Theor Appl Genet (1998) 96: 928–932 

C. E. Bowes · D. J. Wolyn

# Phylogenetic relationships among fertile and petaloid male-sterile accessions of carrot, *Daucus carota* L.

Received: 1 December 1997 / Accepted: 12 December 1997

**Abstract** Mitochondrial (mt) DNA variation for six petaloid cytoplasmic male-sterile (CMS) and three fertile maintainer lines of carrot was assessed to establish genetic relationships. Total DNA was digested with restriction enzymes and probed with six homologous mtDNA cosmid probes. The six CMS accessions derived from wild carrot, four from Guelph, Ontario, one from Orleans, Massachusetts, and one from Madison, Wisconsin, were more closely related with each other ($F = 0.91$) than with fertile maintainer lines derived from cultivated germplasm ($F = 0.62$). The fertile maintainer lines were likewise found to be more similar to each other ($F = 0.78$) than to the sterile lines. Three sterile lines, originating from wild carrot populations within 1 km of each other in Guelph, Ontario, were most closely related ($F = 0.96$). The high degree of similarity among the six petaloid CMS lines which originated from individual wild carrot plants, some from geographically diverse regions, suggests that the cytoplasm responsible for this trait was imported to, or else evolved, only once in North America.

**Key words** Mitochondrial genome · Cytoplasmic male sterility · Petaloidy · Carrot · Phylogeny

## Introduction

Cytoplasmic male sterility (CMS) in carrot is manifested as petaloidy, where stamens are transformed into petal or leaf-like structures which lack pollen-forming tissue (Thompson 1961; Kitagawa et al. 1994). Thompson first described cytoplasmic inheritance of petaloidy in carrot lines initiated from a male-sterile wild carrot plant found near Orleans, Massachusetts, in 1953. Named the Cornell cytoplasm, it has been used to produce the majority of hybrid carrots in the United States (Goldman 1996). A second source of CMS, the Wisconsin cytoplasm, was derived from a petaloid male-sterile wild carrot plant found growing near Madison, Wisconsin, in 1970 (Morelock et al. 1996). In 1991, petaloid carrot plants from wild populations in and near Guelph, Ontario, were collected and sterility exhibited cytoplasmic inheritance (Wolyn and Chahal 1998). Petaloid male-sterile wild carrots have also been observed in southeastern Sweden (McCollum 1966).

Mutations in the plant mitochondrial (mt) genome are associated with CMS in several species (reviewed by Vedel et al. 1994) although the molecular mechanisms involved are not completely understood. Restriction-enzyme analysis of plant mtDNA shows that mt genomes are large, complex, and highly variable between species. The mt genome is able to undergo rapid structural evolution through recombination across repeated sequences, resulting in a mixture of, or else altered, genome arrangements (reviewed in Bonen and Brown 1993). Therefore most mtDNA restriction fragment length polymorphisms result from genome rearrangements rather than from point mutations (Wolfe et al. 1987), which limits their usefulness in strict phylogenetic analyses. Polymorphisms are useful, however, for grouping closely related genomes such as different strains of the same species (Palmer 1992).

Studying the diversity of mitochondrial DNA organization among petaloid CMS carrot lines from geographically distinct wild populations could help determine if these lines were derived from a common ancestor, or if they evolved independently. If all petaloids arose from a common ancestor, the molecular events responsible for this phenotype may be rare occurrences.

Communicated by D. R. Pring

C. E. Bowes · D. J. Wolyn (✉)
Department of Horticultural Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada
Fax: +1 519-767-0755
E-mail: dwolyn@evbhort.uoguelph.ca

The objective of the present study was to characterize the mt restriction fragment patterns of six petaloid CMS accessions originating from wild carrot plants and three fertile maintainer lines from cultivated carrot in order to define phylogenetic relationships. A high degree of similarity was observed among the six sterile lines, suggesting a common progenitor.

## Materials and methods

### Plant materials

Six petaloid CMS carrot lines, originating independently from wild carrot populations, and three fertile maintainer breeding lines were studied (Table 1). The Cornell and Wisconsin sources of CMS were inbred breeding lines. Two petaloid Guelph accessions (Guelph-101, -302) had been backcrossed twice to the W259B fertile maintainer line. For the other two Guelph lines (Guelph-202, -301) petaloid progeny from the original sterile plants in the wild carrot populations were analysed. In subsequent crosses of these petaloid accessions to maintainer lines for the Cornell cytoplasm, only sterile progeny were produced.

