A Second \textit{infA} Plastid Gene Point Mutation Shows a Compensatory Effect on the Expression of the Cytoplasmic Line 2 (CL2) Syndrome in Barley

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The IF1 protein is one of the factors controlling translation initiation in bacteria. This protein is encoded by the \textit{infA} gene, which, in several higher plants, is located in the plastome. Cytoplasmic Line 2 (CL2), an \textit{alboviris} barley mutant, was the first to be proposed as an \textit{infA} gene mutation (T 157 C) in higher plants. This mutant was isolated from a chloroplast mutator genotype (\textit{cpm/cpm}) and was made genetically stable by backcrosses with a wild-type nuclear genotype. In the present work, genetically stable CL2 plants were backcrossed as females by \textit{cpm/cpm} plants in order to regain the mutator activity. Interestingly, a seedling carrying a first leaf blade with a darker green stripe on a typical CL2-mutant background was observed in the F4 generation. The T 157 C transition was confirmed in tissues from the CL2 background, whereas a second transition (A 178 G) was also found in the darker stripe. Two clearly different levels of CL2 syndrome were observed in the seedlings of the F5 and F6 progenies. Those of the greener group carried both transitions. These results suggest a compensatory effect of the second mutation and support the involvement of the \textit{infA} plastid gene in CL2 syndrome, confirming CL2 as the first mutant of this gene reported in higher plants.

\textbf{Key words:} barley chloroplast mutator, compensatory mutation, IF1, \textit{infA} plastid gene, translation initiation factor 1

It is widely accepted that the plastid genome of higher plants, or plastome, has evolved from a cyanobacterium-like genome after endosymbiosis and transfer of a great deal of its genes to the nucleus of the eukaryotic host cell. In support of this idea, there are many structural and functional similarities between plastid and bacterial genomes. Translation initiation in bacteria requires the presence of 3 protein factors: IF1, IF2, and IF3 (Boelens and Gualerzi 2002). In higher plants, the genes encoding homologs of the last two factors reside in the nucleus, whereas in barley and several other plant species, the gene encoding the IF1 homolog (the \textit{infA} gene) is localized in the plastome (Millen et al. 2001). IF1 is an oligonucleotide-binding-fold protein related to the cold shock proteins CspA and CspB and the ribosomal protein S1 (Laursen et al. 2005) and widely conserved in all living organisms (Kyrpides and Woese 1998; Roll-Mecak et al. 2001). In bacteria, IF1 modulates the association/dissociation of ribosomal subunits and the interactions between IF2 and the 30S subunit (Dahlquist and Puglisi 2000; Boelens and Gualerzi 2002; Croitoru et al. 2004; Laursen et al. 2005). However, its functions in chloroplasts are so far only inferred from information in bacteria (Kozak 1983; Millen et al. 2001). Croitoru et al. (2004) reported a collection of \textit{infA} gene mutants in bacteria. In higher plants, a particular chlorophyll-deficient phenotype, designated as cytoplasmic line 2 (CL2) and obtained from a chloroplast mutator genotype (Prina 1996), has been recently attributed to an \textit{infA} gene mutant (Landau et al. 2007). CL2 seedlings carry a peculiar phenotype that shows a time-dependent expression pattern that is observed mainly in the upper part of the first leaf blade (Prina et al. 2003). By transmission electron microscopy, it has been observed that in the CL2 first leaf blade, plastid development and plastid ribosome formation are delayed, and results from pigment analysis indicate that plastid protein synthesis is delayed during CL2 embryogenesis (Prina et al. 2003). Besides, expression results of selected nuclear and plastid genes in CL2 seedlings (Colombo et al. 2008) also support the idea that the \textit{infA} gene is responsible for CL2 syndrome.

