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# Cytoplasmic DNAs and nuclear rDNA restriction fragment length polymorphisms in commercial witloof chicories

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**Abstract** Restriction fragment length polymorphisms of cytoplasmic DNAs and nuclear rDNA were analyzed in several *Cichorium intybus* genotypes, comprising four white inbred lines, eight red witloof experimental lines, and a number of $F_1$ hybrids derived from two white parents. Chloroplast and mitochondrial restriction patterns led to the distinction between two different cytoplasms, called I and II. Southern hybridization using a nuclear rDNA probe revealed that all the lines possessed two types of rDNA repeat units. The shortest unit was 10 kb and was common to all lines. The largest rDNA repeat unit was 10.5 kb in lines I and 10.4 kb in lines II. In addition, a sequence heterogeneity between the 10.5- and 10.4-kb rDNA repeat units was revealed by *Sac*I digestion. A 10-kb rDNA unit was successively cloned, mapped, and used as a probe to check the genetic purity of $F_1$ hybrid seeds between line I and II white parents. We found a 30% average percentage of impurities, originating both from selfing and full-sib crossing, in different open-pollinated hybrid samples.

**Key words** Chloroplast DNA · Mitochondrial DNA · rDNA · RFLP · Witloof chicory

## Introduction

The diploid species *Cichorium intybus* (2n = 2x = 18, genus *Cichorium*, family Compositae), widespread in Europe and in temperate areas of Asia, has long been cultivated for food and for its medicinal properties. The species contains three groups distinguishable by different morphologies. The first group includes some Italian chicories (Treviso, Verona, Chioggia), whereas the second group contains varieties cultivated for their sweet roots, which when roasted supply a coffee substitute. These coffee chicories represent new industrial crops for the production of both fructose syrup and polyfructanes as food fibers. The third group comprises the witloof chicories which have been bred for their root capacity to develop white tuberised buds (chicons) in artificial conditions. Coffee chicories and witloof chicories are thought to derive from Magdebourg chicory, an ancestor of coffee chicory characterized by large roots.

In western European countries, the witloof chicories represent one of the most important vegetable crops. They are mainly allogamous, but seeds can be obtained from about 20% of plants in most varieties by selfing. Several factors, such as unfavourable floral morphology and the occurrence of both autoincompatibility and pollen competition systems, limit selfing. The latter system is responsible for higher growth rates of allopollen tubes than of autopollen tubes. Chicory breeders are, therefore, actively involved in a search for: (1) cytoplasmic male sterility to eliminate selfing and to obtain 100% $F_1$ hybrids, (2) a haploidization technique to accelerate the breeding of pure lines and subsequently of new $F_1$ hybrids, (3) non-selective herbicide resistances to control weeds during chicory culture (Millecamps 1989; Vermeulen et al. 1992), and (4) diversification by crossing witloof with Italian chicories.

Despite its popular success, very little genetic and molecular data is available on witloof chicory (Vermeulen et al. 1994). Strong inbreeding depression, long generation time, and the cross-pollinating nature of chicories are responsible for this lack of knowledge. As a result, the scientific management of chicory breeding is poorly developed in comparison with that of other important vegetable crops. In the present study, we report variations in cytoplasmic and nuclear DNA restriction patterns from several witloof chicory genotypes. The aim of our analysis was varietal identification, the protection of breeder's rights, and the determination of parentage in witloof chicories.

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## Materials and methods

### Plant materials

Heads of the different witloof lines and $F_1$ hybrid seeds were provided by INRA (Centre de Versailles). Heads from four white lines (A, B, C and D genotypes) and eight red lines were analyzed (Table 1). Red lines originated by crossing white witloof chicories and wild red Verona salads, followed by four backcrosses with the white parent. Two types of white $F_1$ hybrid seeds were also analyzed: (1) three $F_1$ hybrids [Flash (A × C), Turbo (B × C) and Bea (B × D)] obtained under strictly controlled conditions, i.e. hand-emasculation of the parent used as female, in a greenhouse, and (2) seven seed samples of the Flash $F_1$ hybrid, representing 2800 plants, produced under open-field conditions.

