Theor Appl Genet (2003) 107:1410–1418
DOI 10.1007/s00122-003-1391-0

ORIGINAL PAPER

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A cytoplasmically inherited mutant controlling early chloroplast development in barley seedlings

Received: 10 November 2002 / Accepted: 25 June 2003 / Published online: 15 August 2003

Abstract Cytoplasmic line 2 (CL2) has been previously reported as a cytoplasmically inherited chlorophyll-deficient mutant selected from a chloroplast-mutator genotype of barley. It was characterized by a localized effect on the upper part of the first-leaf blade. At emergence the CL2 seedlings-phenotype varied from a grainy light green to an albino color. They gradually greened during the following days, starting from the base of the blade and extending to cover most of its surface when it was fully grown. The present results, from both light microscopy and transmission electron microscopy (TEM), confirmed the previously described positional and time-dependent expression of the CL2 syndrome along the first-leaf blade. During the first days after emergence, light microscopy showed a normally developed chloroplast at the middle part of the CL2 first-leaf blade, meanwhile at the tip only small plastids were observed. TEM showed that the shapes and the internal structure of the small plastids were abnormal, presenting features of proplastids, amyloplasts and/or senescent gerontoplasts. Besides, they lack plastid ribosomes, contrasting with what was observed inside chloroplasts from normal tips, which presented abundant ribosomes. Phenotypic observations and spectrophotometric analysis of seedlings produced by mother plants that had been grown under different temperatures indicated that higher temperatures during seed formation were negatively associated with pigment content in CL2 seedlings. In contrast, higher temperatures during the growth of CL2 seedlings have been associated with increased pigment content. Aqueous solution with kanamycin and streptomycin, which are antibiotics known to interfere with plastid gene translation, were used for imbibition of wild-type and CL2 seeds. Antibiotic treatments differentially reduced the chlorophyll content in the upper part of the first-leaf blade in CL2, but not in wild-type seedlings. These results suggest that in the wild-type, plastid-gene proteins which are necessary for chloroplast development and chlorophyll synthesis in the upper part of the first-leaf blade are usually synthesized during embryogenesis. However, under certain circumstances, in CL2 seedlings, they would be synthesized after germination. In addition, a shortening of the sheath has been observed in association with pigment decrease suggesting the existence of plastid factors affecting the expression of some nuclear genes. We consider the CL2 mutant a unique experimental material useful to study biological phenomena and external factors regulating plastid, and nuclear gene expression during embryogenesis and early seedling development.

Keywords Chlorophyll deficiencies · Chloroplast development · Cytoplasmic inheritance · Hordeum vulgare

Communicated by R. Hagemann

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Introduction

Chlorophyll deficient mutants have been utilized extensively to interpret the development and function of chloroplasts (e.g. Gustafsson 1942; Wettstein 1961; Taylor et al. 1987; Wettstein et al. 1995; Leon et al. 1998). Based on the frequency, type and inheritance of chlorophyll-deficient mutants, one chloroplast mutator genotype has been reported in barley (Prina 1992). From that genetically unstable genotype it was possible to select

plants, and later on families, carrying different chlorophyll mutant types. Because of their breeding behavior, they were called cytoplasmic lines (CLs) (Prina 1996). The positional variegation patterns observed on the first-leaf blade of some of them (Prina 1996), suggested that they could be involved in the control of early steps of chloroplast development. At emergence, the seedlings of one of those CLs, named CL2, showed different levels of expression of the chlorophyll deficiency, varying from a grainy light green to an albino color. They gradually greened during the following days, starting from the bottom of the growing blade and extending to cover most of its surface. Even when most of the fully grown first-leaf had a normal-green color, it usually maintained a small white area at the tip and, sometimes, also a fine white line on each margin of the blade. The positional-variegated pattern and time-dependent expression described above, have been confirmed after six generations of natural self-pollination and have proved to be maternally inherited (Prina 1996). Here we present light and transmission electron microscopic (TEM) observations of plastids; results from spectrophotometric analysis of chlorophyll content and morphological measurements were carried out on the first-leaf blade of CL2 and control-seedlings during the first days after seed imbibition. Seeds originated on mother plants grown in different environmental conditions and imbibed either in water or in antibiotic solutions were used.

Materials and methods

Experimental material

Experiments were carried out with CL2 barley mutant and normal control seedlings. The CL2 mutant was previously selected from a chloroplast- mutator genotype originated from mutagenized seeds of MC182 (INTA Castelar accession number), a two-row spring barley (*Hordeum vulgare L.*) genotype (Prina 1992, 1996).

Seeds for all of the experiments were harvested on naturally self-pollinated plants. Mother plants were grown either at the field nursery, in the greenhouse or in growth chambers with control of light and temperature. In growth chambers the mother plants were grown during the entire life cycle with a photoperiod of 16 h of light, 15,000 lux (from white fluorescent tubes and incandescent lamps) at high (20°C dark/26°C light) or low temperatures (8°C dark/15°C light).

