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A mutator nuclear gene inducing a wide spectrum of cytoplasmically inherited chlorophyll deficiences in barley *

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Summary. Some striped plants were observed in plots of a long-grain mutant barley grown at a field nursery. All of the plants of these plots, which were naturally self pollinated, were individually harvested, and most of their progenies (92.5%) segregated seedlings carrying chlorophyll deficiencies (CD) as determined by greenhouse analysis. The majority of the mutant seedlings (84.3%) showed a pattern of longitudinal chlorophyll sectors. The spectrum of CD was wide among the solid mutant seedlings and consisted of three main types (*albina*, *viridis* and discontinuous). In association with some CD types morphological changes were frequently observed. Non-CD-associated morphological changes and diminished seed-set were scarce and, so far, none of them has proved to be inherited. Analysis of CD in reciprocal crosses and backcrosses proved that while CD were transmitted cytoplasmically their induction was controlled by a single nuclear mutator gene, active when homozygous. In addition once the CD were induced, they were expressed independently of the nuclear constitution. The results suggest that the mutator gene induces diverse mutational events on chloroplast (cp) DNA. In barley, as in other monocots, nuclear genes which are inductors of cytoplasmic genetic changes have been reported. However, all of them produced a narrower spectrum of CD and had a more rapid sorting-out of the cytoplasmic mutants than what we observed. On this basis a distinction between chloroplast and mitochondrial (mt) mutator genes is proposed. Accordingly, the chloroplast mutator here described would be the first one reported for monocots. Increased knowledge on this subject can play a fundamental role in elucidating organelle heredity and its interactions with the nuclear genome. Moreover, this material could be a valuable source of variability of the otherwise conservative genetic information encoded in the chloroplast.

Key words: Barley (*Hordeum vulgare*) – Chlorophyll deficiencies – Cytoplasmic inheritance – Mutator gene

Introduction

Since the pioneer experiments of Stadler (1930), chlorophyll deficiencies (CD) have been repeatedly used to estimate rates of both germinal (Constantin and Nilan 1982) and somatic (Prina and Favret 1988) artificial mutations. Spontaneous chlorophyll mutations seldom occur, and in barley their rates have been estimated to range between 0.018 to 0.069 per 100 spike-progenies. (Ehrenberg et al. 1956; Favret and Godeck 1959). In some higher plants, particularly maize, unstable genotypes associated with high frequencies of changes in chlorophyll pigmentation, among other characters, have been reported as originating in diverse chromosome instabilities or being due to nuclear genes that are mutable by controlling elements or by paramutation (Kirk and Tilney-Bassett 1978; Robertson 1978; Grandbastien 1987). Moreover, the highly conservative genetic information encoded in the chloroplasts (Sears 1983; Melzer and Kleinhofs 1987) can be altered by nuclear genes, some of which also seem to affect the genetic stability of the mitochondria. (Kirk and Tilney-Bassett 1978). Some evidence of nuclear genes involved in the induction of mitochondrial genetic changes has been observed in higher plants (Rhoades 1950; Lemke et al. 1988; Mackenzie et al. 1988; Newton 1988), while there is much more information on this subject in yeast (Backer and Foury 1985).

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Nuclear gene mutants which alter chloroplast genetic stability were classified by Kirk and Tilney-Bassett (1978) into two groups according to the width of the spectrum of mutant phenotypes they induce. *Iojap* maize is the classical example of the narrow-spectrum group (Jenkins 1924; Rhoades 1943) to which several barley types also belong (Kirk and Tilney-Bassett 1978). On the other hand, nuclear genes inducing several different cytoplasmically inherited chlorophyll types (wide spectrum) have been observed in *Arabidopsis* (Redei 1973; Redei and Plurad 1973), *Epilobium* (Michaelis 1970), *Oenothera* (Epp 1973), *Nepeta* (Woods and Du Buy 1951) and *Petunia* (Potrykus 1970), but they have not been reported in monocots. In the paper presented here one case in barley, which belongs to this second group of mutator genes, is genetically characterized.