### Isolation of chloroplast and mitochondrial DNAs

Chloroplasts were isolated from green leaves in a medium of high ionic strength (Bookjans et al. 1984). Lysis of chloroplasts and chloroplast (ct) DNA purification by CsCl ultracentrifugation were performed as described previously (Till-Bottraud et al. 1992).

Mitochondria were isolated as reported by Chetrit et al. (1985) with some modifications. White leaves from heads were homogenized at 4 °C in a Waring blender for 3 × 5 s at high speed in 6 ml/g of fresh weight buffer A (0.5 M mannitol, 50 mM Tris-HCl pH 8.0, 3 mM EDTA, 0.1% BSA, 1 mM β-mercaptoethanol). The homogenate was filtered through a 35-μm nylon net and the filtrate centrifuged twice at 1500 g for 10 min. The final supernatant was spun at 12000 g for 15 min (Sorvall, rotor SS34). The pellet (corresponding to 100 g of fresh material) was resuspended using a Potter homogenizer in 30 ml of buffer A containing 10 mM $MgCl_2$ and 100 μg/ml of DNase I. After 1 h of incubation at 4 °C, 3 vol of buffer B (50 mM Tris-HCl pH 8.0, 0.3 M sucrose, 20 mM EDTA) were added and the solution centrifuged at 12000 g for 15 min. The mitochondrial pellet was resuspended in buffer B and the suspension centrifuged at 12000 g for 15 min. The pellet was lysed and the mtDNA purified by CsCl ultracentrifugation as described previously (San et al. 1990).

### Ct- and mt-DNA restriction analysis

One to three micrograms of ct- or mt-DNA was digested in 30 μl of reaction solution with sufficient restriction enzyme (Boehringer Mannheim) to give complete digestion. The restriction fragments were separated by electrophoresis in 0.7% agarose gels (Vedel et al. 1976).

### Molecular cloning of nuclear rDNA

Total DNA was prepared from about 1 g of leaves according to Dellaporta et al. (1983) and purified by CsCl gradient ultracentrifugation as described by Levesque et al. (1990). Five micrograms of DNA were digested with restriction enzymes and fragments separated by agarose electrophoresis as indicated above. Since *Xba*I complete digests showed a high-molecular-weight band containing uncleaved rDNA units, total DNA was partially digested with *Xba*I and fragments of more than 30 kb selected by centrifugation on seven-step (10–15–20–25–30–35–40%) sucrose gradients in buffer TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The selected fragment was ligated to a modified cosmid according to Chetrit et al. (1992). Cosmid DNA was prepared as described by Birnboim and Doly (1979).

### Hybridization

Screening of the cosmid library was performed according to Grunstein and Hogness (1975) by using a $^{32}$P-labelled rDNA probe from *Brassica campestris*, the pBcR1 clone (de Courcel 1989). Total DNA restriction fragments were transferred from agarose to Biodyne A membranes (Pall Inc.) as described by Sambrook et al. (1989). Labelling of cloned rDNA fragments and hybridization conditions were according to manufacturer's instructions (digoxygenin procedure, Boehringer Mannheim).

## Results

### Analysis of cytoplasmic DNAs

The ctDNA isolated from the four white witloof lines showed two distinctive types of restriction patterns with the *Sal*I enzyme only. Type I was found in lines A and B and type II in lines C and D (Fig. 1). Both ctDNA types were equally distributed among the eight red witloof lines analyzed (Fig. 1 and Table 1). Type-I and type-II ctDNAs possessed 49.2- and 15.5-kb *Sal*I-specific fragments respectively. *Bam*HI, *Bgl*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Hpa*II and *Sac*I enzymes failed to distinguish between the different lines (data not shown).

