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Genetic Regulation of Chlorophyll Synthesis Analyzed with Mutants in Barley

Abstract. *Barley seedlings homozygous both for the* xantha-l^{35} *and* tigrina-d^{12} *mutation accumulate magnesium protopophyrins and other precursors of chlorophyllide constitutively in darkness. The homozygous double mutant* xantha-f^{10}, tigrina-o^{34} *produces protoporphyrin constitutively. These results provide evidence for the control of chlorophyllide synthesis in higher plants through the products of regulatory genes in the nucleus.*

Among mutants in barley (*Hordeum vulgare* L.) that affect chloroplast development and greening, several *xantha* (*xan*) mutants and one *albina* (*alb*) mutant have blocks in chlorophyll synthesis (*1*, *2*). A number of *tigrina* (*tig*) mutants accumulate protochlorophyllide in darkness to a greater extent than does the wild type (*3*). The two groups of genes have been interpreted to be structural and regulatory genes, respectively, for chlorophyll synthesis (*3*). On this basis, certain predictions can be made about the phenotypes of double mutants containing both a structural and regulatory gene mutation. We now describe phenotypic characteristics of two double mutant genotypes.

Dark-grown barley seedlings accumulate in their etioplasts during the first 7 days a certain amount of protochlorophyllide, the immediate precursor of chlorophyllide. During further etiolation the quantity of protochlorophyllide declines (*4*). Illumination that results in the reduction of protochlorophyllide to chlorophyllide induces the further synthesis of protochlorophyllide. The following evidence supports the notion that the synthesis of protochlorophyllide from δ-aminolevulinate in etioplasts is regulated by repression and induction of an enzyme that catalyzes δ-aminolevulinate formation. Feeding the porphyrin precursor δ-aminolevulinate to dark-grown seedling leaves results in the accumulation of large amounts of protochlorophyllide and minor amounts of other porphyrins within the etioplasts (*2*, *5*), thus revealing that endogenous δ-aminolevulinate is the limiting factor in protochlorophyllide biosynthesis. The enzyme forming δ-aminolevulinate has a faster turnover than enzymes converting δ-aminolevulinate into protochlorophyllide (*6*). New synthesis of protochlorophyllide after an initial lighting is the result of the induction of δ-aminolevulinate synthesis (*7*, *8*). The induced synthesis of δ-aminolevulinate can be abolished by cycloheximide (*8*), an inhibitor of protein synthesis on cytoplasmic ribosomes (*9*).

While dark-grown structural gene mutants with blocked protochlorophyllide synthesis do not accumulate porphyrin precursors in the absence of exogenous δ-aminolevulinate, they pile up large amounts of different porphyrins and small or only trace amounts of protochlorophyllide when their leaves are supplied with δ-aminolevulinate (Table 1). Alleles at two structural gene loci were used in this investigation. Mutants homozygous for *xan-f*^{10} accumulate protoporphyrin, whereas mutants homozygous for *xan-l*^{35} produce in addition to protoporphyrin large quantities of magnesium protoporphyrin and its monomethyl ester (*2*) (Fig. 1, c and d). We infer provisionally that the *xan-f* gene codes for a protein participating in the insertion of magnesium into protoporphyrin IX and that the *xan-l* gene codes for a protein catalyzing a step between magnesium protoporphyrin monomethyl ester and protochlorophyllide. Since mutant seedlings do not accumulate these porphyrins unless supplied with exogenous δ-aminolevulinate, we conclude that the repression of δ-aminolevulinate synthesis is not affected by the lesions. This is supported by measurements of δ-aminolevulinate pools in the mutants (*8*). Induction of δ-aminolevulinate synthesis upon illumination does not occur in the almost completely blocked mutant *xan-f*^{10}, but takes place to some extent in the leaky *xan-l*^{35} (*8*). The latter result indicates that the photoreduction of protochlorophyllide to chlorophyllide a is a prerequisite for the induction of δ-aminolevulinate synthesis by light.

The *tigrina* mutants listed in Table 2 under regulatory genes accumulate amounts of protochlorophyllide in the dark, which exceed those produced in the wild type by 1.5 to 15 times. The four mutant alleles in the *tig-b* gene differ in the amount of protochlorophyllide produced in the seedling leaves.

Table 1. Structural genes involved in chlorophyll biosynthesis in barley. Data on the origin and genetics of these lethal and sublethal mutants are found in (*10*).