Materials and methods

Seeds of a two-row cultivated barley (*Hordeum vulgare*) genotype, homozygous for the chromosome reciprocal translocation *T (6–7)a*, received several combined treatments of X-rays and sodium azide. One of those treatments consisted of applying 15 kR (carried out at 15 mA, 120 kV) to seeds with 12% humidity, plus a post-soaking in sodium azide aqueous solution (1 m*M*, pH 6, 20 h). In the second generation (M_2) following this treatment, an elongated-grain mutant plant was selected. In M_4 and M_5 plots of that long-grain mutant (MC169 = INTA Castelar accession number) grown at a field nursery, some striped plants of various colors were noticed. This fact led us to analyze chlorophyll deficiences (CD) in later generations of selfing, reciprocal crosses and backcrosses. Observations were made with reference to frequencies and types of CD (Gustafsson 1940).

In the greenhouse most data were registered on second-leaf seedlings, but in one experiment the plants were analyzed from the first to the sixth leaf. At the field nursery all of the leaves were carefully analyzed until heading.

The CD were classified into four main groups:

1) *albina*: white seedlings;
2) *viridis*: homochromous or grainy light-green seedlings;
3) discontinuous or positional variegated: seedlings show different pigmentation between the top and the bottom of the blade (e.g. *virido-albina*, *albo-viridis*, *xantha-alba*). In this type of variegation the different colors observed in one mutant were interpreted as having originated in physiological effects that altered the expression of the chloroplasts in particular regions of the leaves (Kirk and Tilney-Bassett 1978);
4) clonal or cell lineage variegated (striata): this includes all types of CD presented in longitudinal sectors with clearcut margins and without any positional preference. The shapes of the sectors were consistent with cellular proliferation, which in monocots runs parallel to the longitudinal axis of the leaves (Kirk and Tilney-Bassett 1978).

Mutants belonging to groups 1, 2 and 3 were considered to be solid or non-chimerical, while those of group 4 were considered to have a chimerical constitution.

The spectrum of CD was investigated considering only those progenies segregating solid mutant seedlings. We must mention here that all of the M_5 progenies taken into account came from the same M_2 plant; in this way the mutational events which occurred earlier could be represented many times on that spectrum. The same was true for the spectrum made with the F_4 plant progenies, which came from only 1 F_2 plant.

The management from one generation to the next was done in most cases as progeny of individual plants. In one experiment, the testing of progenies from selfing, four spikes from every M_5 plant were harvested. These were separately analyzed in the greenhouse according to the Stadler (1930) method for experimental mutagenesis.

Results

Progenies from selfing

After striped plants had been observed in M_4 and M_5 plots of MC169 barley, all of the M_5 plants, naturally self-pollinated, were individually harvested. Most of their progenies, 303 out of 327 (92.5%), showed CD in a greenhouse analysis at the second-leaf seedling stage. If the results are expressed in numbers of seedlings, CD were observed in 1311 out of 14,617 (9.0%) M_6 seedlings (Table 1).

On each M_5 plant progeny the analysis was carried out separately for the progeny from each spike, making it possible to detect a heterogeneous distribution of CD within the mother plants. Cases of the same CD type segregating in all of the spike progenies from 1 plant, thereby resembling a Mendelian segregation from a non-chimerical heterozygous mother plant, were not observed. Only 1 family was observed in which all of the seedlings in the progenies of the four spikes presented the same kind of CD, they being all solid mutants.

The majority of the M_6 mutant seedlings (84.3%) presented longitudinal chlorophyll deficient sectors (*striata*) that clearly differed from the artificially induced spectra where *striata* usually has been observed as a minor group (Nilan and Konzak 1961; Gaul 1964; Constantin et al. 1974). The spectrum of solid CD registered on the M_6 generation (Table 2) was wide and consisted of three main types: *viridis*, *albina* and discontinuous (see description in Material and methods). Within the *viridis* type there were several degrees of light-green and yellowish-green mutants. This variation in color also occurred with the discontinuous types, which additionally presented several positional patterns.