Two types of mtDNA restriction patterns were found among the four white witloof lines with the *Bam*HI, *Bgl*I, *Sal*I, *Cla*I, *Hin*dIII and *Kpn*I enzymes. Lines A and B possessed an identical type-I mtDNA (Fig. 2) distinct from that of lines C and D (type II). *Hin*dIII patterns revealed the presence of specific 6.6-, 4.4- and 4.0-kb fragments in type-I mtDNAs and of specific 7.1- and 3.2-kb fragments in type-II mt DNAs. A 8.0-kb *Bgl*I fragment was specifically observed in A and B lines, while three *Bgl*I fragments of 38.5, 5.9 and 2.5 kb appeared to be specific to lines C and D. Type-I and type-II mt DNAs were also distinguished by specific 20.9- and 33.0-kb *Sal*I fragments, respectively. The eight red witloof lines contained one or the other mtDNA type, but in accordance with the ctDNA type (Table 1).

**Fig. 1** *Sal*I restriction patterns of ctDNAs from four white lines and eight red lines of witloof chicory. Specific fragment sizes are indicated. The 1-kb ladder (BRL), *Hin*dIII fragments from Lambda DNA (Boehringer), Raoul I (Appligene), and native Lambda DNA were used as molecular-weight standards

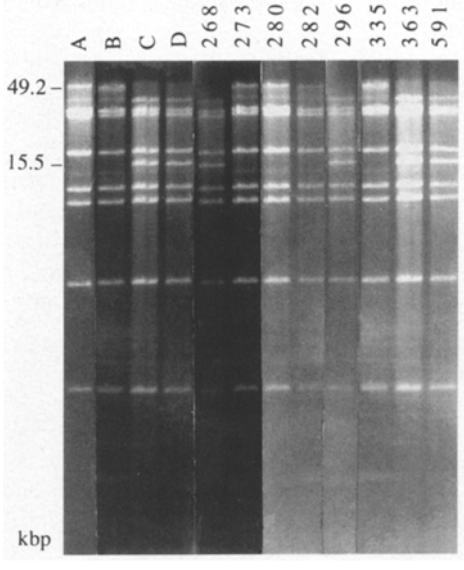


**Table 1** Summary of results showing relationships between genotype and chloroplast, mitochondrial and nuclear rDNA types

| Genotype | ctDNA | mtDNA | rDNA |
|---|---|---|---|
| White lines | | | |
| A | I | I | I |
| B | I | I | I |
| C | II | II | II |
| D | II | II | II |
| Red cultivars[a] | | | |
| RA (S3) 268 | II | II | II |
| RAB (S5) 273 | I | I | II |
| RAH 280 | I | I | II |
| RAH (S3) 282 | I | I | II |
| RP (S4) 296 | II | II | II |
| RU (S5) 335 | I | I | II |
| RUB 363 | II | II | II |
| RP (S4) 591 | II | II | II |

[a] In the case of the red genotypes, the number of selfings after original backcrosses is indicated between brackets