For both spectrophotometric and microscopic analysis, CL2 and normal-green control seedlings were grown in hydropony by the sandwich or growing-rack method (Myhill and Konzak 1967). Environmental conditions in which seedlings were grown for each of the experiments are indicated together with the results.

Spectrophotometric analysis of chlorophyll content

Two samples were harvested from the first-leaf blade. The number of seedlings per sample varied depending on the stage of growth, in order to obtain 500–700 mg. Samples were ground with a mortar and pestle, using sand and $CaCO_3$. Four extractions were made with aqueous acetone (35 ml in total) and, later on, the samples were pooled, cleared by centrifugation, decanted and volumetrically measured (Maclachlan and Zalik 1963). The absorption spectra between 400 and 700 mμ, were recorded with a Beckman DB-6 spectrophotometer, using 1-cm cells. Concentrations of both chlorophyll *a* and *b*, were calculated using equations by Maclachlan and Zalik (1963).

Microscopic analysis

Small segments (2 mm) of first-leaf blades were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 at 4°C, post-fixed in 1% osmium tetroxide in water for 2 h at 4°C, then dehydrated in a graded ethanol-acetone series and embedded in Spurr's resin. Semi-thin sections for light microscopy were stained with 1% Toluidine Blue O (Sigma T 3260 CI 52040), while ultra-thin sections for electron microscopy were mounted on grids, stained with lead citrate followed by uranyl acetate, and examined in a Zeiss EM109T transmission electron microscope. As the CL2 first-leaf presented a positional and time-dependent expression (Prina 1996), three different regions of the blade were used for observations (see diagram Fig. 2) and they were collected at the 3rd, 4th, 5th, 6th and 7th day after emergence.

Antibiotic treatments

Aqueous solutions of the antibiotics streptomycin (100 mg/l) and kanamycin (50 mg/l) were used. Seeds were imbibed either in water or in the antibiotic solution and swirled at 100 r.p.m., at 25°C, for 20 h. After imbibition, they were sown in hydropony and grown for 12 days in similar antibiotic solutions.

Results

I. Experiments with seeds harvested at the field nursery

Seedlings for analysis of chlorophyll content were grown in growth chambers at two different constant temperatures, 14 or 28°C. This experiment showed that CL2 seedlings grown at both temperatures had lower chlorophyll content than normal ones (Fig. 1). Three days after emergence, no chlorophyll could be detected in CL2 seedlings grown at 28°C, and it was near zero after 6 days when they were grown at 14°C. Meanwhile, at the same times, the corresponding control seedlings accumulated considerable amounts of chlorophyll. In comparison with those of the control, CL2 seedlings presented much lower differences in chlorophyll content between the upper and the lower part of the leaf blade (Fig. 1). The main differences between CL2 and control seedlings were observed at lower temperature and mainly in the chlorophyll content of the upper part of the blade.

For microscopic analysis, CL2 and wild-type barley seedlings were grown in a growth chamber at a constant temperature of 18°C. Light microscopy observations carried out on three different parts of the first-leaf blade (Fig. 2) and from the 3rd to the 7th day after germination showed a peculiar pattern of chloroplast development in CL2 seedlings. In contrast with what was observed along the first-leaf blade of wild-type barley seedlings, CL2 ones contained more developed chloroplasts in middle-blade sections than in those from the top, in which only small plastids were observed. In Fig. 2, photomicrographs from a CL2 seedling 5 days after germination are presented. The small plastids of the top can be observed in Fig. 2a, while normal-sized chloroplasts were observed