The most conspicuous morphological change was the narrowness of the leaves in most of the *albina* seedlings and in some of the *virido-albina* types. Non-CD-associated morphological changes have seldom been observed and, so far, none of them has proved to be inherited. The few cases of partial sterility, observed at harvesting as diminished seed-set, have not been inherited either.

Some of the plants carrying CD were transplanted to larger pots for observation until maturity. Most of the solid mutants died before heading, but some of them produced viable seeds. In this way it was possible to observe that their characteristics were transmitted to the

Table 1. Chlorophyll deficiences (CD) in the M_6 generation

	Second leaf seedlings		Plants until heading	
	Normal	Carrying CD	Normal	Carrying CD
Experiment I	13,306	1,311	[a]	[a]
Experiment II	70	8	1	66

[a] Data not registered

Table 2. Spectrum of solid chlorophyll deficiences (CD)

	Number of families segregating each type of CD[a]		
	Viridis	*Discontinuous*	*Albina*
M_5 plant progenies	78	14	10
F_4 plant progenies from crosses (Normal × MC169)	84	5	2

[a] See description of CD types in Materials and methods

Table 3. Chlorophyll deficiences (CD) in the F_1 families derived from reciprocal crosses

	F_1 families carrying CD	Number of analyzed families
MC169 × Normal	16	16
Normal × MC169	0	13

Table 4. Chlorophyll deficiences (CD) per individual leaf in F_2 progenies of (Normal × MC169) crosses

Ordinal number of the leaves on the main stem	Striped leaves[a]	*n*
First	$1\,VS_{10}+1\,VS_5+1\,AS_1(1)$	1,005
Second	$1\,VS_{10}(1)$	1,005
Third	$1\,VS_{20}+1\,AS_1$	827
Fourth	$1\,VS_1+2\,AS_1$	827
Fifth	$2\,VS_{10}+1\,VS_5+1\,VS_1(2)+1\,AS_1$	827
Sixth	$1\,VS_{20}+2\,VS_{15}+2\,VS_{10}+2\,VS_5+1\,VS_5(2)+3\,VS_1$	827

[a] VS, *Viridis* striped leaf; AS, *Albina* striped leaf; subscripts indicate roughly the percentage of leaf area carrying CD; in brackets, equal numbers indicate the striped leaves belong to the same plant

progenies when selfing. We must mention here that most of the CD observed in second-leaf seedlings as solid mutants showed additional longitudinal streaks when they were analyzed at later stages. The streaks were either darker or lighter than the color of the mutant background.

Most of the viable solid CD plants were markedly weaker than MC169 normal green plants. However, some *viridis* and discontinuous types showed such vitality that in subsequent years it was possible to multiply them directly under field conditions. High vitality has been mainly observed in those CD types that recovered, at least partially, the normal-green color when heading.

In another small experiment with MC169, 8 out of 78 M_6 second-leaf seedlings showed CD (Table 1). The same material transplanted to the field nursery and analyzed until heading showed CD on 66 out of 67 plants. Several of them showed a pattern of isolated and narrow clonal streaks unevenly distributed within the plant. Some of those plants had as few as one minute streak of a few cells in only one of the leaves.

Reciprocal crosses

MC169 plants carrying solid CD, or tillers with CD comprising more than 50% of their tissues, were used for hybridization (Table 3). Sixteen MC169 plants were crossed as female with normal-green and genetically stable plants of 3 different genotypes. Among the resultant F_1 (MC169 × Normal) progenies, 10 out of 16 presented solid and/or sectorial CD of the same kind as that observed on the corresponding maternal tiller. On the other 6 F_1 progenies, CD were also present, but they did not correspond exactly with the kind of CD observed in the mother plant. Thirteen crosses using MC169 plants as male were carried out with normal-green plants of seven different genotypes. None of those F_1 (Normal × MC169) progenies showed any evidence of CD, though high tillered plants were carefully analyzed from the first leaf stage until heading.

