The *rpl23* gene and pseudogene are hotspots of illegitimate recombination in barley chloroplast mutator seedlings

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Previously, through a TILLING (Targeting Induced Local Lesions in Genomes) approach applied on barley chloroplast mutator (*cpm*) seedlings a high frequency of polymorphisms in the *rpl23* gene was detected. All the polymorphisms corresponded to five differences already known to exist in nature between the *rpl23* gene located in the inverted repeats (IRs) and the *rpl23* pseudogene located in the large single copy region (LSC). In this investigation, polymorphisms in the *rpl23* gene were verified and besides, a similar situation was found for the pseudogene in *cpm* seedlings. On the other hand, no polymorphisms were found in any of those loci in 40 wild type barley seedlings. Those facts and the independent occurrence of polymorphisms in the gene and pseudogene in individual seedlings suggest that the detected polymorphisms initially arose from gene conversion between gene and pseudogene. Moreover, an additional recombination process involving small recombinant segments seems to occur between the two gene copies as a consequence of their location in the IRs. These and previous results support the hypothesis that the CPM protein is a component of the plastome mismatch repair (MMR) system, whose failure of the anti-recombination activity results in increased illegitimate recombination between the *rpl23* gene and pseudogene.

The plastid genome or plastome is considered more conserved evolutionarily in comparison to the nuclear genome in terms of its structural organization and gene content. However, comparative molecular analyses revealed complex patterns of mutational changes¹ suggesting the existence of failures of DNA replication, recombination and/or repair (DNA-RRR) systems in the plastome².

It is known that the stability of the plastome can be markedly affected by nuclear gene mutations, some of them also altering the genetic stability of the mitochondrion³–⁵. Investigations of these mutants could improve the limited knowledge about the mechanisms that maintain the integrity of plant organellar DNA.

The barley chloroplast mutator (*cpm*) mutant is the first example in monocots that induces a wide spectrum of cytoplasmically inherited mutations⁶–⁸, which serve as a rich resource for the study of the mechanisms for the maintenance of plastome integrity. Previously, we proved that the plastome was the location of a number of mutations isolated from *cpm* plants⁹–¹² and recently¹³, we extended the molecular analysis of the plastome in *cpm* seedlings through a TILLING (Targeting Induced Local Lesions in Genomes) approach. Following this strategy, we identified a wide range of plastome genes containing polymorphisms, consistent with our previous hypothesis about the involvement of the Cpm gene in maintaining plastome stability¹⁴. The spectrum of molecular changes observed in *cpm* seedlings mostly consisted of point mutations and small indels in microsatellite regions, which were explained as originating from at least 61 independent mutational events. However, in the case of the *rpl23* gene, we observed a peculiar pattern of variation, which consisted of combinations of one to several of the same five polymorphisms. In the grass family there are two copies of the *rpl23* gene that are located in the IRs, while

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there also exist a non-functional version, the \textit{rpl23} pseudogene, located in the large single copy (LSC) region (see Fig. 1).

Coincidently, the five \textit{rpl23} gene polymorphisms mentioned above correspond to the polymorphic variation already existing between the \textit{rpl23} gene and pseudogene in nature, suggesting that they arose from recombination events between these two loci. In support of this hypothesis, Bowman et al.\textsuperscript{15} suggested that the \textit{rpl23} pseudogene has been converted by the functional \textit{rpl23} gene, and Morton and Clegg\textsuperscript{16} had provided additional support for a gene conversion model between the \textit{rpl23} pseudogene and its functional counterpart in at least two lineages of the grass family.

In the present work, we performed a deeper analysis of the \textit{rpl23} gene and included the \textit{rpl23} pseudogene using the same DNA samples of \textit{cpm} seedlings previously investigated by Landau et al.\textsuperscript{13}. Moreover, both regions were also studied in two groups of control genotypes. One included several families derived from the parental line from which the \textit{cpm} mutant was isolated\textsuperscript{6}. The other group consisted of several barley genotypes representing a wide range of origins.

It was concluded that the nuclear \textit{cpm} mutant induces not only a high rate of plastome point mutants, as previously reported\textsuperscript{13}, but it also stimulates recombination between the \textit{rpl23} gene and pseudogene, via gene conversion.

Our findings support previous hypotheses, which postulated that the \textit{Cpm} gene is involved in plastome DNA repair\textsuperscript{6} as a component of the mismatch repair system\textsuperscript{13}.