### Plant culture

Plants were grown from seed at the Cambridge Research Station, Cambridge, Ontario, from May to August. Carrots were uprooted, vernalized for 10 weeks at 4°C, and planted in pots in a greenhouse at Guelph, Ontario. Plants were grown at 18°C/15°C (day/night) with a 16-h photoperiod until flower stalks appeared, when temperatures were raised to 22°C/18°C. Leaves from the flowering stalks were collected, frozen with liquid $N_2$ and stored at −80°C. Two plants of each genotype were sampled with the exception of Guelph-202 and W33B where only one plant was assayed.

### Probe sources and preparation

Heterologous probes for mt genes *atpA* (Makaroff et al. 1990), *atp6* (Dewey et al. 1985a), *atp9* (Dewey et al. 1985b), *cob* (Boer et al. 1985), and *nad1a* (Chapdelaine and Bonen 1991) were used to screen a carrot mtDNA cosmid library from A. Chahal (Chahal et al. 1998). Membrane disks containing immobilized DNA from bacterial colonies were prepared following the procedure of Sambrook et al. (1989). Probe labelling and hybridization were conducted according to The DIG System User's Guide for Filter Hybridization (Boehringer Mannheim 1993). Selected cosmids were mapped for *Bam*HI restriction sites using partial digestion (FLASH non-radioactive gene mapping kit, Stratagene 1994). A total of six cosmids identified with the heterologous mt genes were used as probes in Southern blots of carrot DNA.

### DNA isolation, digestion and Southern hybridization

Total DNA was isolated from 5 g of frozen leaf tissue according to Dellaporta et al. (1983), and quantified by spectrophotometry. DNA digestion with *Bam*HI or *Hin*dIII and gel electrophoresis (10 μg/lane, 0.6% agarose) were conducted according to standard protocols (Sambrook et al. 1989). Blotting of DNA gels was performed using a PosiBlot Pressure Blotter according to the manufacturer's instructions (Stratagene 1990). Southern hybridization and detection were carried out according to The DIG System User's Guide for Filter Hybridization (Boehringer Mannheim 1993).

### Data analysis

The proportion of shared fragments (F) was calculated according to the formula $F = 2n_{XY}/(n_X + n_Y)$ (Nei and Li 1979), in which $n_X$ and $n_Y$ are the numbers of restriction fragments in lines X and Y, respectively, whereas $n_{XY}$ is the number of fragments shared by the two lines. RESTSITE, a phylogenetic program that sorts raw restriction data (Miller 1991), was used to calculate the F value shared between each pair or group of individuals. A (1-F) distance matrix was constructed and used in the NEIGHBOR and DRAWTREE programs from the PHYLIP package (Felsenstein 1993) to plot an unrooted phylogenetic tree.

## Results

### Mitochondrial genome variability among carrot accessions

Carrot accessions differed for the mtDNA restriction fragments observed after hybridizing *Bam*HI- or

**Table 1** Carrot lines used for mitochondrial DNA analysis

| Cytoplasm | Nuclear genotype | Designation | Origin | Source |
|---|---|---|---|---|
| Petaloid CMS lines: | | | | |
| Cornell | W259 | W259A | Wild carrot near Orleans, Massachusetts, USA (Thompson 1961) | Dr. W. H. Gabelman, University of Wisconsin |
| Wisconsin | 2566 | 2566S | Wild carrot near Madison, Wisconsin, USA (Morelock et al. 1996) | Dr. P. W. Simon, USDA/University of Wisconsin |
| Guelph-101 | W259 (BC2) | Guelph-101 | Wild carrot in and around Guelph, Ontario, Canada | Dr. D. J. Wolyn, University of Guelph |
| Guelph-302 | W259 (BC2) | Guelph-302 | | |
| Guelph-202 | Wild | Guelph-202 | | |
| Guelph-301 | Wild | Guelph-301 | | |
| Fertile maintainer lines: | | | | |
| – | W259 | W259B | Cultivated carrot | Dr. W. H. Gabelman |
| – | W33 | W33B | Cultivated carrot | Dr. W. H. Gabelman |
| – | 2566 | 2566M | Cultivated carrot | Dr. P. W. Simon |

*Hin*dIII-digested total DNA with six homologous mtDNA probes (data not shown). The six petaloid and three fertile lines not only had distinguishing restriction fragments, but also shared many fragments of identical sizes, ranging from 1.2 to 22 kb. The number of fragments for an individual plant/enzyme/probe combination varied from 6 to 17; more fragments were observed in *Hin*dIII than *Bam*HI digests due to the greater A/T than G/C content of the mt genome (Ward et al. 1981) and the *Hin*dIII recognition sequence. In total, over 3000 bands were analysed from the 24 Southern blots. Although each carrot accession was characterized by a distinct mt genome organisation, the sterile and fertile lines were more similar within, rather than between, groups.