In the F_2 and later generations those progenies that originated in the last type of crosses mentioned above (Normal × MC169) were the most enlightening with respect to the origin of the CD because they came from F_1 plants free of CD. Table 4 presents CD per individual leaf (from the first to the sixth leaf of the main stem) in F_2 (Normal × MC169) plants. CD comprised only a small percentage of the F_2 plants' tissues. Because of the difficulties in accurately classifying streaks of small sizes, we grouped the leaves in only two types, *viridis* or *albina*. Solid CD were not observed at the plant level or at the leaf level. Only 2 plants showed CD in more than 1 leaf, indicated in Table 4 by identical subscripts, and only 1 of them had the same kind of CD in both leaves. Considering the data per individual plant at the second-leaf stage

Table 5. Chlorophyll deficiences (CD) in the F_2 generation of (Normal × MC169) crosses

	Number of second-leaf seedlings		Number of plants until heading	
	Normal	Carrying CD	Normal	Carrying CD
Experiment I	1,002	3	[a]	[a]
Experiment II	34	0	21	7

[a] Data not registered

Table 6. Chlorophyll deficiences (CD) in progenies from selfing of (Normal × MC169) crosses analyzed at second-leaf stage

F_3 generation

	F_3 seedlings				F_2 plants' progenies		
	Carrying CD		Normal	% with CD	Carrying CD	Normal	% with CD
	Striata	Solid					
	77	3	3,403	2.3	24	56	30.0

F_4 generation

Family[a]	F_4 seedlings				F_3 plants' progenies		
	Carrying CD		Normal	% with CD	Carrying CD	Normal	% with CD
	Striata	Solid					
1	37	3	360	10.0	14	1	93.3
2	43	2	444	9.2	15	0	100.0
3	67	14	844	8.8	28	4	87.5
4	47	10	706	7.5	27	0	100.0
5	21	4	489	4.9	17	4	81.0
6	33	3	867	4.0	17	6	73.9
7	10	1	613	1.8	4	22	15.4
8	8	1	567	1.6	4	15	21.1
9	11	1	794	1.5	6	18	25.0
10	10	0	820	1.2	6	20	23.1
11	8	0	715	1.1	8	14	36.4
12	4	0	394	1.0	3	12	20.0
13	4	0	662	0.6	1	18	5.3
14	4	0	1,124	0.4	3	35	7.9
15	0	0	461	0.0	0	19	0.0
16	0	0	842	0.0	0	24	0.0
17	0	0	882	0.0	0	29	0.0
18	0	0	513	0.0	0	20	0.0

[a] Data grouped in families decendent from each F_2 plant

(Table 5), we can state that seedlings carrying CD were seldom observed.

There were also results from other F_2 (Normal × MC169) progenies. At the second-leaf stage, 34 seedlings analyzed in the greenhouse did not show any CD, but the very same individuals transplanted to the field nursery segregated 7 plants carrying CD out of 28 (Table 5). Those 7 plants showed a CD pattern of isolated clonal streaks, usually limited to one or a few narrow streaks per plant.

In F_3 plants solid CD appeared (Table 6) and in F_4 plants they were observed more frequently. In the F_4 18 families arising from 18 F_2 plants could be classified in three clearly separate groups of high (6 families), medium (8 families) and null (4 families) CD frequencies. This classification was valid for both F_4 seedlings and F_3 plant progenies, and the results did not differ from a 1:2:1 ratio. On average the high frequency families carried CD on 88.7% of the plant progenies and 7.1% of the seedlings, 12.7% of them being solid mutants. These values approximated those given above for the M_6 generation (92.5%, 9.0% and 15.7%, respectively). Some of the F_4 families were homogeneous recombinants between grain shape and level of CD frequencies, both characters which were found to mutate together in MC169.