The first isolated CL2 mutant was made genetically stable by crossing CL2 plants as females with normal pollen, and, afterward, in the subsequent segregating generations, genetically stable families, that is without showing chlorophyll clonal stripes, were looked for (Prina 1996). Those genetically stable CL2 progenies, which were assumed to carry a normal (nonchloroplast mutator) nuclear genotype, were multiplied...
by natural self-pollination during several generations, and clonal streaks were never observed in the first leaf blades, all of which carried the CL2 phenotype. In the present work, we placed the chloroplast mutator genotype back into plants belonging to one of those stable families in order to regain the mutator action. Thus, CL2-stable plants were crossed, as females, with pollen from homozygous chloroplast mutator plants. Results presented here were obtained from F1 to F6 generation seedlings coming from those crosses and plants. Results presented here were obtained from F1 to F6 generation seedlings coming from those crosses and consisted of glasshouse observations of phenotypic reversions (normal or darker green stripes in a CL2-mutant background). In the F4 generation, only 1 of approximately 1500 seedlings showed a phenotypic reversion of this kind. The infA gene was sequenced in both portions of the clonally variegated leaf. In tissues of the mutant background, the mutation (T 157 C) that had been previously observed in CL2 (Landau et al. 2007) was confirmed, whereas an additional transition (A 178 G), 21 bp downstream the first transition, was found in tissues of the darker green stripe. This second mutation in the infA gene was also confirmed in darker green seedlings of the F3 and F6 generations. It corresponded to a second amino acid change in the IF1 protein, which would cause a partial recovery of its functionality.

Materials and Methods

Plant Material

Two-row spring barley (Hordeum vulgare L.) was used as experimental material.

CL2 was isolated several years ago from the barley chloroplast mutator genotype (Prina 1992, 1996). The experimental material was hybridized and handled to regain chloroplast mutator activity and keep the CL2-mutant plastome, according to the scheme shown in Figure 1.

Glasshouse Observations

The glasshouse analysis from the F1 to the F6 generations consisted in recording the number of seedlings with regard to typical CL2 or darker green phenotypes, which appeared either as solid homogeneous or as clonally variegated (striata seedlings) presenting both types of longitudinal stripes, carrying typical CL2 or darker green tissues. No other chlorophyll deficiencies were taken into account. It is important to remark that barley CL2 syndrome has a positional and time-dependent expression mainly manifested in young first leaf blades (Prina et al. 2003), and consequently, CL2 phenotypes must be recorded during the first days after emergence.

Gene Amplification and Sequencing

Total DNA was extracted from leaves following the micromethod protocol described by Dellaporta 1994. The sequence of the infA gene was amplified by polymerase chain reaction (PCR) with primers designed on the basis of published information of the chloroplast genome DNA sequence of barley (H. vulgare; GenBank NC_008590). A 934-bp fragment was amplified using the primer rpl36þ, 5'-CCCCTGTGTTTGGTTATGCTCG-3', located on the rpl36 gene and the primer rps8−, 5'-CGAGAGGTTTATTGAAGTGTTCCA-3', on the rps8 gene. Amplification was performed in a final volume of 50 μl, using 80 ng of total DNA, 5 μl Pfu DNA polymerase 10× reaction buffer with MgSO4, 1 μl dNTPs mix (10 mM each nucleotide), 1 μl of each primer (10 μM), and 0.5 μl Pfu DNA polymerase (3 U/μl). After denaturation at 94°C for 5 min, the reaction mixtures were heated to 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min, in 30 cycles.

The PCR products were sequenced using the PCR primers.

Results

The experimental material originated according to the scheme of Figure 1 consisted of:

- 52 F1 plants.
- 1022 F2 seedlings (corresponding to 52 F1 plant progenies).
- 1408 F3 seedlings (corresponding to 209 F2 plant progenies).
- Approximately 1500 F4 seedlings (corresponding to 142 F3 plant progenies).
- 59 F5 seedlings (corresponding to the progeny of 1 F4 striata seedling) and 358 F5 seedlings (corresponding to the progeny of 6 F4 plants carrying typical CL2 phenotype.
- 292 F6 seedlings (corresponding to the progeny of 12 F5 plants coming from the F4 striata seedlings).