**Results**

**Analysis of \textit{rpl23} gene in polymorphic \textit{cpm} seedlings.** A chloroplast TILLING (cpTILLING) analysis of the region corresponding to the \textit{rpl2} amplicon in 304 \textit{cpm} seedlings previously showed that 92 were polymorphic for the \textit{rpl23} gene\textsuperscript{13}. In the present study, the same \textit{cpm} seedlings were further analyzed using the region corresponding to the \textit{rpl23} amplicon (see Fig. 2A). The \textit{rpl23} amplicon digestions showed three pairs of bands, as they did in the \textit{rpl2} amplicon (Supplementary Fig. S1). However, the product bands of \textit{rpl23} amplicon digestion were better resolved in the analytical gel (Fig. 2B) and, therefore, the different combinations of \textit{rpl23} gene...
polymorphisms, i.e. genetic variants, could be more precisely evaluated. In fact, all of them were later confirmed by sequencing. These assessments showed that 30% (92/304) of the \( \text{cpm} \) seedlings were polymorphic for the \( \text{rpl23} \) gene.

The sequencing of the \( \text{rpl23} \) amplicon confirmed that the different digestion patterns always represented combinations of five DNA molecular changes in the \( \text{rpl23} \) gene that correspond to the following mutations: 2 transitions (G118A: missense; A203G: missense), 2 transversions (G115T: missense; T132A: silent), and 1 nucleotide deletion (G at position 133: frameshift mutation generating a premature stop codon). As depicted in Fig. 2 and detailed in Table 1, these five changes were always observed in three groups or blocks named \( A \), \( B \), or \( C \), while the absence of molecular changes (corresponding to the wild type sequence) is represented by the symbol + in each of the blocks. Block \( A \) comprised two single base mutations (G115T and G118A), as did block \( B \) (T132A and DelG133), while block \( C \) consisted of only one molecular change (A203G). The different block combinations (\( ABC, AB+ \), etc.) can give seven potential genetic variants, in addition to the wild type sequence in all the three blocks (+++).

All potential combinations, with the exception of \( A + C \), were observed for the \( \text{rpl23} \) gene (Table 1). The most abundant variant was ++C, which was observed in all the six families and in 41 out of the 92 \( \text{rpl23} \) gene polymorphic seedlings. It was followed by variant \( ABC \), which was observed in five families and 21 seedlings.

### Screening and identification of \( \text{rpl23} \) pseudogene polymorphisms in \( \text{cpm} \) seedlings.

All 304 \( \text{cpm} \) seedlings previously analyzed by Landau et al. to isolate the 92 \( \text{rpl23} \) gene polymorphic seedlings were tested in the present investigation for \( \text{rpl23} \) pseudogene polymorphisms, by using a similar strategy of CJE digestion of the \( \text{rpl23} \) pseudogene amplicon (see Fig. 4A). As observed for the \( \text{rpl23} \) gene, combinations of three pairs of bands were identified (Fig. 4B), whose sizes were similar to those of the \( \text{rpl23} \) gene.

The sequencing of \( \text{rpl23} \) pseudogene amplicons giving differential digestion patterns revealed that all the genetic variants corresponded to combinations of the five molecular changes observed in \( \text{rpl23} \) pseudogene:
two transitions (A115G and G199A), two transversions (T112G and A129T) and 1 nucleotide insertion (G at position 130). Moreover, as observed for the rpl23 gene, the five mutations in the rpl23 pseudogene appeared to be grouped in three blocks, which were named D, E and F. Block D contained two molecular changes (T112G and A115G), as did block E (A129T and InsG130), while block F contained only one molecular change (G199A) (see Fig. 4). Also similar to the rpl23 gene, the potential combinations of these blocks would give seven genetic variants for the rpl23 pseudogene besides the combination composed of the wild type pseudogene sequences in all three blocks +++ (Fig. 5).

The number of seedlings carrying the different rpl23 pseudogene variants in each family is shown in Table 2. The rpl23 pseudogene was polymorphic in 65% of the seedlings (197 out of 304). Although seedlings polymorphic for the rpl23 pseudogene were approximately twice than those polymorphic for the rpl23 gene, only three of the seven potential genetic variants were detected. Most of rpl23 pseudogene polymorphic seedlings (128 out of 197) carried the DEF combination with changes in all three blocks. The variant DE + was observed in 66 out of 197 seedlings, while only three seedlings showed the variant D + F. This means that almost all rpl23 pseudogene polymorphic seedlings (194 out of 197) carried only two of the potential combinations.

Table 1. Number of seedlings carrying different rpl23 gene variants in the two groups of cpm families. *The number of homoplastic seedlings is shown in brackets. A, B and C represent the polymorphic positions in the cpm rpl23 gene (blocks A, B and C). + represents the wild type sequences.