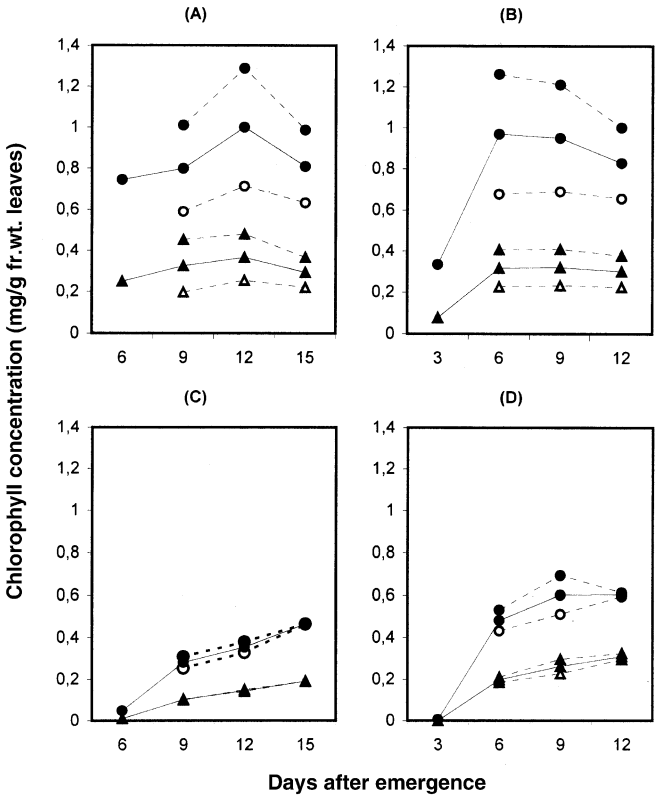


Fig. 1A–D Chlorophyll concentration in extracts of first-leaf blades from normal and CL2 seedlings, grown at two temperatures, (**A** and **B**, control seedlings grown at 14°C and 28°C, respectively. **C** and **D**, CL2 seedlings grown at 14°C or 28°C, respectively) and a photoperiod of 18 h. *Filled symbols on full lines* represent chlorophyll *a* (*circles*) or *b* (*triangles*) contents in whole first-leaf blades. *Filled symbols on dotted lines* indicate contents in the upper portion of the blades. *Blanch symbols on dotted lines* indicate the lower portion

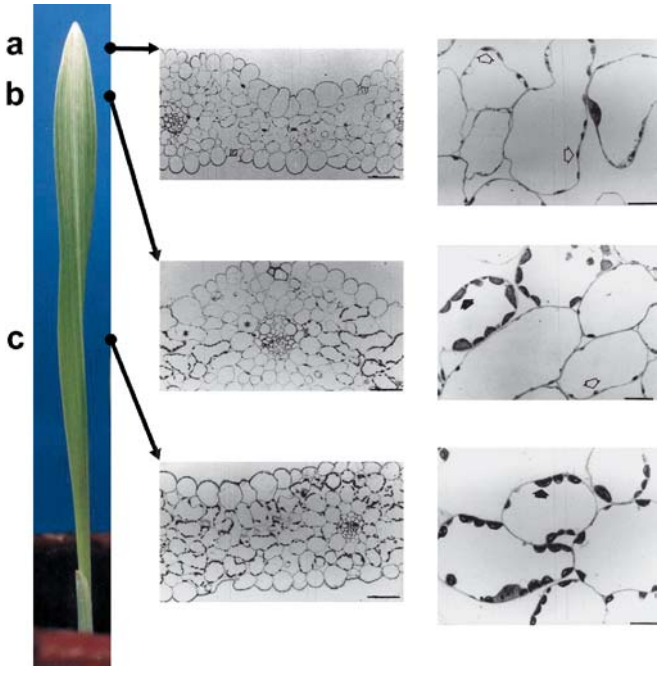


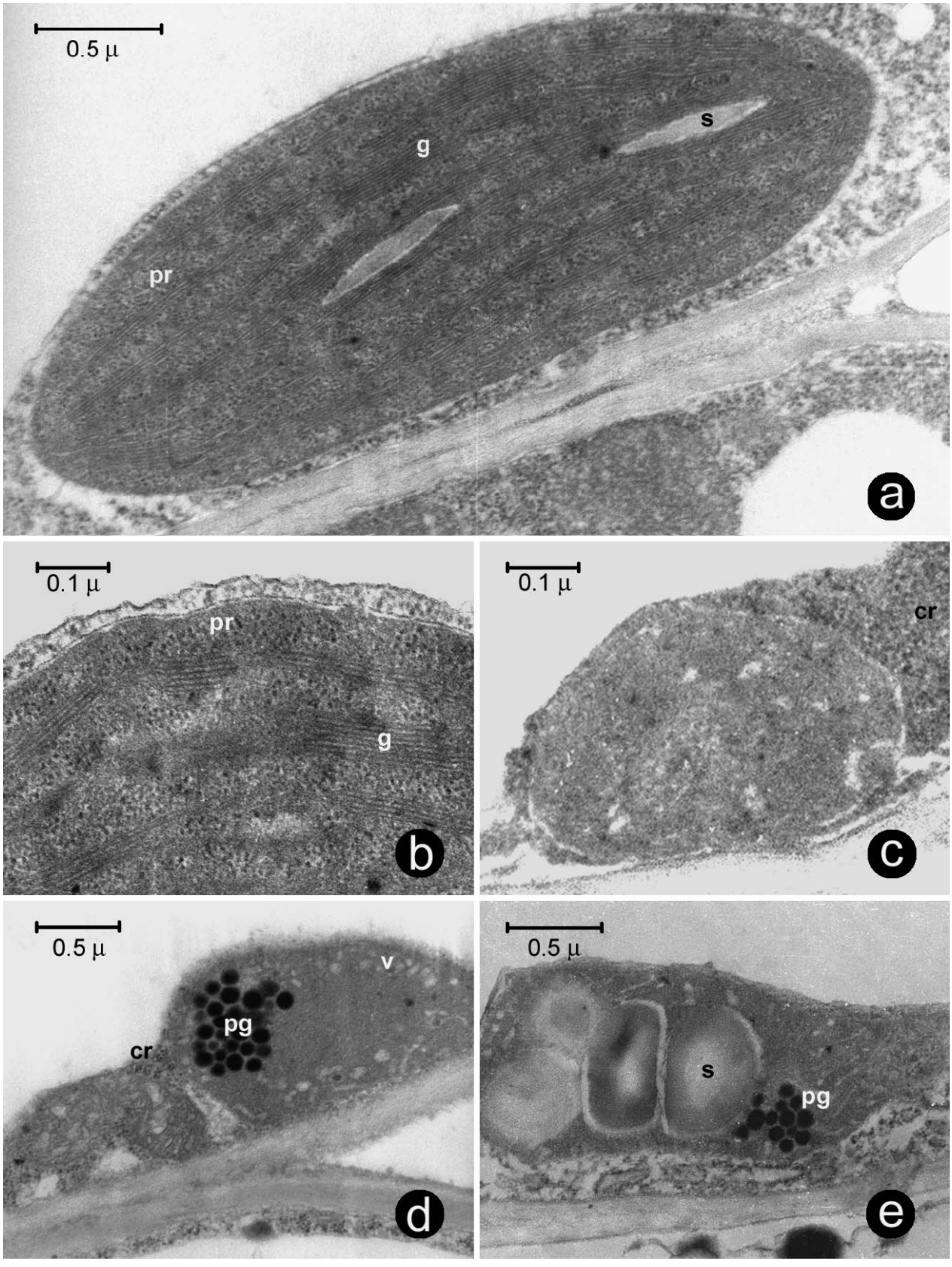
Fig. 2 Transverse sections of the first-leaf blade in CL2 seedlings at the 5th day after emergence. Seedlings were cultivated in hydropony at a constant temperature of 18°C and a photoperiod of 18 h. * Indicates some of the cells with under-developed chloroplasts; filled or blanch arrows indicate normal or underdeveloped chloroplasts, respectively. Left column: Bar = 25 μm, right column: 5 μm

