i>Chlorina-125 (Clo/clo)</i>	F, H	317	18.9
	F, G	35.5	1.21
	None	201	16.7
<i>Chlorina-157 (Clo/clo)</i>	F, H	294	14.9
	F, G	152	4.70
	None	81.2	6.59
<i>Chlorina-161 (Clo/clo)</i>	F, H	314	15.6
	F, G	71.0	2.16
	None		

Wild-type 42-kDa subunits (H) were separated into a high-speed supernatant from pelleted wild-type 70-kDa subunits (G). The 140-kDa subunit (F) was present in both fractions. These wild-type subunits were supplied to reconstitution assays of Mg-chelatase activity. The amounts of protein used in 50- μ l assays were 260 μ g of supernatant (F, H), 680 μ g of pellet (F, G), 150 μ g of *Chlorina-125 (Clo/Clo)*, 300 μ g of *Chlorina-125 (Clo/clo)*, 159 μ g of *Chlorina-125 (clo/clo)*, 60 μ g of *Chlorina-157 (Clo/Clo)*, 399 μ g of *Chlorina-157 (Clo/clo)*, 420 μ g of *Chlorina-157 (clo/clo)*, 255 μ g of *Chlorina-161 (Clo/Clo)*, 411 μ g of *Chlorina-161 (Clo/clo)*, and 300 μ g of *Chlorina-161 (clo/clo)*.

has an absolute requirement for ATP and proceeds in two steps (22, 23). In a first activation step, the 70- and 42-kDa subunits interact, resulting in ATP hydrolysis as well as synthesis of ATP from ADP by the intrinsic phosphate-exchange activity of the 42-kDa subunit (9).

The mutations in the three semidominant barley mutants *Chlorina-125*, *-157*, and *-161* and in the tobacco *Sulfur* mutation cause four different amino acid substitutions in amino acid residues that are conserved in all 14 known primary structures of the 42-kDa subunit. Western blot analyses of the three barley mutants indicate that the mutated protein is maintained stably in the plastids, and the absence of Mg-chelatase activity in the yellow homozygous *Chlorina (Clo/Clo)* mutant seedlings proves that the 42-kDa XANTHA-H subunit is mutated into an inactive form. The heterozygous barley seedlings have only 25–50% of the Mg-chelatase activity observed in the wild type. The expression of the active and the inactive 42-kDa subunit in the heterozygous barley seedlings can lead to two types of heterodimers between the strongly interacting 42-kDa and 70-kDa subunits. One heterodimer will consist of wild-type 42-kDa and 70-kDa subunits (H + G), and the other will consist of mutated 42-kDa and wild-type 70-kDa subunits (h + G). Therefore, the reduced Mg-chelatase activity would be explained by the capacity of the heterodimers consisting of mutated 42-kDa protein and wild-type 70-kDa protein to bind to the 140-kDa subunit. However, because these heterodimers cannot carry out the activation step, their association with the third subunits (h + G + F) leads to inactive Mg-chelatase. If the 42-kDa and 70-kDa subunits were to dissociate after the activation step and an activated 70-kDa subunit alone is to interact with the 140-kDa subunit as suggested (8), we would not expect a codominant action of the inactive 42-kDa subunit, a reduction in Mg-chelatase activity, and a diminished chlo-

rophyll content. Under this hypothesis, the heterodimers of an inactive 42-kDa with a 70-kDa subunit would fail to be activated and to dissociate. In heterozygotes, this failure would leave only the activated 70-kDa subunits that had been associated with wild-type 42-kDa subunits to interact with the 140-kDa subunit. Such a recessive action actually occurs in heterozygotes containing mutated 140-kDa subunits and in heterozygous *Xantha-h* mutants that fail to produce a 42-kDa subunit protein.

In contrast to the *Chlorina* mutants, the originally isolated and analyzed *xantha-h* mutants are recessive. The heterozygotes of the recessive mutants *xantha-h*³⁰, *-h*³⁸, *-h*⁵⁶, and *-h*⁵⁷ are normal green, are indistinguishable from wild type, and have no reduced Mg-chelatase activity. The homozygous lethal mutants lack the 42-kDa subunits, as determined by Western blot analysis. Of the three recessive *xantha-h* mutants analyzed by Northern blotting, two were devoid of transcripts of the gene, and the third had a truncated transcript. In the absence of a nonfunctional 42-kDa protein in the heterozygotes, only wild-type 42-kDa subunits are present, and these interact in the standard way with the 70-kDa and 140-kDa subunits. (In view of these results, the three *Chlorina* mutants should be renamed *Xantha-h*^{clo 125}, *-h*^{clo 157}, and *-h*^{clo 161}.)