| Genetic variants in the rpl23 gene* | A++ | +B+ | ++C | AB+ | +BC | A+C | ABC | ++++ |
|-----------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| **Group A**                       |     |     |     |     |     |     |     |     |
| F2                               | 0   | 4 (1) | 25 (23) | 4 (0) | 6 (0) | 0 | 12 (5) | 108 |
| M2                               | 0   | 0 | 5 (5) | 0 | 0 | 0 | 1 (0) | 17 |
| Subtotal                         | 0 | 4 (1) | 30 (28) | 4 (0) | 6 (0) | 0 | 13 (5) | 125 |
| **Group B**                       |     |     |     |     |     |     |     |     |
| F2-1                             | 0 | 0 | 3 (2) | 0 | 1 (0) | 0 | 1 (0) | 21 |
| F2-2                             | 0 | 0 | 1 (0) | 0 | 3 (2) | 0 | 0 | 12 |
| F2-3                             | 0 | 1 (0) | 2 (0) | 4 (0) | 1 (0) | 0 | 4 (0) | 27 |
| F2-4                             | 1 (0) | 5 (0) | 5 (1) | 0 | 0 | 0 | 3 (0) | 27 |
| Subtotal                         | 1 (0) | 6 (0) | 11 (3) | 4 (0) | 5 (2) | 0 | 8 (0) | 87 |
| **Total**                         | 1 (0) | 10 (1) | 41 (31) | 8 (0) | 11 (2) | 0 | 21 (5) | 212 |

Screening for polymorphisms in the rpl23 gene and rpl23 pseudogene in wild type control seedlings. No polymorphisms for the rpl23 gene or the rpl23 pseudogene were detected in 40 DNA samples coming from individual wild type seedlings of the two groups of accessions used as controls using CJE digestions (Supplementary Figs S2 and S3). The absence of polymorphisms was further corroborated by sequencing both amplicons in some randomly selected control seedlings.

Determination of the homo- or hetero-plastomic state for variations in the rpl23 gene and rpl23 pseudogene. The homo- or hetero-plastomic state of the variants in the rpl23 gene and the pseudogene was estimated through the observation of a single or double peak, respectively, in the electropherograms after the amplicons were sequenced. In addition, CJE digestions were also performed. As a criterion, the blocks were considered to be homoplastic when the digestion products were only detected in DNA samples previously mixed with wild type DNA, while they were considered to be heteroplastic when the digestion bands were observed in the DNA sample analyzed alone. Only the seedlings carrying all the blocks in homoplastic state were considered homoplastic.
Thirty nine out of the 92 rpl23 gene polymorphic seedlings were determined as homoplastomic by the two methods mentioned above; most of them (31 out of 39) had the variant combination ++C. The other variants determined as homoplastomic were: ABC, +BC and +B+, which were found in five, two and one seedling respectively.

In contrast to what was observed in the rpl23 gene, homoplastomic seedlings were much more frequent in the pseudogene, but the genetic variants were fewer. In fact, the majority of the rpl23 pseudogene polymorphic seedlings were homoplastomic (123 out of 197) being the proportion of seedlings in homo- or heteroplastomic state not homogeneous between gene and pseudogene ($\chi^2 = 10.23$; df = 1; p = 0.0014). Most of the rpl23 pseudogene homoplastomic seedlings (81 out of 123) had the genetic variant DEF, 40 seedlings had the variant DE+ and only two seedlings had D+F.

Regarding the proportion of homoplastomic seedlings with respect to the two groups of cpm families analyzed (see Materials and Methods), in Group A the gene was observed homoplastomic in 34 out of 57 seedlings (0.60) versus 5 out of 35 (0.14) in Group B. While in Group A, the pseudogene was observed homoplastomic in 81 out of 113 seedlings (0.72) versus 42 out of 84 (0.50) in Group B. That is to say, the proportion of homoplastomic seedlings was higher in Group A than in Group B for both loci, gene and pseudogene.

Figure 4. The rpl23 pseudogene amplicon. (A) Polymorphisms detected in the rpl23 pseudogene. (B) CJE digestions of rpl23 pseudogene amplicons on a nondenaturing 3% polyacrylamide gel. The cpm samples show different digestion patterns because they carry several combinations of polymorphisms: seedlings 1, 4 and 11 do not carry any polymorphism; seedlings 2, 3 and 8 carry T112G, A115G, A129T and InsG130; seedings 5, 6, 7, 9 and 10 carry T112G, A115G, A129T, InsG130 and G199A. *The samples run slower than the molecular weight marker probably due to the components of the CJE digestion mixture.
Analysis of albino seedlings carrying polymorphisms in the \textit{rpl23} gene.

Among the 304 \textit{cpm} seedlings, 20 had an albino phenotype, and twelve of these were polymorphic for the \textit{rpl23} gene, with different genetic variants. Eight of these seedlings were homoplastomic: five had the ABC combination (see digestions of samples 1 and 5 in Fig. 2B), two had the +BC variant and only one had the +B variant (see digestion of sample 2 in Fig. 2B and the electropherogram in Supplementary Fig. S4). The remaining four albino seedlings were heteroplasmic for blocks A and C but not for block B (see digestions of samples 3, 4 and 6 in Fig. 2). Moreover, it was observed that none of the \textit{rpl23} polymorphic seedlings with a normal green phenotype was homoplastomic for block B.