The descendents of 2 of the recombinant families mentioned above were also analyzed in the F_5 generation, as a progeny test of the F_3 plants. One of them was family no. 3 of Table 6, which had both high CD frequencies and normal grain shape. In the F_5, all 29 F_3 plant families segregated CD. The frequency of seedlings carrying CD ranged from 2.5% to 23.2% (8.9% on an average of 6506 analyzed seedlings). At maturity all of the F_5 plants of these families showed normal grain shape (nearly 1,000 plants were analyzed). Furthermore, F_5 and F_6 descendents of family 3 have been utilized to select different CD types in plants with normal grain shape. In these generations a wide spectrum of CD types was observed, similar to the results of the M_6 analysis (Table 2). Only one difference was noticed and that was the presence of a family segregating *xantha-alba* seedlings, a discontinuous mutant not observed in the M_6.

Contrary to what was found in family no. 3, family no. 15 of Table 6 showed null CD frequencies and long-grain shape in the F_5 generation. None of the families, descendent from 16 F_3 plants, segregated CD either in the greenhouse (2738 second-leaf F_5 seedlings were analyzed) or in the field nursery (nearly 600 high tillered F_5 plants were analyzed).

Backcrosses

CD were absent in both kinds of backcrosses of (Normal × MC169) F_1 plants by normal genotypes, as female or as male, in all of the progenies (Table 7). On the contrary, (Normal × MC169) F_1 plants crossed as female by MC169 produced progenies segregating plants with CD that showed a pattern of isolated clonal streaks. These plants segregated in frequencies statistically not differing from a 1:1 ratio. On the other hand, one solid mutant of the discontinuous *virido-xantha* type was

Table 7. Chlorophyll deficiences (CD) in progenies of backcrosses

Backcrosses	Second-leaf seedlings		Plants until heading	
	Normal	Carrying CD	Normal	Carrying CD
Normal (Normal × MC169)	49	0	47	0
(Normal × MC169) Normal	28	0	22	0
(Normal × MC169) MC169	26	1	5	14[b]
[(VX-MC169 × Normal) Normal] Normal	0	14 VX[a]	0	12

[a] VX, *Virido-xantha* type
[b] With a pattern of isolated clonal streaks

crossed and backcrossed twice by normal genotypes, using the normal genotypes as male (Table 7). All of the plants of those progenies were of the original *virido-xantha* type without any clonal streak.

Discussion

Transmission and expression of the chlorophyll deficiencies (CD)

The high frequency of M_6 seedlings with clonal patterns of variegation (*striata*) and the general occurrence of heterogeneous segregations among M_5 spike progenies were the first indications of a non-Mendelian inheritance of CD. Subsequently, the cytoplasmic transmission of CD was proven by several experiments involving reciprocal crosses. The *striata* mutants observed in (MC169 × Normal) F_1 plants suggested the occurrence of heteroplastic zygotes. This fact and results from repeated backcrosses of plants carrying solid CD used as the female indicated that the expression of the CD was independent upon the nuclear constitution of the plant.

Induction of CD

Though CD showed a cytoplasmic transmission and an expression independent of nuclear constitution, their induction was proven to be under nuclear control. Results from F_2 and later generations of (Normal × MC169) crosses indicated that the induction of CD is controlled by a single nuclear gene. The need of homozygocity for the activity of the inductor or mutator gene was indicated by the absence of CD in (Normal × MC169) F_1 plants and in their reciprocal backcrosses by normal genotypes. As a counterpart, we observed CD in F_2 progenies derived from plants free of them belonging to crosses (Normal × MC169) and backcrosses of the kind [(Normal × MC169) × MC169].

Manifestation of mutator activity and spectrum of mutants

The mutator gene manifested itself through the expression of the CD it induced and it was evident in 9.0% of the M_6 second-leaf seedlings and in most of the high tillered plants. Similar observations have been reported by Redei (1973) in an *Arabidopsis* mutant.