Structural genes	Mutant alleles		Phenotype of homozygous mutant seedlings in the dark	
	Number	Nature	Porphyrins accumulated upon feeding δ-aminolevulinate (*2*)	Carotene content (*11*)
xan-f	8 (2 leaky)	Recessive	Protoporphyrin	
xan-g	5 (2 leaky)	Recessive	Protoporphyrin	
xan-h	4	Recessive	Protoporphyrin	
xan-l	1 (leaky)	Recessive	Mg-protoporphyrins + protoporphyrin	
xan-u	1 (leaky)	Recessive	Protoporphyrin + uroporphyrinogen	Blocked in β-carotene synthesis, accumulate aliphatic polyenes
alb-e	1 (leaky)	Recessive	Protoporphyrin	Reduced content

The relaxed synthesis of protochlorophyllide in these mutants is considered to be the result of mutations in regulatory genes leading to a more or less constitutive synthesis of protochlorophyllide in the dark. Gough and Granick (*8*) have in fact secured evidence that the endogenous synthesis of δ-aminolevulinate in *tig-d*^{12}, *tig-o*^{34}, and *tig-b*^{23} mutants is not repressed in the dark, as it is in the wild type. This indicates that the repression of a δ-aminolevulinate–forming enzyme is defective in the mutants. With regard to protochlorophyllide accumulation, wild-type seedlings supplied with exogenous δ-aminolevulinate are phenocopies of the constitutive *tigrina* mutants.

Dark-grown seedlings of double mutants containing both a *xantha* and a *tigrina* mutant gene in homozygous form are expected to accumulate porphyrins constitutively. More specifically, the double mutants should accumulate without an external supply of δ-aminolevulinate the same porphyrins as the homozygous, single structural gene mutants when the latter are supplied with δ-aminolevulinate.

In a series of diallelic crosses between *xantha* and *tigrina* mutants, F_1 plants heterozygous for *xan-l*^{35} and *tig-d*^{12} were selected by progeny testing. Kernels of these plants were germinated in the dark (*2*), and the 6-day-old dark-grown seedlings were sorted into four classes by removing apical 1-cm segments of the primary leaves and then illuminating them overnight in a moist chamber. The leaf segments of seedlings in class 1 green overnight; those in class 2 remain yellow; those in class 3 are green in darkness (accumulated protochlorophyllide) and bleach in the light; and those in class 4 are yellow and bleach in the light. Among 189 seedlings 118 belonged to class 1, 25 to class 2, 32 to class 3, and 14 to class 4—a ratio not significantly differing from a dihybrid segregation ($\chi^2 = 5.3$; $.2 > P > .1$ for a 9 : 3 : 3 : 1 ratio). Class 4 comprises the double mutants, class 3 seedlings are homozygous for *tig-d*^{12}, class 2 seedlings are homozygous for *xan-l*^{35}, and class 1 seedlings contain at least one wild-type allele in both loci. Seedlings heterozygous for *tig-d*^{12} and homozygous or heterozygous for the *xan* wild-type allele could be recognized by a yellow tip on the greening leaf segment. Only 36 such seedlings were counted, which indicates the identification to be unreliable, and they are therefore pooled with the other two genotypes in class 1.

A progeny from F_1 plants heterozygous for *xan-f*^{10} and *tig-o*^{34}, consisting of 177 dark-grown seedlings, could be sorted into three classes: 104 greening seedlings, 20 green seedlings bleaching to white in the light, and 53 remaining yellow in the light ($\chi^2 = 7.21$; $P \sim .025$ for a 9 : 3 : 4 ratio). The greening seedlings contain at least one wild-type allele in both loci; the protochlorophyllide accumulating seedlings which bleach by photooxidation to white are homozygous for *tig-o*^{34}; but the yellow seedlings comprise all genotypes homozygous for *xan-f*^{10} including the double mutants expected to accumulate protoporphyrin. Double mutants could be identified by spectrophotometry through their absorption characteristic in the 635-nm region (see below).