Furthermore, we looked for \textit{striata-albina} seedlings in one of the families that segregated the albino seedlings mentioned above. The \textit{striata} seedlings allowed us to separately isolate samples from normal green or albino tissues of the same seedling (Supplementary Fig. S5). When sequencing the \textit{rpl23} amplicon in samples isolated from three individual seedlings, those from albino tissues showed the presence of block B in homoplasmic state, while those from normal green tissues showed the wild type sequence of the \textit{rpl23} gene or the sequence corresponding to block B in heteroplasmic state (combination of variants ++ + and +B+).

All these data show that there is an association between the homoplasmic state of block B (DelG133) and albinism.

Analysis of RbCl protein level in albino and normal green seedlings by Western blot.

The deletion at position 133 of the \textit{rpl23} gene causes a frameshift generating a premature stop codon and a truncated protein of only 47 amino acids (Supplementary Fig. S6), which is probably non-functional. This led us to investigate the level of the Rubisco large subunit (RbCl), to check if the chloroplast protein accumulation due to impairment of the chloroplast translation was affected in albino seedlings containing the DelG133 in homoplasmacy. We also evaluated a nucleus-encoded chloroplast-targeted cpRecA protein, as a control. The presence or absence of these two proteins was determined by Western blots of progeny of \textit{rpl23} polymorphic seedlings from two different families (see Materials and Methods) segregating albino and normal green seedlings. RbCl was missing in the albino seedlings of both families. In contrast, RbCl was detected in samples from green seedlings. On the other hand, the chloroplast targeted version of the nuclear-encoded protein cpRecA was present in both, albino and normal green seedlings (Fig. 6).
Comparison of wild type sequences of the rpl23 gene and pseudogene. The alignment of the barley wild type sequences of the rpl23 gene and rpl23 pseudogene showed five polymorphic positions in addition to the absence of ATG at the beginning of the rpl23 pseudogene. Notably, these positions coincide exactly with blocks A, B, C, D, E and F mentioned above. The molecular changes identified in the rpl23 gene of cpm seedlings match exactly with the corresponding sequence of the wild type pseudogene. Similarly, the molecular changes identified in the pseudogene of cpm seedlings match exactly with the corresponding sequence of the wild type gene of barley (Fig. 7).

In contrast to the high frequency of seedlings carrying the molecular changes corresponding to the genetic variants described above, notably no other polymorphisms were found in the rpl23 gene or rpl23 pseudogene loci. However, two different polymorphisms were found in both amplicons, but outside the gene or the pseudogene regions. One of them was a C302T substitution in the rpl23 amplicon, which was found in only one seedling. The other one, which was also found in only one seedling, was a T856C substitution in the rpl23 pseudogene amplicon.

Coexistence of rpl23 gene and rpl23 pseudogene polymorphisms in individual seedlings. The coexistence of polymorphisms, i.e.: existence of polymorphisms in the rpl23 gene and the rpl23 pseudogene in individual seedlings, is presented in Table 3. Data were compiled per locus disregarding the variants or the heteroplasmic or homoplasmic state of the polymorphisms. The major proportion of seedlings had polymorphisms only in the pseudogene. Statistically, the occurrence of polymorphisms in the gene or the pseudogene are independent ($\chi^2 = 0.47; \text{df} = 1; p = 0.49$).
| rpl23 gene | rpl23 pseudogene | Number of seedlings |
|------------|------------------|--------------------|
| polymorphic | polymorphic | 57 |
| polymorphic | wild type | 35 |
| wild type | polymorphic | 140 |
| wild type | wild type | 72 |

Table 3. Number of seedlings with polymorphic rpl23 gene and/or rpl23 pseudogene.

| Number of seedlings | rpl23 gene | rpl23 pseudogene | Identical sequences |
|---------------------|------------|------------------|--------------------|
| 77                  | +++        | D E F            | yes                |
| 23                  | +++        | D E +            | no                 |
| 12                  | ++C        | D E +            | yes                |
| 11                  | ++C        | +++              | no                 |
| 5                   | ABC        | +++              | yes                |
| 2                   | +BC        | +++              | no                 |
| 1                   | +B+        | D + F            | yes                |
| 1                   | +++        | D + F            | no                 |

Table 4. Genetic variants of the rpl23 gene and rpl23 pseudogene in homoplastomic seedlings.

A total of 132 seedlings were homoplastomic both in the rpl23 gene and in the rpl23 pseudogene (see Table 4). Most of these double homoplastomic seedlings (101 out of 132), carried the wild type sequence +++ in the rpl23 gene, while 77 carried the DEF variant in the pseudogene. Moreover, 18 seedlings had the wild type sequence +++ in the rpl23 pseudogene and five of these seedlings carried the variant ABC in the gene. Finally, 13 seedlings had sequences different to wild type both in the gene and in the pseudogene, i.e.: 12 seedlings carried variant ++C in the gene and variant DE+ in the pseudogene; and one seedling had the combination of + B+ in the gene and D + F in the pseudogene.