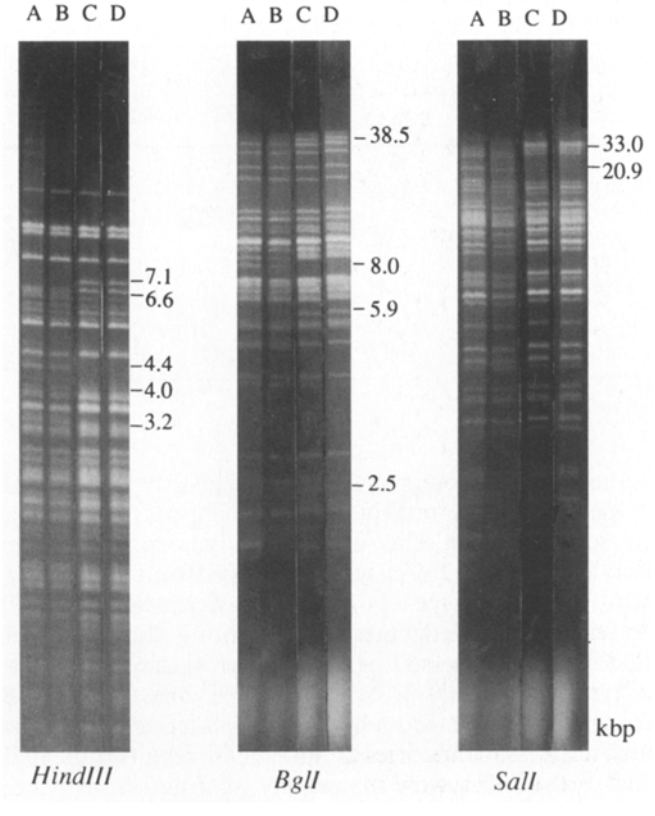
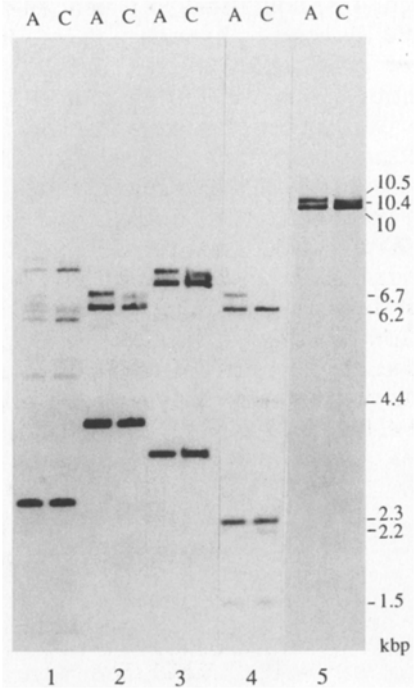



**Fig. 2** *Hin*dIII, *Bgl*I and *Sal*I restriction patterns of mtDNAs from four lines of white witloof chicory. Specific fragment sizes are indicated. Molecular-weight standards are as in Fig. 1

## rDNA analysis

Two groups of white witloof lines, group I (A, B) and group II (C, D), were distinguished by probing different restriction digests of genomic DNA with a complete *B. campestris* rDNA repeat unit (see Materials and methods). Both groups contained two rDNA size classes, as illustrated in Figs. 3–5. Only one *Xba*I site was located in the rDNA repeat units, producing two fragments which represent the size of each rDNA size class. Lines I and II both contained the smallest class of 10 kb. The largest rDNA size class was 10.5 kb in lines I and 10.4 kb in lines II. Lines I and II can also be distinguished by 6.8- and 6.7-kb *Eco*RI fragments, respectively, and by 7.5- and 7.4-kb *Eco*RV fragments, respectively, (Fig. 3, lanes 2, 3). *Bam*HI and *Sac*I patterns appeared more complex than those described above. Lines I showed four *Bam*HI-specific fragments (7.9, 6.7, 6.4 and 5.1 kb), while lines II were distinguished by three fragments of 7.8, 6.3 and 5.0 kb. In higher plants, the occurrence of rDNA size classes in both unmethylated and methylated forms was shown to be responsible for complex *Bam*HI profiles (Flavell 1986). The *Sac*I patterns appeared to be the most useful to discriminate between lines I and II. They showed three common fragments of 6.2, 2.3 and 1.5 kb, which corresponded to the 10-kb rDNA repeat unit. The 6.2-kb fragment contained the intergenic sequence (IGS), with the 2.3- and 1.5-kb fragments corresponding to the coding sequences. Type-I lines were characterized by a 6.7-kb *Sac*I fragment belonging to the 10.5-kb rDNA repeat unit. Type-II lines showed two *Sac*I-specific fragments, 4.4 and 2.2 kb, belonging to the 10.4-kb rDNA repeat unit. This latter unit thus contained an additional *Sac*I site. The different rDNA size classes were not cut by *Hin*dIII, *Bcl*I, *Pst*I or *Sca*I enzymes.