in the middle-blade section (Fig. 2c). Curiously, in sections from an intermediate zone, below the white top (Fig. 2b), it was possible to observe that cells of the middle mesophyll had more developed chloroplasts than did sub-epidermal ones and those located adjacent to the vascular bundle. This pattern of plastid development in relation with the vascular bundle was not observed in any of the control samples, which had a homogeneous chloroplast development in each transverse section. By transmission electron microscopy (TEM), normally developed chloroplasts were observed in tips of wild-type seedlings, which contained well-organized internal structures and showed normal-shaped (elongated) starch grains and abundant ribosomes in the stroma. All these characteristics can be observed in a chloroplast from the tip of a 3-day old normal first-leaf blade (Fig. 3a). In Fig. 3b, with a bigger magnification than in 3a, it is possible to appreciate the abundant ribosomes in the stroma of a chloroplast from a 4-day old normal seedling. In contrast, the small plastids observed in CL2 tips did not differentiate any internal membranes, they lacked plastid ribosomes and contained round-shaped starch grains, internal vesicles and plastoglobules (Fig. 3c–e). In Fig. 3c and d, the lack of plastid ribosomes contrasted with the presence of abundant ribosomes in the adjacent cytoplasm. The plastid of Fig. 3d also showed numerous internal vesicles that resembled a peripheral reticulum.

II. Experiments with seeds harvested on mother plants grown under controlled environmental conditions

Seeds were harvested from mother plants grown in two different temperature regimes (low = 8°C dark/15°C light,

Fig. 3a–e Transmission electron micrographs in tips of the first-leaf blade, either from normal or CL2 seedlings. **a** Chloroplast from the tip of a 3-day old normal first-leaf blade. It shows a well-developed membrane organization, normal shaped starch grains (*s*) and abundant plastid ribosomes (*pr*) in the stroma. **b** Partial view of a chloroplast from a 4-day old normal seedling showing abundant plastid ribosomes (*pr*) in the stroma. **c** Small plastid from the tip of the first-leaf blade in a 4-day old CL2 seedling. It lacks internal membrane organization and plastid ribosomes; in contrast, outside the plastid abundant cytoplasmic-ribosomes (*cr*) can be noticed. **d** Plastid from the tip of a 6-day old CL2 seedling, without showing internal membrane organization and plastid ribosomes. It presents plastoglobules (*pg*) and abundant internal vesicles (*v*) that resemble a peripheral reticulum. Cytoplasmic ribosomes (*cr*) can be noticed outside the plastid. **e** Plastid from the tip of a 7-day old CL2 seedling. It lacks internal membrane organization and plastid ribosomes. It presents plastoglobules (*pg*) and abnormal (round-shaped) starch grains (*s*)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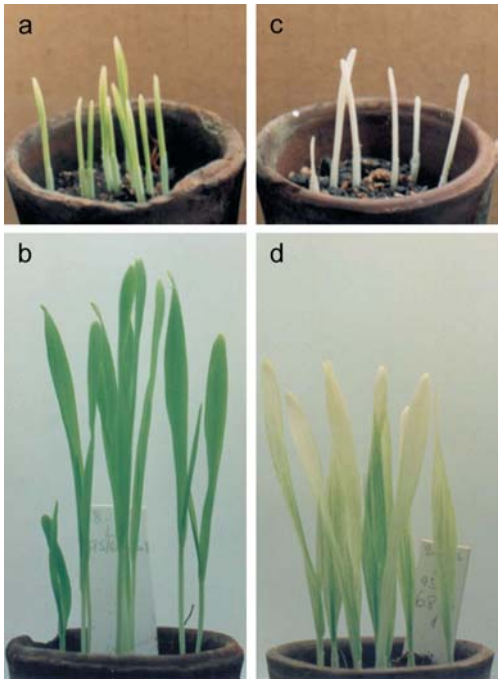