In silico analysis for the presence of homologous sequences of the rpl23 gene or the pseudogene amplicons in the nuclear and mitochondrial genomes of barley. We performed an in silico analysis to check for the presence of the complete rpl23 gene and pseudogen amplicons in the barley genome. Both sequences were blasted against the H. vulgare mitochondrial and nuclear DNA. No hits were found when both amplicons were blasted to the mitochondrial genome of H. vulgare while the alignments showed that the amplicons would be contained completely in the nuclear genome. The rpl23 amplicon matched against chromosomes 1, 2, 5, 6 and 7, and it was observed that some of the nuclear sequences were identical to the wild type plastome, while others were polymorphic. In total, 55 polymorphisms were identified and among them only two (G118A and A203G) corresponded to the five detected in cpm seedlings by CJE digestion and sequencing. In the nuclear sequences, those two polymorphisms were always accompanied by others, but none of them corresponded to those detected by CJE digestion and sequencing. Moreover, the only polymorphism found in the rpl23 amplicon that located outside the gene region did not correspond to any of the 55 polymorphisms identified by blast in the nuclear genome.

The rpl23 pseudogene amplicon matched against chromosome 6 and the nuclear sequence contained the five changes (T112G, A115G, A129T, InsG130, G199A) identified in the rpl23 pseudogene of cpm seedlings.

Discussion

The high frequency of polymorphisms detected by CJE digestion in the cpm rpl23 gene and/or pseudogene arose from illegitimate recombination of pre-existing differences in wild type barley between these two loci. A high proportion of cpm seedlings (92/304) were polymorphic for the rpl23 gene (Table 1) and the proportion was even higher (197/304) for the rpl23 pseudogene (Table 2). However, not a single polymorphism was detected among 40 wild type control seedlings, which included a wide range of barley entrances and also one H. spontanea, a fact that strongly supports the influence of the cpm gene in the occurrence of these polymorphisms.

Given that by a blast analysis some of these polymorphisms were found in nuclear DNA sequences, it is appropriate to discuss if the polymorphisms detected by CJE digestions in cpm seedlings correspond to molecular differences that raised under the influence of the cpm genotype in the plastid rpl23 gene and/or the pseudogene or, on the contrary, if we detected polymorphisms already existing in the nucleus. First of all, the sensitivity of detection of CJE/Cel I digestion of mismatches after heteroduplex formation in a mixture of different DNA molecules depends on the detection method. When using fluorescent dye detection (Li-cor), which is more sensitive than ethidium bromide, the pools of individuals are eightfold at most because higher dilutions of a polymorphism make it very hard to be detected by this technique. Furthermore, chloroplast genes are in much greater proportion than nuclear genes due to the high-copy number of plastids per cell. Genomic DNA extracts are naturally enriched for plastids and thus the plastome would be an easier target than low-copy nuclear genes for sequencing and amplification. In this sense, the identification of homoplastomic seedlings carrying polymorphisms in
the rpl23 gene and pseudogene is another situation difficult to explain under the hypothesis of the nuclear origin of the polymorphisms.

Regarding the blast analysis, among the 55 polymorphisms found in the nuclear DNA for the rpl23 amplicon, only two of them were identified by CJE digestion and sequencing in cpm seedlings and in this sense, it is worth mentioning that one of them, G118A, was always found together with G115T, which was not found in the nuclear sequences. For the rpl23 pseudogene amplicon, all the five polymorphisms were found together in chromosome 6, while when detected by CJE digestion and sequencing they showed with and without polymorphism G199A.

It is important to remark that all the polymorphisms observed by CJE digestions and sequencing corresponded to different combinations of the very same five polymorphisms already existing in wild type barley between the rpl23 gene and pseudogene. Besides, the five different polymorphisms located in similar positions in gene and pseudogene segregated as three blocks in correspondence with the distance between the different polymorphisms. The combination of the three blocks originated different genetic variants in both, gene and pseudogene. All these results strongly support the hypothesis that the polymorphisms in those regions, which were only detected in cpm seedlings, arose from increased illegitimate recombination of polymorphisms pre-existing in nature between the rpl23 gene and pseudogene.

Independent occurrence of polymorphisms in the rpl23 gene or the pseudogene suggests that they originated in gene conversion. The analysis of both loci in the same seedling showed that the presence of polymorphisms in one of them was statistically independent from the presence of polymorphisms in the other. This result suggests that the polymorphisms more likely arose from gene conversion rather than from reciprocal exchanges, which would simultaneously affect both loci. In wheat and corn, Bowman et al. suggested that the sequence of the rpl23 pseudogene undergoes a conversion process from DNA sequences of the rpl23 gene, because of the lower divergence in pseudogenes in comparison to the surrounding non-coding regions. Subsequent work based on a phylogenetic analysis provided additional support to a model of gene conversion between the rpl23 pseudogene and its functional counterpart in at least two lineages of the grass family. Our results support previous hypothesis and also show that gene conversion occurred in both directions, gene to pseudogene and vice versa.