**Fig. 3** Autoradiogram showing hybridization of the $^{32}$P-labelled cloned rDNA transcription unit of *Brassica campestris* (pBcR1 insert, de Courcel 1989) to different total DNA restriction digests from two white witloof chicory lines. Digests were obtained with *Bam*HI (*1*), *Eco*RI (*2*), *Eco*RV (*3*), *Sac*I (*4*) and *Xba*I (*5*)



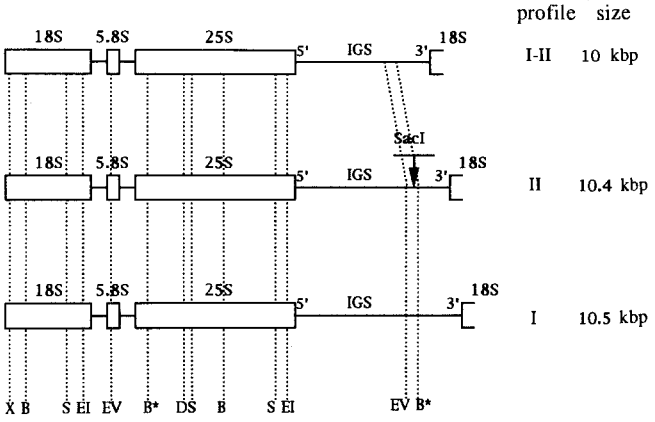



**Fig. 4** Size and restriction-site comparison between the three classes of rDNA units of the white witloof chicory lines. *B, D, EI, EV, S* and *X* are cleavage sites for *Bam*HI, *Dra*I, *Eco*RI *Eco*RV, *Sac*I and *Xba*I, respectively; (*) methylated-*Bam*HI sites

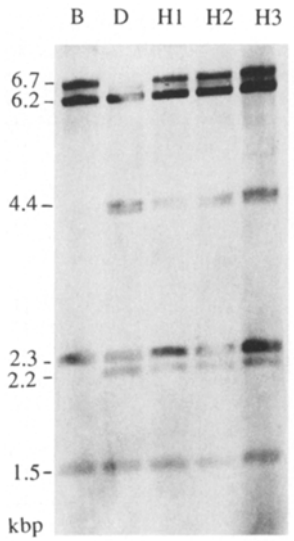



**Fig. 5** Autoradiogram showing hybridization of the $^{32}$P-labelled cloned rDNA unit of *Cichorium intybus* (pCiR1 insert) to *Sac*I total-DNA digests from B and D white lines and from the three hybrids Flash (*H1*), Turbo (*H2*) and Bea (*H3*)

All the red witloof lines possessed the same two rDNA size classes as the white B lines (data not shown).

Restriction maps (Fig. 4) were established for each rDNA size class by comparison with the highly conserved restriction sites in higher-plant rDNA maps and after cloning and mapping of a 10-kb rDNA repeat unit (belonging to the pCiR1 clone). Such a unit was cloned using *Xba*I and mapped according to the multienzyme method. Length polymorphism between the three rDNA size classes resulted in length variation of the IGS. The additional *Sac*I site specific for the 10.5- and 10.4-kb repeat units in lines I and II, respectively, was located in the IGS.

**Table 2** Compilation of rDNA analysis in seven samples of open-pollinated $F_1$ hybrids originating from A × C crosses

| Sample no. | Parent A | | Parent C | | Hybrid | |
|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % |
| 84830 | 56 | 14.0 | 70 | 17.5 | 274 | 68.5 |
| 41403 AC | 48 | 12.0 | 38 | 9.5 | 314 | 78.5 |
| 01568 | 75 | 18.7 | 31 | 7.7 | 294 | 73.5 |
| 8008 | 48 | 12.0 | 33 | 8.2 | 319 | 79.7 |
| 8049 | 41 | 0.2 | 133 | 33.2 | 226 | 56.5 |
| 8054 | 77 | 19.2 | 24 | 6.0 | 299 | 74.7 |
| 8117 | 54 | 13.5 | 77 | 19.2 | 269 | 67.2 |
| Total | 399 | 14.25 | 406 | 14.5 | 1995 | 71.25 |