The mutator activity was scarcely manifested in F_2 seedlings from (Normal × MC169) crosses, but it increased notably in high tillered plants, resembling a 1:3 ratio. The small size of the streaks observed in this generation could be explained by a slow sorting-out of the mutant chloroplasts (Kirk and Tilney-Bassett 1978; Sears 1983), though mutational events commence at the earliest stages of the development of the homozygous embryo. The idea of a slow chloroplast sorting out, a process probably accelerated during meiosis, is in agreement with the observation of a few solid mutant seedlings in the F_3 and an increased number of them in the F_4 generation.

The wide spectrum of CD cytoplasmically inherited suggests a mutator action on many different chloroplast genes. On the other hand, the lack of inheritable sterility suggests that the mitochondrion was not affected.

In comparison with mutagenic treatments, it is remarkable that the mutator gene reported here induces a wide cytoplasmic variability on a homogeneous nuclear background. In this way, both the isolation and the analysis of the plastome mutants are facilitated.

Comparison with mutants previously described and conclusions

The results presented here support the existence of a recessive nuclear gene responsible for the high mutation rate observed in MC169. This gene leads to a wide spectrum of CD that is transmitted to the progeny solely by the female parent and expressed independently of the nuclear constitution, suggesting that the cpDNA has undergone diverse mutational events. Examples resembling this have been previously reported for some dicots (see Introduction).

In barley, several genes inducing a narrow spectrum of maternally inherited chlorophyll mutants have been described. They were designated as "Okina mugi" (Imai 1928, 1936), *white* (*w*) (Arnason et al. 1946; Arnason and Walker 1949), *albostrians* (*as*) (Hägemann and Schölz 1962; Börner et al. 1976), *striata-4* (von Wettstein and Eriksson 1965) and *white-streak-3* (*ws-3*) (Takahashi and Moriya 1969). Similar cases have also been reported in maize (Jenkins 1924; Rhoades 1943; Stroup 1970; Coe et al. 1982; Thompson et al. 1983) and rice (Imai 1928; Kirk and Tilney-Bassett 1978). All of them differed from our results, not only in their CD spectrum, but also in

their breeding behavior, showing a much faster sorting-out of the cytoplasmic mutant than that we observed.

Additional observations on some of the mutants mentioned above showed that the morphological alterations were not restricted to chloroplasts but were also present in the mitochondria (von Wettstein 1961; Wettstein and Eriksson 1965) and that cytoplasmic male sterility and mtDNA alterations were present (Rhoades 1950; Lemke et al. 1988). However, so far no cpDNA alterations have been detected (Walbot and Coe 1979; Börner and Sears 1986). If all the aforesaid is taken into consideration as well as the rapid sorting-out expected for mitochondria but not for chloroplasts (Lonsdale et al. 1988), it is tempting to hypothesize that the cases previously reported were related to the induction of mitochondrial genetic changes. In such cases, the defective chloroplasts would originate secondarily as a consequence of mitochondrial abnormalities, such as has been earlier proposed by von Wettstein and Eriksson (1965) for some of those barley mutants. Therefore, the nuclear gene described here would be the first "chloroplast mutator" reported for monocots. We must make it clear that the highly mutagenic mutator system of maize has been reported to induce a wide spectrum of mutants, but they usually are nuclear recessive (Masterson et al. 1986; Mourad et al. 1989).

A general discussion on organelle mutator genes was presented by Kirk and Tilney-Bassett (1978). Mutations either in a DNA polymerase or in a DNA repair enzyme were some of the proposed mechanisms. Transposable elements residing in the chloroplast and activated by a nuclear gene was another theory proposed later (Sears 1983). According to Hasting et al. (1976) the recessiveness of a mutator gene is highly indicative that it is affecting the DNA repair capabilities.

Improved knowledge of mutator genes like the one described here can play a fundamental role in elucidating organelle heredity and its interactions with the nuclear genome. Moreover, they would be a valuable source of variability of the otherwise highly conservative genetic information encoded in the chloroplasts (Sears 1983; Melzer and Kleinhofs 1987). This variability would include not only CD types, but also mutants in agronomically valuable characters like photosynthetic efficiency or herbicide tolerance.

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