Higher diversity of genetics variants in the rpl23 gene suggests that between the two copies of the gene there exist an additional recombination process apart from that recombining gene and pseudogene. The rpl23 gene polymorphic seedlings carried six of the seven potential combinations of polymorphisms (Table 1), while the rpl23 pseudogene polymorphic seedlings carried only three of the seven potential combinations (Table 2). Moreover, in the gene, 59 seedlings carried genetic variants that involve recombinations between block B and C, which are separated by 70 bp, while 22 seedlings carried genetic variants involving recombinations between blocks A and B, which are separated by only 14 bp. Our interpretation is that in cpm seedlings, recombination between the two copies of the gene occurs much more frequently than it does between the two copies of the gene and the pseudogene. Previous reports about homologous recombination of foreign DNA through gene conversion concluded that it is extremely rare in plastid sequences smaller than 50–100 bp. However, it is worth to remark that recombination in the inverted repeats (IRs) have been several times proposed to occur not only by gene conversion. On the one hand, a copy correction mechanism like gene conversion between the IRs within a molecule is needed to maintain sequence identity between them. On the other hand, a high frequency of intramolecular recombination, probably crossing over between the IRs, was inferred in Chlamydomonas, ferns and land plants from the fact that chloroplast genomes carrying two IRs, exist as an equimolar mixture of two isomers differing only in the relative orientation of their single-copy regions. On this basis, it can be hypothesized that the numerous recombinants involving small segments observed in the rpl23 gene were a consequence of its location in the IRs. Whether the high recombination rates observed between the two copies of the gene have been increased under the influence of the cpm remains an open question.

Regarding the process of gene conversion between gene and pseudogene, it is reasonable to think that the genetic variants observed in the pseudogene would be more similar to those that originally arose from illegitimate recombination, than those observed in the gene, since the latter could be modified by selection pressure and/or additional recombination between the two copies of the gene. In such a case, we can speculate that probably gene conversion between gene and pseudogene frequently occurs by replacement of the entire DNA sequence in each locus and, recombinants between blocks separated by short segments would originate in the gene by crossing over between two copies of the gene, which are located in the IRs.

A greater proportion of homoplastomic seedlings in the pseudogene in relation to the gene is in agreement with the idea regarding the higher rates of new allele fixation in the single copy regions compared to that in the IRs. As expected, homoplastomic seedlings for both gene and pseudogene were higher in Group A, which carried the cpm mutant during many more generations of self-pollination than Group B and therefore, mutants originated in early generations would have more opportunities of sorting out.

Besides, our results show that cpm seedlings polymorphic for the pseudogene (Table 2) had a greater proportion of homoplastomic seedlings than those polymorphic for the gene (Table 1), which is in agreement with the widely accepted idea that new alleles in the single copy regions have a higher rate of fixation than those in the IRs. In this sense, the double copy number of genes located in the IRs compared to that of genes located in the single copy regions has been used to explain the lower frequency of mutations estimated for the former. Several double homoplastomic seedlings show concerted evolution and lead to a dead end of potential variability arising from recombination between gene and pseudogene. The most
common combination of double homoplastomic seedlings in both, gene and pseudogene, was ++++ in the gene and DEF in the pseudogene (Table 4). As the sequences corresponding to blocks D, E and F in the pseudogene are identical to wild type sequences in the gene, both loci ended up having identical sequences. Therefore, such a genetic composition leads to a dead end in terms of potential variability through recombination between these loci. Moreover, if it is considered that sequences corresponding to blocks A, B and C in the gene are identical to wild type sequences in the pseudogene, the same situation occurs in other double homoplastomic seedlings i.e.:+++C in the gene and DE++ in the pseudogene; ABC in the gene and ++++ in the pseudogene and +B+++ in the gene and D++F in the pseudogene (see Table 4). These findings can be considered an experimental verification of what has been defined as concerted evolution4,35.

Relationship between block B (DelG133) and albinism suggests that some albino phenotypes could be attributed to the malfunction of the plastid ribosomes due to the lack of Rpl23 protein. Several data support the hypothesis that some albino phenotypes are due to the detrimental effect of block B (Del133) in homoplastomic state. All 12 seedlings that carried block B (DelG133) in homoplastomy were albino and none of the seedlings carrying the wild type phenotype were homoplastomic for block B. Additionally, in striata seedlings carrying albino and normal green longitudinal stripes, block B in homoplastic state was only observed in albino tissues. Moreover, albino seedlings homoplastomic for block B lacked the plastome encoded RbcL protein (Fig. 6), a fact that could be attributed to the malfunction of the plastid ribosomes due to the lack of Rpl23 protein, whose essentiality was determined in tobacco even in heterotrophic conditions36. Similar observations have been made of an albino rice mutant having a substitution in the nuclear gene prpl12, which encodes the chloroplast ribosome protein PRPL1242 and the albostrians mutant of barley, which lacks plastid ribosomes and RbcL protein in albino tissues3.4,39.