Fig. 4 CL2 seedlings originated in seeds harvested on mother plants grown in two different temperature regimes: *a* and *b* low temperature (8°C dark/15°C light), *c* and *d* hight temperature (20°C dark/26°C light). They were sown in pots and cultivated at a temperature of 21°C light/16°C dark and a photoperiod of 16 h. Pictures were taken from seedlings at the 7th (*a* and *c*) and the 11th (*b* and *d*) day after sowing. The same experimental material was grown in hydropony for analysis of pigment content (see Fig. 5)

high = 20°C dark/26°C light). For visual analysis, seeds from both environments were sown in small pots with soil and were cultivated at intermediate temperatures (16°C dark/ 21°C light). From each origin the expression of the CL2 syndrome was more homogeneous than that we usually observed in CL2 seedlings derived from mother plants from non-controlled environments. During the first days after germination, 72 seedlings derived from plants grown in the low temperature regime, presented a grainy light-green color in the basal part, while the upper part was white (Fig. 4a). They gradually greened to cover all or almost all of its surface with a normal green color by the 11th day (Fig. 4b). From seeds coming from high temperatures, 77 seedlings were obtained, and they were albino during the first days after emergence (Fig. 4c) and, in contrast with those described above, they greened much later. By the 11th day, they maintained white tissues on most of the half upper part of the blade, reaching a grainy light-green color in the rest, with increasing green-intensity towards the basal part (Fig. 4d). Curiously, the sheaths were shorter in this last material. It can be noticed in Fig. 4, in which second leaves can also be observed, indicating that the different lengths of the sheaths can not be explained based on retarded development. In addition, seeds of the same two origins were sown in hydroponics in order to analyze their chlorophyll content. Results are shown in Fig. 5, and agree with the

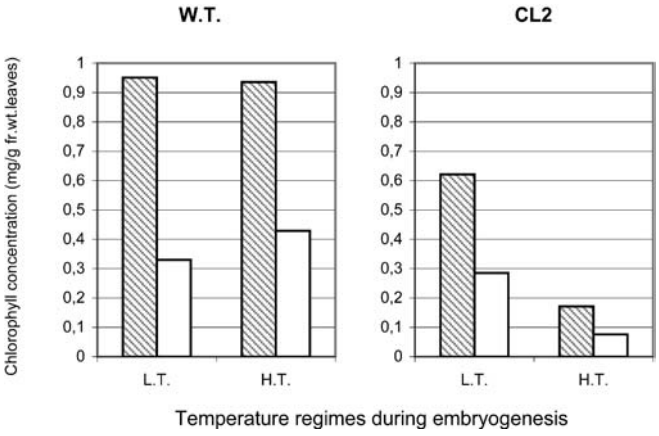

Fig. 5 Chlorophyll content in CL2 and wild-type (WT) barley seedlings, originated from mother plants grown either at low (L.T.: 8°C dark/15°C light) or high (H.T.: 20°C dark/26°C light) temperature regimes. Analyzed seedlings were grown at a temperature of 21°C (light)/16°C(dark). *Dashed bars:* chlorophyll *a*; *empty bars:* chlorophyll *b*

above phenotypic descriptions. Thus, the seedlings coming from seeds harvested at higher temperatures showed a much-lower chlorophyll content.

III. Experiments with antibiotic-treated seeds

In these experiments, in addition to CL2 and wild-type field-harvested seeds, CL2 seeds harvested in the greenhouse were also used. After being imbibed during 20 h in water or in antibiotic solutions, seeds were sown in hydroponics with similar solutions and cultivated in a growth bench at 16°C dark/21°C light. Results of chlorophyll content in the upper part of the first-leaf blade of wild-type and CL2 seedlings from both origins (field and green house) are presented in Fig. 6. Wild-type seedlings did not show any effect of the antibiotic treatments on pigment content; on the contrary, when antibiotics were applied to CL2 seeds, the seedlings derived from both origins had diminished chlorophyll content. In Fig. 7, measurements of sheath length of these seedlings are presented. Results showed that, in addition to a decrease in pigment content, antibiotic treatments produced morphological changes in CL2 seedlings. Figure 8 shows that in response to kanamycin or streptomycin, wild-type seedlings suffered a drastic loss of the normal green color only in the basal part of the blade, meanwhile the upper part was not affected. In contrast, CL2 seedlings from greenhouse-grown mother plants lost most of their green color all over the blade, indicating that at the upper part of the first-leaf blade the genotypes are differentially affected by the antibiotic treatments. Photos of CL2 seedlings from field-harvested seeds are not shown, but it is worth mentioning here that, in accordance with the results presented in Fig. 6 and 7, both antibiotic treatments resulted in an intermediate