Mutations in the *Xantha-f* gene like *xantha-f*¹⁰ that produce an inactive but stable 140-kDa subunit (Fig. 3; ref. 4) are recessive and thus show no interference with association of wild-type subunits in the heterozygotes. Considering the observation that major deletions of 70-kDa protein domains still can support functional Mg-chelatase activity, we expect some mutated 70-kDa subunits to show recessive behavior (8). However, mutations in the 70-kDa subunit that affect the interaction with the 42-kDa subunit are likely to be codominant. The recessive *xantha-g*²⁸ mutant investigated here lacked

only the 70-kDa subunit (Fig. 3), and Mg-chelatase activity can be reconstituted by supplying this subunit (5). The *xantha-h*⁵⁶ mutant preparation analyzed here seems to lack both the 42- and 70-kDa protein, although exclusively supplying the 42-kDa protein readily compensated for the lack of activity in the stroma of *xantha-h*⁵⁷ (5). We interpret this inconsistency to indicate that, in the absence of a 42-kDa subunit, the 70-kDa subunit may have reduced stability and may be sensitive to proteolytic degradation in certain mutants and during certain developmental stages.

A nucleotide phosphate binding site of the type GX₄GKS has been recognized in the 42-kDa subunit (4). This subunit has unique properties. By itself, the subunit catalyzes the exchange of one phosphate group of ATP to an ADP molecule, generating ADP and ATP, respectively (9). The binding of the 42- and 70-kDa subunits elicits a strong ATPase activity, hydrolyzing ATP to ADP plus free phosphate. This hydrolysis is characteristic of the activation reaction. Inhibitor studies indicate that both subunits contribute to the active site. The 140-kDa subunit that binds the protoporphyrin IX substrate has a weak ATPase activity by itself and seems to have two ATP binding sites that react differently to NaF inhibition (9).

An enhancement of ATPase activity is characteristic for the association in *Escherichia coli* of the 70-kDa DnaK heat-shock protein with the 41-kDa DnaJ heat-shock protein. These proteins are used as chaperones in refolding denatured polypeptides (24, 25) but also function in the conversion of, for example, the 32-kDa RepA initiator protein from a dimer to a monomer that binds to the origin of replication of the P1 plasmid (26, 27). In both cases, a third heat-shock protein, the 22-kDa GrpE protein, interacts with the other two. The three proteins are also essential for the replication of λ -phage DNA (28). In this case, ATPase activity of DnaK is stimulated up to 50-fold in the simultaneous presence of the DnaJ and GrpE proteins. The GrpE protein alone increases the rate of release of bound ATP or ADP without affecting the rate of hydrolysis and is thought to expedite the exchange of ADP to ATP on DnaK. In eukaryotic chaperones, the required phosphate-exchange activity postulated in *E. coli* for the GrpE protein has been identified recently as an intrinsic activity in the bovine heat-shock protein Hsp70 (13). In Hsp70, the interaction with protein substrate is regulated by ATP hydrolysis and ADP-to-ATP exchange. When bound to ADP, Hsp70 binds tightly to protein substrates, but when Hsp is bound to ATP, the peptides are released (29). ATP binding, but not ATP hydrolysis per se, is essential for substrate dissociation (30). In the binding and release cycle, ATP hydrolysis and ADP-to-ATP exchange are the methods by which Hsp70 cycles between the ADP-Hsp70 and ATP-Hsp70 complexes.

Although there is no obvious homology between the primary structure of Hsp70 and the 42-kDa subunit of Mg-chelatase, the similar use of ATP and ADP by these proteins provides an incentive to explore the functions of the 42-kDa subunit in controlling the folding and conformation of its own and the other two subunits. The result of the codominant action on the Mg-chelatase activity in heterozygotes of wild-type and mutant 42-kDa subunits with single amino acid changes leads us to the following working hypothesis. The ATP-to-ADP exchange reaction promotes the binding of the 42-kDa subunit to the 70-kDa subunit in a manner similar to the binding of Hsp70 with an unfolded protein. Once the two subunits are bound, the ATPase activity makes it possible for

these subunits to bind the 140-kDa subunit. All three subunits bound together insert Mg²⁺ into protoporphyrin with ATP hydrolysis.