Illegitimate recombination between the rpl23 gene and pseudogene is increased in cpm seedlings probably due to failures in the anti-recombination activity of a DNA mismatch repair (MMR) protein. The cpm seedlings investigated here were previously analyzed by a cpTILLING approach directed to a wide range of genes and intergenic regions, and those results support previous postulations regarding the involvement of the Cpm gene product in maintaining plastome DNA stability13. The spectrum of plastome polymorphisms detected in cpm seedlings mostly consisted of substitutions and small indels in microsatellites. Additionally, a peculiar pattern of polymorphisms observed in the rpl23 gene and the presence of some big indels suggested that an increase of recombination rates also occurs in cpm seedlings15. In the present investigation, the hypothesis regarding the recombinational origin of rpl23 gene polymorphisms in cpm seedlings was fully confirmed.

The spectrum of polymorphisms previously observed in cpm seedlings13 suggests the malfunctioning of a gene involved in the MMR system. The MMR system is critical for keeping mutation rates at low levels by correcting DNA replication errors. Besides, it contributes to genome stability by blocking recombination between divergent DNA sequences, such as the case of the rpl23 gene and its pseudogene. Eukaryotic MMR proteins have been studied extensively in yeast and mammalian cells40–42, while knowledge about their role in maintaining plant genome stability, including plant genome organelles, is more scarce43–46.

Conclusions and perspectives. It was confirmed that rpl23 gene and rpl23 pseudogene polymorphisms observed in cpm seedlings arose from increased rates of illegitimate recombination by gene conversion between these two loci. It is proposed that illegitimate recombination occurs in cpm seedlings as a consequence of failure of the MMR system anti-recombination activity that is usually in charge of preventing promiscuous recombination42. In the gene, the availability of polymorphisms acquired from the pseudogene allowed us to distinguish that of the MMR system anti-recombination activity that is usually in charge of preventing promiscuous recombination42. Additionally, a peculiar pattern of polymorphisms observed in the rpl23 gene and the presence of some big indels suggested that an increase of recombination rates also occurs in cpm seedlings15. In the present investigation, the hypothesis regarding the recombinational origin of rpl23 gene polymorphisms in cpm seedlings was fully confirmed.

The overall landscape of polymorphisms previously observed in cpm seedlings also includes increased rates of substitutions and small indels in microsatellites, all supporting the postulation of the Cpm gene as a member of the DNA-MMR system15. The cpm mutant offers an interesting experimental material to investigate the mechanisms involved in maintaining the integrity of plant organelar DNA47,48 and their role in plant evolution49. Besides, as it was pointed out several times13,42,50, unstable genotypes like the cpm mutant can provide an alternative to the limited capacity of traditional techniques for inducing and isolating new plastome mutations.

Materials and Methods
Plant material. Most of the plant material consisted of DNA samples coming from 304 barley chloroplast mutant (cpm) seedlings8. This experimental material was previously used to conduct a plastome TILLING strategy for an extensive scanning of 33 plastome genes and a few intergenic regions13. These seedlings belong to six families maintained by natural self-pollination, which carried the cpm genotype through different numbers of generations. They were grouped into: Group A, consisting of two families that carried the homozygous mutant genotype (cpm/cpm) through 12 to 17 generations, and Group B consisting of four families that carried the mutant genotype for five generations43. In that investigation, 92 samples turned out to be polymorphic for the rpl23 gene and they were used in the present work to further analyze the rpl23 gene polymorphisms. In addition, all 304 seedlings mentioned above were tested in this work for rpl23 pseudogene polymorphisms. Wild type seedlings belonging to two different control groups were also analyzed for polymorphisms in both the rpl23 gene and the pseudogene. Group 1 consisted of 20 individual seedlings coming from different families derived from the very
same parental genotype from which the cpm mutant was isolated after a mutagenic treatment. Group 2 consisted of 20 individual seedlings representing a very wide range of barley accessions, which included malting and fodder commercial varieties and also some wild barley populations (Supplementary Table S1). In addition, we analyzed a few seedlings corresponding to progenies coming from rpl23 polymorphic plants that segregated solid or striata-albina together with normal green seedlings.

All the seedlings were grown in a greenhouse for observation at the second leaf stage and for tissue extraction; later on they were transplanted to the field nursery and grown to maturity for observation and seed multiplication.

**DNA isolation.** Genomic DNA was isolated from one or two leaves of individual seedlings using the micromethod described in Dellaporta with modifications. The tissue was ground with Dellaporta isolation buffer in the Fast Prep®-24 Instrument (MP Biomedicals, USA) and extracted with chloroform before DNA precipitation. DNA concentrations were measured using a spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE, USA) and standardized to a concentration of 80 ng/µl.