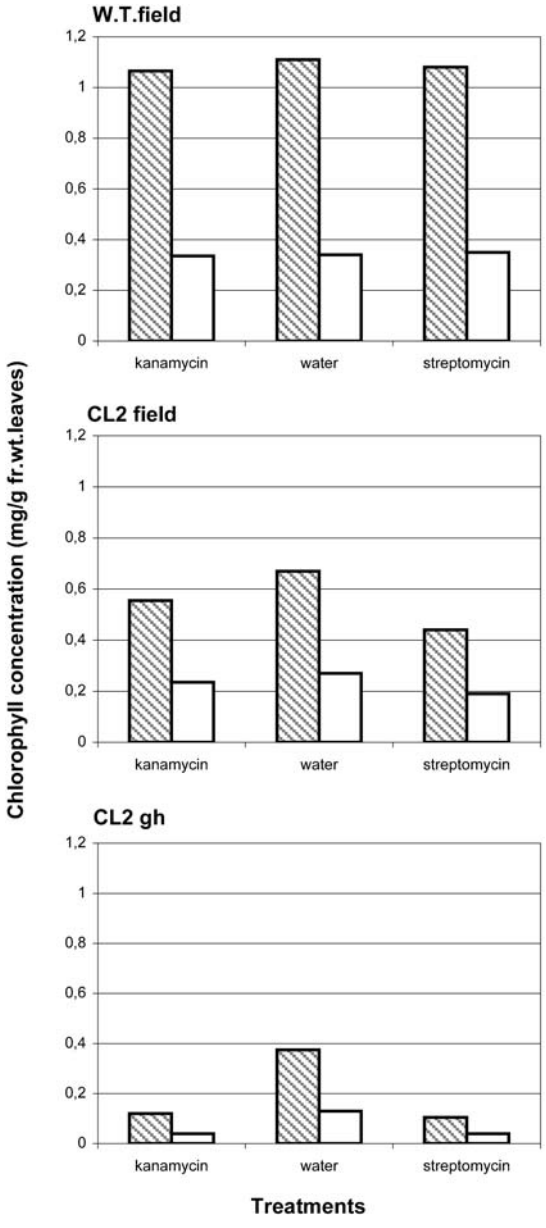

Fig. 6 Chlorophyll content in the half upper part of the first-leaf blade at the 12th day after imbibition. Wild-type (W.T.) and CL2 barley seedlings originated either in mother plants grown at the field nursery (CL2 f) or in the green house (CL2 gh.). Seeds were imbibed during 20 h either in water or in antibiotic solutions, and seedlings were cultivated in hydropony with a similar solution at 21°C (light)/16°C (dark) under a photoperiod of 16 h. *Dashed bars:* chlrophyll *a*; *empty bars:* chlorophyll *b*

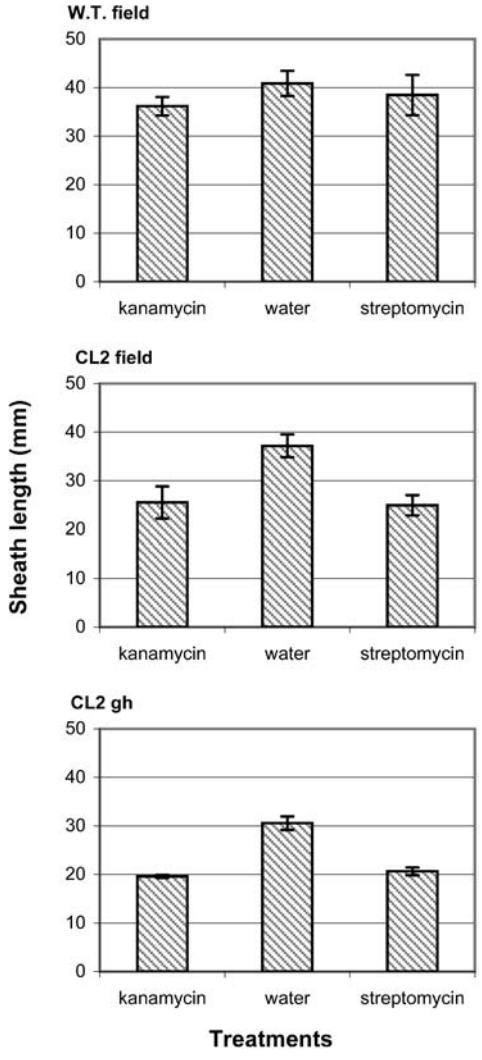

Fig. 7 First-leaf sheath length in CL2 and wild-type barley seedlings at the 12th day after imbibition. Experimental material corresponds to that described in Fig. 6

phenotypic effect between those observed in the wild-type and CL2-greenhouse harvested seeds.