When analyzed in the glasshouse, from seedling emergence to the second leaf stage, no phenotypic reversions were detected until the F4 generation in which only 1 of approximately 1500 analyzed seedlings carried a darker green clonal stripe on a CL2 phenotype background (Figure 2A). Samples from both kinds of tissue, that is, the darker stripe and the CL2 background, were collected in order to sequence the infA gene. In the sample of the last kind, the CL2-mutant gene was confirmed, that is, it carried the infA gene. In the sample coming from the greener stripe, an additional mutation, an A 178 G transition (A 178 G), 21 bp downstream the first transition, was observed mostly as clonally variegated pattern similar to that observed in the F4 mother plant (Table 1A). With reference to the 58 seedlings showing solid phenotypes, 42 carried the typical CL2 phenotype, that is, white or almost white first leaf blades during the first days after emergence (Figure 2B,C)
almost normal green except for the tip when they were fully grown, whereas the other 16 emerged much greener than typical CL2 seedlings and showed normal green fully grown first leaf blades afterward (see segregation of both types in Figure 2D,E). Sequencing of the infA gene in both kinds of solid seedlings revealed that those carrying the typical CL2 phenotype had sequences identical to that of the original CL2 mutant (Landau et al. 2007), whereas darker green seedlings showed both above-mentioned transitions. Furthermore, the progenies of 12 F5 seedlings (6 darker green and 6 typical CL2) were analyzed in the glasshouse (see Table 1B). The progenies coming from 4 F5 plants which showed the typical CL2 phenotype (plants 1–4) consisted of typical CL2 seedlings only, although 2 different segregations were observed in the progenies of plants 5 and 6. The progeny of plant 6 segregated 11 darker green versus 17 typical CL2 seedlings, whereas that from plant 5 segregated 27 typical CL2 and 2 striata seedlings carrying clonal stripes of typical CL2 and darker green tissues, similar to the striata seedlings observed in the F4 generation. On the other hand, the progenies of 5 plants which were darker green at seedling stage (plants 7–11) consisted of darker seedlings only, whereas that from plant 12 segregated 15 darker green versus 10 typical CL2 seedlings.

Although reciprocal crosses between the restituted green CL2 and the original CL2 line (which would show the cytoplasmic origin of the compensatory effect) were not performed, the peculiar segregation of phenotypes observed in the F4, F5, and F6 generations (see Table 1), which was also tightly associated with the segregation of the infA gene mutations, could hardly be explained on a different genetic basis.

**Discussion**

Phenotypic plastid reversion or restitution, especially in plastome mutator lines, has been known for several decades in many different plant taxa (cf. Kirk and Tilney-Bassett
Figure 2. (A) F4 seedling carrying a darker green clonal stripe on a CL2 phenotype background. (B) and (C) Typical CL2 seedlings, 6 or 8 days after sowing, respectively. See in (D) that greening has slightly started in a few seedlings. (E) Progeny of the F4 clonally variegated seedling shown in Figure 1. It shows the segregation of typical CL2 seedlings (light green or white) and much greener seedlings, 6 or 8 days after sowing, respectively.

1978). In this respect, it is important to point out that very different rates of phenotypic reversions have been previously observed in chlorophyll mutants derived from the barley chloroplast mutator (Prina 1996). In some of these mutants, even after carrying a mutator nucleus (cpm/cpm) during several generations of self-pollination, phenotypic reversions are either not observed or rare, whereas in others most of the seedlings present phenotypic reversions (Prina 1996). In the present study, CL2 showed no phenotypic reversions in the F2 and the F3 generations, whereas only 1 of more than a 1000 F4 seedlings carried evidences of reversion. This observation markedly differs from previous results obtained from crosses of a highly reverting line designated as CL4. In crosses of CL4 nonmutator (Cpm/Cpm) plants as females by mutator ones (cpm/cpm), evidences of phenotypic reversions were already observed in the F2 generation and in as many as 79 of 431 F2 seedlings (Prina 1996), suggesting that phenotypic reversions were present in most of the F2 seedlings carrying the cpm/cpm mutator nuclear genotype. These contrasting results about reversion rates observed in different mutant lines under the action of the same mutator gene probably depend on the genetic constitution underlying in each mutant line. In agreement with these results, reversion rates differing in several orders of magnitude have been early on noticed for different mutants in diverse organisms like the bacteriophage T4 (Benzer 1957), the yeast Saccharomyces cerevisiae (Das et al. 1988), Escherichia coli (Calos et al. 1978), and Neurospora crassa (Rambosek and Kinsey 1984).