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- Gibson, L. C., Willows, R. D., Kannangara, C. G., von Wettstein, D. & Hunter, C. N. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 1941–1944.
- Willows, R. D., Gibson, L. C. D., Kannangara, C. G., Hunter, C. N. & von Wettstein, D. (1996) *Eur. J. Biochem.* **235**, 438–443.
- Jensen, P. E., Gibson, L. C. D., Henningsen, K. W. & Hunter, C. N. (1996) *J. Biol. Chem.* **271**, 16662–16667.
- Jensen, P. E., Willows, R. D., Petersen, B. L., Vothknecht, U. C., Stumann, B. M., Kannangara, C. G., Henningsen, K. W., von Wettstein, D. & Henningsen, K. W. (1996) *Mol. Gen. Genet.* **250**, 383–394.
- Kannangara, C. G., Vothknecht, U. C., Hansson, M. & von Wettstein, D. (1997) *Mol. Gen. Genet.* **254**, 85–92.
- Papenbrock, J., Gräfe, S., Kruse, E., Hänel, F. & Grimm, B. (1997) *Plant J.* **12**, 981–990.
- Walker, C. J. & Willows, R. D. (1997) *Biochem. J.* **327**, 321–333.
- Gräfe, S., Saluz, H.-P., Grimm, B. & Hänel, F. (1999) *Proc. Natl. Acad. Sci. USA* **96**, in press.
- Hansson, M. & Kannangara, C. G. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 13351–13356.
- Henningsen, K. W., Boynton, J. E. & von Wettstein, D. (1993) *R. Dan. Acad. Sci. Lett. Biol. Skr.* **42**, 1–349.
- Nguyen, L. V. (1995) Ph.D. thesis (North Carolina State Univ., Raleigh, NC).
- Kjemtrup, S., Sampson, K. S., Peele, C. G., Nguyen, L. V., Conkling, M. A., Thompson, W. F. & Robertson, D. (1998) *Plant J.* **14**, 91–100.
- Hiromura, M., Yano, M., Mori, H., Inoue, M. & Kido, H. (1998) *J. Biol. Chem.* **273**, 5435–5438.
- Simpson, D. J., Machold, O., Høyer-Hansen, G. & von Wettstein, D. (1985) *Carlsberg Res. Commun.* **50**, 223–238.
- Simpson, D. J. & von Wettstein, D. (1993) *Barley Genet. Newsletter* **21**, 102–108.
- Jende-Strid, B. (1978) *Barley Genet. Newsletter* **8**, 55–57.
- Murray, H. G. & Thompson, W. F. (1980) *Nucleic Acids Res.* **8**, 4321–4325.
- Hansson, M., Gough, S. P., Kannangara, C. G. & von Wettstein, D. (1997) *Plant Physiol. Biochem.* **35**, 827–836.
- Fling, S. P. & Gregerson, D. S. (1986) *Anal. Biochem.* **155**, 83–88.
- Schägger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379.
- von Wettstein, D., Kahn, A., Nielsen, O. F. & Gough, S. P. (1974) *Science* **184**, 800–802.
- Walker, C. J., Hupp, L. R. & Weinstein, J. D. (1992) *Plant Physiol. Biochem.* **30**, 263–269.
- Walker, C. J. & Weinstein, J. D. (1994) *Biochem. J.* **299**, 277–284.
- Hendrick, J. P. & Hartl, F.-U. (1993) *Annu. Rev. Biochem.* **62**, 349–384.
- Szabo, A., Langer, T., Schröder, H., Flanagan, J., Bukau, B. & Hartl, F.-U. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 10345–10349.
- Wickner, S. H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2690–2694.
- Wickner, S., Hoskins, J. & McKenney, K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7903–7907.
- Liberek, K., Marszałek, J., Ang, D., Georgopoulos, C. & Zylicz, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2874–2878.
- Lewis, M. J. & Pelham, H. R. B. (1985) *EMBO J.* **4**, 3137–3143.
- Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J. & Fink, A. L. (1993) *Nature (London)* **365**, 664–666.