**Analysis of the rpl23 gene and rpl23 pseudogene by celery juice extract (CJE) digestions.** The 92 cpm DNA samples polymorphic for the rpl23 gene were previously detected by amplification and celery juice extract (CJE) digestion of the rpl23 amplicon (1,216 bp) obtained using the primers: rpl2F 5′-CACTTGCGCTGGTGTT-CAA-3′ and rpl2R 5′-TGGAGTTGCGAATTTTCTGCA-3′. The polymorphisms were determined by sequencing of the rpl23 amplicon as described in Landau et al. In the present work, these 92 samples were re-amplified with the rpl23 amplicon and then subjected to CJE digestion. The primers used for amplification of the rpl23 amplicon (831 bp) were: rpl23F 5′-ATGGATGGAACTCATACTGCA-3′ and rpl22F 5′-CACTTTGCGCTGGTGTT-CAA-3′. The rpl23F primer is located at the beginning of the rpl23 gene and in combination with the rpl22F primer amplifies both copies of the rpl23 gene.

The screening for rpl23 pseudogene polymorphisms was done by amplification and CJE digestion of the rpl23 pseudogene amplicon (1,126 bp) in the 304 cpm seedlings. The primers used were: rpl23F 5′-ATGGATGGAACTCATACTGCA-3′ and hotspotR 5′-CATCCTCATGGCCTTTCTTAC-3′. The rpl23F primer is located at the beginning of the rpl23 pseudogene.

The rpl23 amplicon and the rpl23 pseudogene amplicons were also used to identify polymorphisms by CJE digestion of DNA samples from wild type seedlings of the control groups.

In order to favor the heteroduplex formation during the screening assays for detection of rpl23 gene and pseudogene polymorphisms, each sample was mixed with wild type DNA in a 1:1 ratio. The wild type DNA used for this purpose was the same used in our previous work. In the analyses of the rpl23 amplicon, the cpm DNA samples were also subjected to CJE digestion alone, without mixing with wild type DNA, to identify the homo- or hetero-plastomic state of the polymorphisms. The homo- or hetero-plastomic state was also estimated by the observation of one or two peaks in the electrophograms coming from the amplicons sequencing.

Primer design was based on the chloroplast genome sequence of barley (Hordeum vulgare) [GenBank: NC_008590.1] using Primer3 software (v. 4.0.0, http://primer3.ut.ee/).

The PCR amplification conditions, slow re-annealing step, CJE digestion and polyacrylamide electrophoresis were performed according to Landau et al.

**Identification of polymorphisms in the rpl23 gene and the rpl23 pseudogene.** The rpl23 gene and the rpl23 pseudogene amplicons showing digestion with CJE were analyzed by standard sequencing (Macrogen Inc, Korea) and the polymorphisms relative to the wild type sequences were determined.

Assembly and graphics were done with Vector NTI 10.0 Software (Thermo Fisher Scientific Inc, USA) and sequences were aligned with Clustal O (1.2.4). The barley plastome figure was done with OGDraw online software.

**Protein extraction and Western blot.** The soluble protein fraction of the thylakoid isolation protocol was obtained according to Guimet et al. The concentration of soluble proteins was assessed by micro BCA protein assay kit (Thermo Fisher Scientific Inc, USA). Soluble fraction proteins were separated by SDS-PAGE and immunoblotted with rabbit anti Rubisco large subunit (RbcL) antiserum (Agrisera, Sweden) and rabbit E. coli RecA antiserum (Abcam, UK) to determine the presence of the chloroplast-encoded subunit of the Rubisco (RbcL) and the chloroplast-targeted ortholog of the E. coli RecA protein (cpRecA). Twenty micrograms of soluble proteins were electrophoretically separated in 13% (w/v) SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with rabbit antibodies against RbcL and cpRecA proteins. The immunodetection was performed with SuperSignal West Dura extended duration substrate (Thermo Fisher Scientific Inc, USA) according to the manufacturer’s description.

**In silico analyses for the presence of homologous sequences of the rpl23 gene and pseudogene amplicons in the nuclear and mitochondrial genome of barley.** The presence of the rpl23 gene and pseudogene amplicons in the nuclear and mitochondrial genomes of barley was investigated by Blastn. The sequences of both amplicons were blasted against each of the seven chromosomes of Hordeum vulgare sub. vulgare cv. Morex from EnsemblPlants (ftp://ftp.ensemblgenomes.org/pub/release-43/plants/fasta/hordeum_vulgare/ dna/) and IPK barley blast server (https://webblast.ipk-gatersleben.de/barley_ihscl) using barley pseudomolecules masked apr2016 as database. For the mitochondrial genome, the Genbank sequence of Hordeum vulgare sub. vulgare cv. Haruna Nijo (https://www.ncbi.nlm.nih.gov/nuccore/AP017301) was used.

**Data Availability**
All data generated or analyzed during this study are included in this published article (and its Supplementary Information Files).
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Author Contributions
F.L. and A.M.L. designed and performed the experiments; M.E.P. collaborated in performing some experiments; M.G.P. and K.K. have made contributions to the conception of the work; A.R.P. developed the experimental material and organized the isolation of samples; F.L., A.M.L. and A.R.P. drafted the manuscript.

Additional Information
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