### Genetic relationship based on mtDNA analysis

Based on the proportion of shared fragments (F-values) the highest similarity among lines was observed for Guelph-301 and Guelph-302 while the lowest was between W33B and 2566S (Table 2). The F values among steriles (F = 0.84–0.98) and among fertiles (F = 0.70–0.92) were consistently higher than the values observed between sterile and fertile accessions (F = 0.57–0.67).

Genetic relationships were found to be associated with geographic origin and phenotype when F-values within and between the groups "Cornell sterile" (W259A), "Wisconsin sterile" (2566S), "Guelph sterile" (Guelph-101, -202, -301, -302) and "fertile maintainer" (W259B, 2566M, W33B) were calculated. The Guelph sterile accessions showed a high within-group similarity (F = 0.94). Comparisons between Cornell and Wisconsin (F = 0.90), Cornell and Guelph (F = 0.89) and Wisconsin and Guelph (F = 0.88) sterile accessions revealed that the genetic distances were approximately equal among these three groups. The proportions of shared fragments among sterile groups were significantly higher than those found when these lines (Cornell, Wisconsin and Guelph) were compared to the fertile maintainer group (F = 0.61, 0.60 and 0.63 respectively). Within the fertile maintainer group, lines were more closely related to each other (F = 0.78) than to sterile accessions. Further grouping of the carrot lines into two categories, sterile and fertile, resulted in the following F-values: within sterile, 0.91; within fertile, 0.78; and between sterile and fertile, 0.62.

### Dendrogram based on mtDNA diversity

When F-values from Table 2 were used as measures of similarity to generate a dendrogram, sterile and fertile lines were clearly divided on the tree (Fig. 1). Five major branches separated the nine carrot accessions Three Guelph sterile accessions (Guelph-101, -301, -302) were grouped together near a branch terminus, while the fourth was on a separate branch (Guelph-202). A third branch contained the Cornell and Wisconsin sterile accessions. The fertile maintainer W259B was by itself on a fourth branch and the remaining fertiles (2566M, W33B) were grouped together on the final branch.

## Discussion

In this investigation, carrot mt genome organization was related to plant geographic origin and sterile vs fertile phenotype. Petaloid CMS lines originating from wild plants found in and around Guelph, Ontario, within 10 km of each other, shared a high proportion of

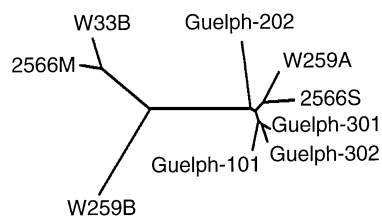


**Fig. 1** Relationships among mitochondrial genomes of fertile (W259B, 2566 M, W33B) and CMS (W259A, 2566S, Guelph-101, -202, -301, -302) carrot lines, based on Li and Nei's (1979) estimate of genetic divergence

**Table 2** Proportion of shared hybridizing fragments (F) between petaloid CMS (W259A, 2566S, Guelph-101, -202, -301, -302) and fertile (W259B, 2566M, W33B) carrot lines

| Line | 2566S | Guelph-101 | Guelph-202 | Guelph-301 | Guelph-302 | W259B | 2566M | W33B |
|---|---|---|---|---|---|---|---|---|
| W259A | 0.90 | 0.90 | 0.84 | 0.90 | 0.90 | 0.63 | 0.62 | 0.59 |
| 2566S | | 0.88 | 0.86 | 0.89 | 0.89 | 0.61 | 0.61 | 0.57 |
| Guelph-101 | | | 0.87 | 0.95 | 0.96 | 0.63 | 0.63 | 0.64 |
| Guelph-202 | | | | 0.92 | 0.88 | 0.59 | 0.59 | 0.67 |
| Guelph-301 | | | | | 0.98 | 0.63 | 0.63 | 0.62 |
| Guelph-302 | | | | | | 0.64 | 0.64 | 0.64 |
| W259B | | | | | | | 0.74 | 0.70 |
| 2566M | | | | | | | | 0.92 |