The nuclear genes that alter plastome stability were early classified in 2 groups by Kirk and Tilney-Bassett (1978), according to the spectrum width of the mutant phenotypes they induce. InfA 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, the barley chloroplast mutator is the only one inducing a wide spectrum in monocots (Prina 1992). Additionally, all the aforementioned monocot mutators also differ from the barley chloroplast mutator in their breeding behavior, showing a faster sorting out of the cytoplasmically inherited mutant (see Prina 1992). Nuclear genes inducing several different cytoplasmically inherited chlorophyll types (wide spectrum) have been reported in dicots. However, in some of the chlorophyll-deficient mutants induced by them, it has been observed that morphological alterations are restricted not only to chloroplasts but also to mitochondria. Besides, cytoplasmic male sterility and mitochondrial DNA (mtDNA) alterations have also been observed in progenies coming from those mutators. In contrast, in mutants isolated from the barley chloroplast mutator, no cases of mitochondrial morphological changes and/or male sterility have been observed (Prina 1992, 1996; Prina et al. 2003; Landau et al. 2009). However, because no mtDNA molecular analyses were performed on these experimental materials, we cannot discard that some changes in the chondriome could have been induced by the barley chloroplast mutator genotype. Several hypotheses have been put forward to explain the underlying mechanisms of nuclear genes inducing plastome mutations. For those inducing a wide spectrum, the most straightforward explanations are based on failures in the nuclear genes that encode plastid DNA polymerases or plastid DNA repair enzymes (Hagemann 1986). The example better characterized at the molecular level is the mutator genotype of Oenothera, which induces deletions and duplications in the plastome through a replication slippage mechanism (Stoike and Sears 1998).

Plastome spontaneous variability is particularly scarce, and artificial induction of mutations by physical or chemical treatments has been moderately successful (reviewed by Prina et al. forthcoming). Based on this background, plastome mutator genes inducing a wide spectrum of mutants appear as an interesting alternative to increase plastome variability. With regard to the barley chloroplast mutator (Prina 1992), the new mutational change in the inf-I gene here reported is the 20th plastome transition isolated from plants carrying the barley mutator genotype, plus one base insertion (Landau et al. 2009; Prina et al. 2009). The subtle molecular changes induced by this mutator in plastid DNA point it out as a promising novel tool to explore the
The isolation of a clonally variegated F4 seedling (from both point mutations observed in the greener stripe of the F4 mother plant, which suggests a chimerical (heteroplastomic) plastid genome. The 2 levels of expression of CL2 syndrome were later observed in seedlings of CL2 background, whereas an additional transition (A 178 G) T 157 C transition (Landau et al. 2007) was confirmed in the F4 variegated plant, only 1 of 59 F5 seedlings showed only the original mutational change T 157 C. In the progeny of the F4 variegated seedling, with 2 different sequences, depending on the phenotype of the sector. The T 157 C transition (Landau et al. 2007) was confirmed in the CL2 background, whereas an additional transition (A 178 G) was also observed in the greener stripe. The 2 levels of expression of CL2 syndrome were later observed in seedlings of the F5 and F6 generations. Interestingly, we were able to confirm that the seedlings carrying the greener phenotype had the original mutational change T 157 C. In the progeny of the F4 variegated seedling, only 1 of 59 F5 seedlings showed a clonally variegated pattern similar to that observed in the F4 mother plant, which suggests a chimerical (heteroplastomic) condition. Nevertheless, it must be taken into account that nonstriped first leaf blades phenotypes are no guarantee of the otherwise highly conserved plastid genome.