### Analysis of commercial hybrids

Under greenhouse conditions in which self-pollination is impossible, the three $F_1$ hybrids were characterized by the expected hybrid rDNA patterns (Fig. 5). In open-field conditions, this was not the case. Large-scale rDNA analysis was performed on seven field-produced Flash seed samples, using a rapid DNA isolation procedure (de Courcel 1989; Levesque et al. 1990), and *Sac*I digests combined with pCiR1 probing. For each of these samples, 400 plants were analyzed. Compilation of the results obtained from the 2800 plants (Table 2) demonstrated that the percentage of hybrids was between 56.5 and 79.7%, depending on the sample. Selfing was in favor of one or the other parent, or was equivalent between the two. Over the seven samples, the average percentage of hybrids was 71.25, thus indicating 29.75% of selfing: a value far from satisfactory for the plant breeder.

## Discussion

Two types of cytoplasms were found in the four white witloof lines A, B, C and D. They were designated I and II by reference to the nuclear genotypes I and II respectively. The mtDNA polymorphisms were easier to identify than the ctDNA polymorphisms. The two types of ctDNAs were identified only with *Sal*I, while the two types of mtDNAs were distinguished by each of the enzymes used. Among the eight red lines, four possessed a type-I cytoplasm and four a type-II cytoplasm. Since the red lines originated from crosses between white witloof chicories as female and red Italian chicories as male, these results indicated that cytoplasms were maternally inherited from white chicories.

We also attempted to distinguish between the different witloof lines by using rDNA RFLP markers. We found polymorphisms between the two groups of genotypes, i.e. I (A and B) and II (C and D). In addition to a common 10-kb rDNA size class, type-I and -II lines contained a specific rDNA class of 10.5 kb and 10.4 kb respectively. Comparison between detailed restriction maps of the three rDNA classes showed that the differences were due to variations in IGS size. The three $F_1$ hybrids, Flash (A × C), Turbo (B × C) and Bea (B × D), obtained from controlled pollination (in a greenhouse)

contained specific rDNA *Sac*I fragments from both parental lines. An rDNA analysis of red witloof lines indicated that the eight lines possessed the two rDNA classes of group-II white lines (C and D). This result suggested that red chicories were backcrossed with group-II white lines. Unfortunately, rDNA profiles of the red parent(s) used as the male in the initial crosses are unknown, these parent(s) being difficult to maintain.

Production of commercial $F_1$ hybrid seeds in witloof chicory is normally performed in the field, taking advantage of factors limiting selfing (see Introduction). RFLP markers would be very valuable to check sample purity, thereby saving space, time, and the resources available to the breeder (Beckman 1988). Using rDNA RFLP, we tried to estimate the percentage of impurities in seven seed samples of open-pollinated Flash $F_1$ hybrids (A × C). Only 70% of plants from $F_1$ seeds showed a hybrid rDNA pattern, the remainder possessed an rDNA similar to one or the other parent. From these results, it seems probable that the production of witloof $F_1$ hybrid seeds under open-pollinated conditions leads to a high percentage of impurities (originating from selfing), so hampering commercialization. To-date, only nuclear male-sterile plants have been observed in *C. intybus*, and the production of pure $F_1$ hybrid seeds requires hand emasculation of the parent used as female. Since this process is both time-consuming and of a relatively low efficiency, chicory breeders are currently seeking CMS to eliminate selfing. Recently, male-sterile chicory plants were obtained by fusion of coffee chicory protoplasts and CMS sunflower protoplasts (Rambaud et al. 1993). An mtDNA analysis of the male-sterile cybrids indicated that mtDNA rearrangements had occurred between both parents.

Cytoplasmic DNA and nuclear rDNA analysis led respectively to a distinction between only two cytoplasms which appeared to be associated with two nuclear genotypes. Variations between A and B, and between C and D, lines were not observed in either type of analysis, indicating a narrow genetic variability. We are now trying to distinguish between the four white witloof lines by using RAPD-PCR markers.

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