Figure 1 shows absorption spectra recorded from single, dark-grown leaves with a Cary 17 spectrophotometer equipped with the scattered transmission accessory and an EMI 9659 QB photomultiplier tube. The identifications of pigments in this study rest on the correlations established previously (*1, 2, 10*) between spectra made in vivo and the chromatographic and spectroscopic identifications of the pigments extracted from single gene mutant and wild-type seedling leaves grown in darkness with and without the feeding of δ-aminolevulinate. The spectrum of a leaf from the leaky *xan-l*^{35}/*xan-l*^{35} mutant (Fig. 1a) reveals the presence of nearly wild-type amounts of protochlorophyllide. The mutant *tig-d*^{12}/*tig-d*^{12} accumulates large amounts of inactive protochlorophyllide (Fig. 1a). The double mutant, *xan-l*^{35}/*xan-l*^{35}, *tig-d*^{12}/*tig-d*^{12} obtained in the F_2 from a cross between a heterozygous *xan-l*^{35} plant with a heterozygous *tig-d*^{12} plant shows a mixture of

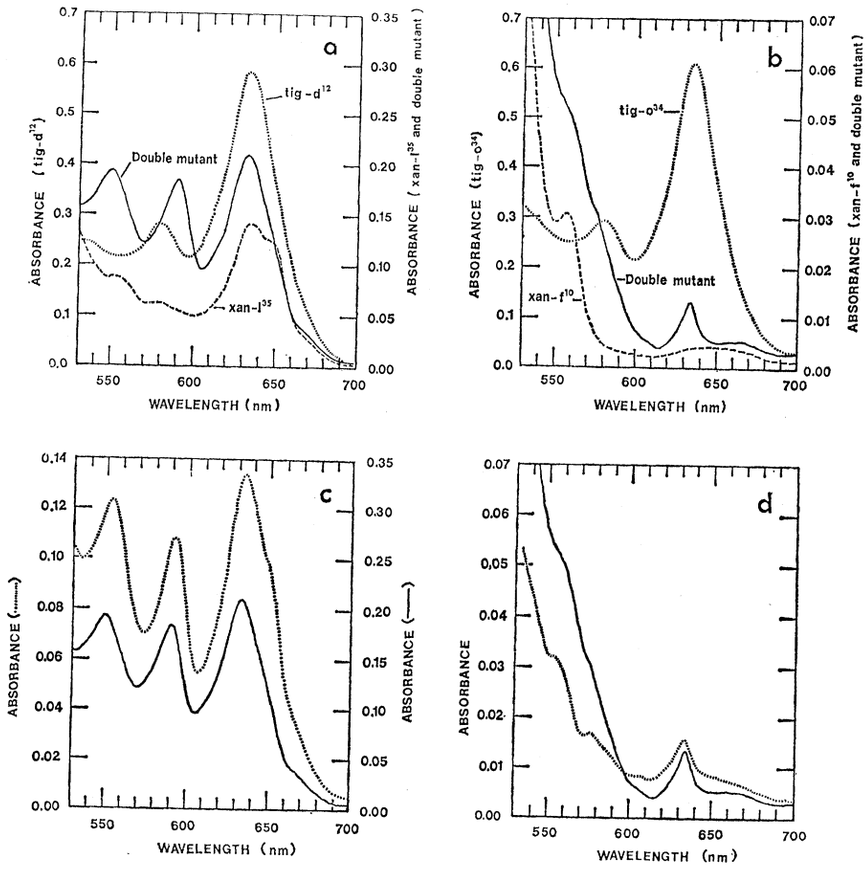

Fig. 1. Absorption spectra of single primary leaves of barley seedlings grown for 7 days in darkness. (a and b) No feeding with δ-aminolevulinate; genotypes of seedlings as designated. (c) *xan-l*^{35}/*xan-l*^{35}, *tig-d*^{12}/*tig-d*^{12} (——) and its phenocopy, *xan-l*^{35}/*xan-l*^{35} after uptake of $5 \times 10^{-3}M$ δ-aminolevulinate for 9 hours by the latter only (· · · ·). (d) *xan-f*^{10}/*xan-f*^{10} *tig-o*^{34}/*tig-o*^{34} (——) compared with *xan-f*^{10}/*xan-f*^{10} after uptake of $5 \times 10^{-4}M$ δ-aminolevulinate for 16 hours by the latter only (· · · ·).

Table 2. Regulatory genes involved in the regulation of chyorophyll biosynthesis in barley (*10*).