Several previous evidences support the idea that the infA gene mutations originate the peculiar CL2 phenotype (Prina 1996; Prina et al. 2003, 2009; Landau et al. 2007; Colombo et al. 2008). The isolation of a clonally variegated F4 seedling (from crosses of CL2 genetically stable plants as females by chloroplast mutator ones) suggests that the CL2 mutant is either reverted or suppressed by a new plastome mutation probably induced by the chloroplast mutator genotype, which regained its action in the segregating generations. Sequencing the infA gene in samples from the 2 types of tissue of the F4 variegated seedling revealed 2 different sequences, depending on the phenotype of the sector. The T 157 C transition (Landau et al. 2007) was confirmed in the CL2 background, whereas an additional transition (A 178 G) was also observed in the greener stripe. The 2 levels of expression of CL2 syndrome were later observed in seedlings of the F4 and F5 generations. Interestingly, we were able to confirm that the seedlings carrying the greener phenotype had both point mutations observed in the greener stripe of the F4 variegated plants and that the typical CL2-mutant seedlings had only the original mutational change T 157 C. In the progeny of the F4 variegated seedling, only 1 of 59 F5 seedlings showed a clonally variegated pattern similar to that observed in the F4 mother plant, which suggests a chimerical (heteroplastomic) condition. Nevertheless, it must be taken into account that nonstriped first leaf blades phenotypes are no guarantee of homoplasmy in those F5 seedlings. This was tested in the F6 generation, by analyzing in the glasshouse 12 plant progenies coming from F5 plants which were classified according to the first leaf blade phenotype, as solid mutants (6 typical CL2 and 6 darker green). Nine of these progenies (4 coming from typical CL2 and 5 from darker green ones—see Table 1B) consisted of only one kind of mutant. However, 3 of these families segregated the 2 types of mutant tissues, indicating that the corresponding F5 mother plants were in fact heteroplastomic. Two of the progenies (coming from plants 6 and 12) segregated solid mutants of both kinds but no striata, whereas in the third progeny (from plant 5), most of the seedlings were typical CL2 solid mutants but 2 were striata (Table 1B). In conclusion, a rapid sorting out of the 2 kinds of plastids (carrying either 1 or 2 mutations in the infA gene) was observed in most cases between the F4 and F6 generations. However, in some cases, this sorting out was much slower, that is, the speed of sorting out among different progenies was variable, as it can be expected as a consequence of the highly polyploid and relaxed plastid genome (Birky 2001).

In an attempt to better understand the effect of these mutations in the function of the IF1 protein, we subjected an alignment of the wild type, the CL2 mutant, and the CL2 infA double mutant (DM). Figure 3. (A) Alignment of infA gene sequences from wild-type barley, CL2 mutant, and CL2 infA double mutant. (B) Alignment of IF1 protein sequences from wild-type barley, CL2 mutant, and CL2 infA double mutant (DM).
Table 1  Glasshouse analysis of progenies coming from the F4 striata seedlings shown in Figure 2 by self-pollination: Segregation of seedlings phenotypes regarding typical CL2, darker green, or clonally variegated (striata seedlings) presenting typical CL2 and darker green tissues together.

A: data from the F4 and F5 generations

<table>
<thead>
<tr>
<th>F4 mother plants</th>
<th>F4 seedlings phenotype</th>
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</thead>
<tbody>
<tr>
<td>Typical CL2</td>
<td>Darker green</td>
</tr>
<tr>
<td>Striata</td>
<td></td>
</tr>
<tr>
<td>1 (Typical CL2)</td>
<td>52</td>
</tr>
<tr>
<td>2 (Typical CL2)</td>
<td>63</td>
</tr>
<tr>
<td>3 (Striata)</td>
<td>42</td>
</tr>
<tr>
<td>4 (Typical CL2)</td>
<td>55</td>
</tr>
<tr>
<td>5 (Typical CL2)</td>
<td>61</td>
</tr>
<tr>
<td>6 (Typical CL2)</td>
<td>60</td>
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<tr>
<td>7 (Typical CL2)</td>
<td>67</td>
</tr>
</tbody>
</table>

B: data from the F5 and F6 generations

<table>
<thead>
<tr>
<th>F5 mother plants</th>
<th>F6 seedlings phenotype</th>
</tr>
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<tbody>
<tr>
<td>Typical CL2</td>
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<tr>
<td>Striata</td>
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<td>67</td>
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No other chlorophyll deficiencies were taken into account. A: data from the F4 and the F5 generations. B: data from the F5 and the F6 generations. 

avitating the corresponding F5 seedling phenotype.

that a mutation in another gene causing the CL2 phenotypic reversion casually occurred together with the newly induced transition in the \textit{infA} gene, which was observed only in the greener stripe of this rare F4 striped seedling and only in the darker green seedlings of its progeny. In conclusion, our results support the involvement of the \textit{infA} plastid gene in the CL2 phenotype. At present, if we assume that knockouts of the \textit{infA} gene are not obtainable by plastid transformation because no plant species harboring \textit{infA} are amenable for this technique, the CL2 lines are the only experimental material available by which the function of the \textit{infA} gene can be studied in higher plants.

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**References**


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