Discussion

The present results, from both light microscopy and TEM, confirm the previously described positional and time-dependent expression of the CL2 syndrome along the first-leaf blade (Prina 1996). We observed that normally developed chloroplasts where mostly found on the middle part of the CL2 first-leaf-blade, while at the tip, only small plastids were observed. This observation contrasts with the gradient of chloroplast-development usually found in monocot leaves, in which older cells and more-developed chloroplasts are located near the tip and progressively younger cells are located towards the leaf base (Mullet 1988, 1993). This rupture of the normal association between plastid development and cell age observed in CL2 seedlings offers the possibility of a new approach to study the control of plastid gene expression and its true relationship with cell development. Results of chlorophyll content agreed with expectations from both the visual pattern of variegation and microscopic observations. The youngest tissues, located toward the blade base, were the first in greening, and the rate of greening diminished toward the top. The tip, which carries the oldest cells in a normal monocot blade (Mullet 1988),

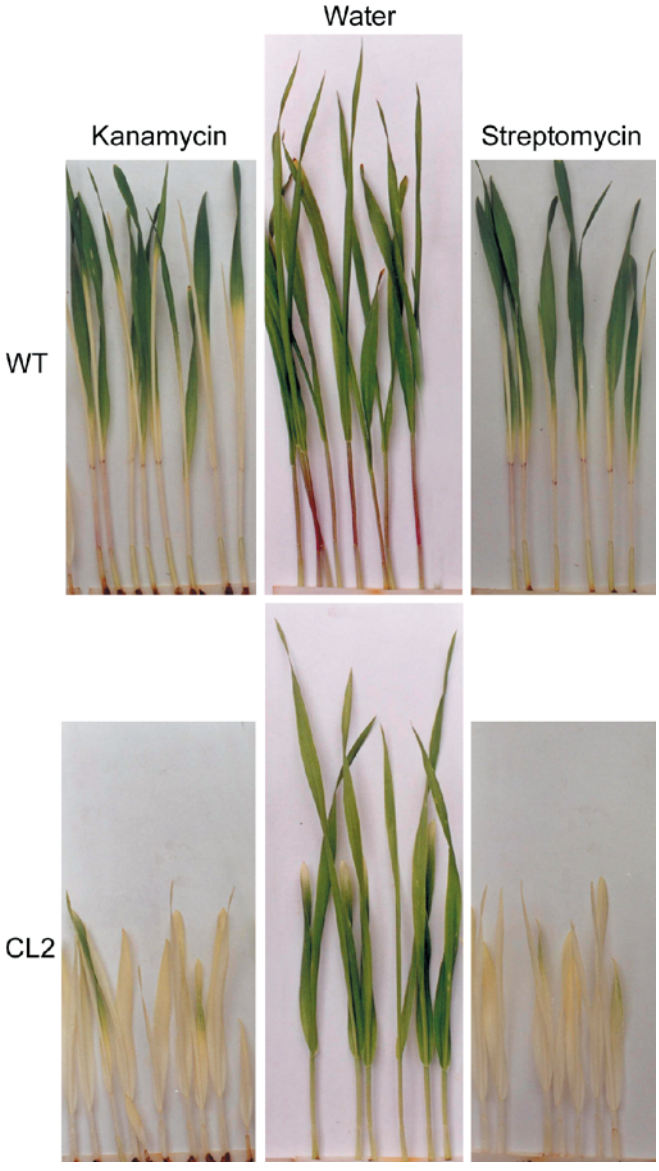

Fig. 8 Wild-type (W.T.) and CL2 barley seedlings, coming from seeds which were imbibed either in water (center) or in antibiotic solutions: kanamycin (left) or streptomycin (right). Seedlings were cultivated in hydropony in the similar solution in which seeds were imbibed. They were grown at a temperature of 16°C (dark)/21°C (light) and under a photoperiod of 16 h. Experimental materials correspond to wild-type and CL2-gh (greenhouse harvested seeds) seedlings described in Fig. 6. Pictures were taken at the 10th day after imbibition

usually remained white in CL2 plants. This can be interpreted as the younger cells have a higher ability to recover from the CL2 syndrome. Abnormal shapes and internal structures observed by TEM in the aberrant plastids of the CL2 white-tip, some of them presenting features of proplastids, amyloplasts or senescent gerontoplasts, suggest that those plastids lost the capability of following the normal program of chloroplast development and consequently they degenerated. In the chloroplast from the tips of normal seedlings abundant plastid ribosomes were observed. In contrast, plastids from the tip of CL2 first-leaf blades lacked ribosomes in their stroma, meanwhile outside them cytoplasmic ribosomes were abundant. It suggested that the overall plastid-gene proteins synthesis would be affected by the CL2 syndrome.