Regulatory genes	Mutant alleles		Phenotype of homozygous seedlings in the dark	
	Number	Nature	Protochlorophyllide accumulated (*3*)	Carotene content (*11*)
tig-b	4	Semidominant	2 to 10 times wild type, depending on allele	Accumulate ζ-carotene
tig-d	1	Semidominant	15 times wild type	Same as wild type
tig-n	1	Recessive	1.5 times wild type	Increased content, accumulate lycopenic pigments
tig-o	1	Recessive	5 times wild type	Accumulate lycopene

porphyrins with adsorption maxima at 550, 590, and 635 nm (Fig. 1a); these are typical for absorption spectra of pigment mixtures containing primarily protoporphyrin, magnesium protoporphyrin and its monomethyl ester, and, in addition, protochlorophyllide (*1, 2*). In Fig. 1c the absorption spectrum of a double mutant is compared with that of its phenocopy—that is, a homozygous *xan-l*^{35} mutant fed with δ-aminolevulinate. There is good qualitative agreement between the spectrum of the phenocopy known to contain protoporphyrin, magnesium protoporphyrin, and magnesium protoporphyrin monomethyl ester as well as protochlorophyllide, and the spectrum of the double mutant.

The double mutant isolated after crossing heterozygous *xan-f*^{10} with heterozygous *tig-o*^{34} accumulates protoporphyrin constitutively as evidenced by the spectrum in Fig. 1b. Three characteristics make the spectrum of the double mutant diagnostic for protoporphyrin IX: the ratio of absorption at 635 nm to that at 578 nm, the extreme symmetry of the 635-nm peak giving an absorption minimum at 615 nm, and an adjacent shoulder in the 605-nm region. Figure 1b also shows the absence of detectable quantities of protochlorophyllide in *xan-f*^{10} and the large amount of protochlorophyllide in the other parent, *tig-o*^{34}. The spectrum of the double mutant in the 635-nm region (Fig. 1d), where protoporphyrin has a large absorption, resembles closely the spectrum of homozygous *xan-f*^{10} fed δ-aminolevulinate. The latter phenocopy of the double mutant has been produced by choosing a suitable low concentration and time for δ-aminolevulinate feeding. The red shift of the spectrum of the double mutant with reference to the spectrum of the δ-aminolevulinate fed *xan-f*^{10} in the 530- to 590-nm region is presumably due to the accumulation of lycopene caused by the *tig-o*^{34} allele (*11*).

The results obtained with the homozygous double mutants *xan-l*^{35} *tig-d*^{12} and *xan-f*^{10} *tig-o*^{34} support the hypothesis that the nuclear genes *tig-d* and *tig-o* are regulatory genes for δ-aminolevulinate formation, and more specifically for a δ-aminolevulinate synthesizing enzyme. The *xan-l* and *xan-f* nuclear genes appear to be structural genes for components of the pathway between δ-aminolevulinate and protochlorophyllide.

All mutant alleles of the structural genes are recessive (Table 1). The mutant alleles in at least two of the regulatory genes are semidominant as may be expected, if their products interact with other gene loci. The semidominance is expressed in darkness as increased, initially inactive, protochlorophyllide content in heterozygous seedlings, relative to homozygous wild-type seedlings. In light-dark cycles the leaf tips of such heterozygous seedlings are frequently separated from the rest of the leaf by a necrotic band.

The four regulatory genes specify molecules with different functions and probably different target genes. This is revealed by the carotene content and the ultrastructure of the etioplasts in dark-grown homozygous mutants of these genes (*3, 11*) (Table 2). The carotene content and the ultrastructure of the etioplast membranes are indistinguishable from wild type in *tig-d*^{12}. In alternating light and dark cycles the primary leaves of this mutant are zebra-striped (that is, tigrina) and the mutant is a seedling lethal. In continuous light the mutant is viable and uniformly green. This suggests that the product of the *tig-d* gene functions exclusively in the repression of δ-aminolevulinate synthesis in darkness and is dispensable in light. It is a good candidate for the repressor of a δ-aminolevulinate–forming enzyme in etioplasts. Of course there are additional possibilities; *tig-d*^{12} could be an operator or promoter constitutive mutant adjacent to a gene coding for the δ-aminolevulinate–forming enzyme. The other regulatory gene mutants for protochlorophyllide synthesis have lesions in the synthesis of carotenes when grown in the dark. Intermediates of the β-carotene pathway accumulate, and are accompanied by characteristic membrane aberrations (*3, 11*). The products of these genes apparently play a role in the coordinated function of the chlorophyll and carotene pathways in plastid development. The close physiological connection between the formation of these two classes of photosynthetic pigments is also indicated by *xan-u*^{21}, which simultaneously is blocked in porphyrin synthesis and the synthesis of β-carotene in darkness (*2, 11*).

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