During the first days after germination, another positional effect was observed by light microscopy in transverse sections of the blade. The cells of the middle mesophyll layers contained more-developed chloroplasts than those carried by cells surrounding the vascular bundles and most of the sub-epidermal ones. This spatial distribution of chloroplast development somewhat resembles the cell-spacing pattern of chloroplast dimorphism seen in Kranz anatomy (Langdale and Nelson 1991), and suggests that such a pattern could be largely based on a single mutant gene.

In experiments with seeds harvested from mother plants grown in controlled environments, the seedlings showed more homogeneous expression of the CL2 syndrome than was usually observed in CL2 seedlings from seeds harvested from the field. This suggests that the usual varied expression of the CL2 syndrome could be due to environmental factors acting during seed formation. Moreover, in CL2, higher temperatures during embryogenesis were negatively associated with pigment content in the derived seedlings, indicating that the temperature during seed formation had a marked influence on the production or stability of a product necessary for accomplishing chlorophyll synthesis in the upper part of the first-leaf blade of CL2 seedlings. Curiously, when the effects of temperatures during seedling development were tested (Fig. 1), the higher temperature was observed to be associated with increased pigment content.

Leon et al. (1998) reviewed the range of mutants known to have altered chloroplast development, concluding that almost every step of plastid development depends on the direct action of nuclear-encoded genes. They pointed out that although many developmental mutants have been isolated, few of them affect initial events in organelle biogenesis. Mache et al. (1997) summarized results concerning nuclear gene expression during the early steps of plastid development after germination. They concluded that nuclear gene expression precedes that of plastid-encoded genes, emphasizing that the early signals for chloroplast development originate from the nucleus. Since the translational machinery of plastids resembles that of prokaryotes, it is sensitive to most antibiotics which affect prokaryotic ribosomes (cf. Börner and Sears 1986). For this reason, we treated CL2 and normal barley seeds with streptomycin or kanamycin, which are antibiotics known to be inhibitors of plastid translation. Visual observations and analysis of chlorophyll content in the wild-type indicated that this function was not essential for most, or all, the chlorophyll synthesis carried out in the upper part of normal first-leaf blades. These results are in agreement with the general conclusions of the literature mentioned above. In

contrast, the same antibiotic treatments applied to CL2 seeds considerably reduced the chlorophyll content in the same portion of the first-leaf blade. This indicates that, under certain environmental conditions, the synthesis of at least some plastid-encoded proteins, which usually occurs during embryogenesis in a normal seedling, is delayed in CL2 seedlings, in such a way that it became a necessary step to carry out the chlorophyll synthesis in that portion of the blade.

In addition to a marked decrease in chlorophyll content, phenotypic observations and sheath-length measurements showed that CL2 seedling morphology was also affected by the antibiotic treatments. A similar effect on CL2 seedling morphology and decreased chlorophyll content was observed when seeds harvested at high temperatures were used. These results would indicate that the expression of some nuclear genes, not only those that control chlorophyll synthesis, but also some involved in leaf morphology, are influenced by the CL2 gene. Therefore, CL2 provides a new approach to study the existence of plastid factors controlling the expression of nuclear genes, a fact that has several times been previously proposed (cf. Bradbeer et al. 1979; Börner 1986, Oelmüller et al. 1986; Taylor 1989, Hess et al. 1994, 1997; Green and Salter 1996; Churin et al. 1999; Somanchi and Mayfield 1999; Rodermel 2001). The molecular basis of the CL2 syndrome expression is being the subject of further investigations, under the hypothesis that the overall CL2 mutant behaviour could be explained as it originated in a delay of plastid gene translation.

There still exist in the literature several unclear aspects about the regulation of plastid gene expression, in which both, transcriptional and post-transcriptional mechanisms are involved (Reviewed by Krupinska and Apel 1989; Baumgartner et al. 1993; Mullet 1993; Stern et al. 1997; León et al. 1998; Mayfield and Cohen 1998; Bruick and Mayfield 1999; Hess and Börner 1999). With respect to this we consider the CL2 mutant to be unique experimental material, which could be useful to study the biological phenomena and external factors interacting in the regulation and coordination of plastid and nuclear gene expression during embryogenesis and early seedling development.

Acknowledgements The authors thank Prof. Barbara Sears (Michigan State University, USA) for critical reading of the manuscript, Mrs. Edith García, Mr. Abel Moglie, Mr. Roberto Civitillo and Mr. Miguel Díaz for their skilfull technical assistance. They also thank the financial support from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina), PIP 96/4414 and FONCYT- SeCyT (Fondo Nacional para la Ciencia y la Tecnología-Secretaría de Ciencia, Tecnología e Innovación Productiva, Argentina), PICT 08/04841.

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