mtDNA restriction fragments with each other (F = 0.94). Three of these lines (Guelph-101, -301, -302) originated from plants found within 1 km of each other (F = 0.96), while the fourth (Guelph-202) was found 10 km away. Plants from petaloid CMS lines originating from wild plants found near Orleans, Massachusetts, (W259A) and Madison, Wisconsin, (2566S) also shared a high proportion of fragments with each other (F = 0.90) and with the Guelph steriles (F = 0.88, 0.89). The three geographic locations from which CMS plants were isolated, Guelph, Ontario, Madison, Wisconsin, and Orleans, Massachusetts, span 1500 km, yet the proportions of shared hybridizing fragments among lines from these areas were nearly identical. The high similarity among mtDNAs of the sterile accessions from these locations suggests that petaloid CMS was imported to, or else evolved, only once in North America, and then spread across the continent; the small differences among accessions most likely resulted from specific sequence rearrangements over time in each geographical area. An alternative explanation for the low diversity observed among petaloid accessions could be that the petaloid CMS trait arose independently in different populations, but evolutionary forces only allowed the observed type of genome arrangement to exist in the presence of CMS. Since the different sterile lines can be maintained and restored to fertility by the same genotypes/genes (Wolyn and Chahal 1998) a common origin for the petaloids is most plausible.

These findings agree with previous studies, which compared the mtDNAs of petaloid CMS, brown anther CMS and fertile lines. A unique mtDNA organisation was observed between lines with distinct CMS phenotypes and between petaloid and fertile lines ($F_{petaloid/brown\ anther} = 0.79$, $F_{petaloid/fertile} = 0.60$; Ronfort et al. 1995). Comparisons of carrot lines within the two sterile phenotypic classes indicated high similarities (F = 0.91 for two petaloid lines, F = 0.98 for two brown anther lines; Pingitore et al. 1989).

Within-group similarity was less among fertile (F = 0.78) than among sterile lines (F = 0.91), and fertile-plant mt genomes were more similar to each other than to any of the sterile plants (F = 0.62). These differences are not unexpected considering the origins of the groups. CMS lines were selected from wild carrot based on phenotype, while fertile maintainers were selected from cultivated carrot lines based on their ability to maintain male-sterile inbreds. The mtDNA diversity observed between and within cultivars of carrot led other investigators to hypothesize that ancestral plants transmitted divergent mt genomes to present-day cultivars (Ichikawa et al. 1989; Steinborn et al. 1992). The differences observed between fertile maintainer lines (Pingitore et al. 1989; this study) could reflect the population of genomes in the open-pollinated cultivars from which they were initiated.

The highly variable nature of mtDNA in plants has been useful in determining relationships at very low taxonomic levels. Further comparisons of petaloid CMS carrot mtDNAs in North America and around the world will help in understanding the origins of this important trait.

**Acknowledgments** This research was funded by the Natural Science and Engineering Research Council of Canada. The authors thank Dr. W. H. Gabelman, University of Wisconsin-Madison, and Dr. P. W. Simon, USDA-ARS, University of Wisconsin-Madison, for supplying inbred carrot lines. We also thank Dr. L. Bonen, University of Ottawa, Dr. C.S. Levings, North Carolina State University, and Dr. C. A. Makaroff, Miami University-Ohio, for supplying DNA probes.

## References

Boer PH, McIntosh JE, Gray MW, Bonen L (1985) The wheat mitochondrial gene for apocytochrome b: absence of a prokaryotic ribosome binding site. Nucleic Acids Res 13:2281–2292

Bonen L, Brown GG (1993) Genetic plasticity and its consequences: perspectives on gene organization and expression in plant mitochondria. Can J Bot 71:645–660

Chahal A, Sidhu HS, Wolyn DJ (1998) A fertile revertant from petaloid cytoplasmic male sterile carrot has a rearranged mitochondrial genome. Theor Appl Genet (in press)

Chapdelaine Y, Bonen L (1991) The wheat mitochondrial gene for subunit 1 of the NADHdehydrogenase complex: a trans-splicing model for this gene-in-pieces. Cell 65:465–472

Dellaporta SL, Wood J, Hicks JB (1983) A plant minipreparation: version II. Plant Mol Biol Rep 1:19–21

Dewey RE, Levings CSIII, Timothy DH (1985a) Nucleotide sequence of the ATPase subunit 6 gene of maize mitochondria. Plant Physiol 79:914–919

Dewey RE, Schuster AM, Levings CSIII, Timothy DH (1985b) Nucleotide sequence of the F0-ATPase proteolipid (subunit 9) gene of maize mitochondria. Proc Natl Acad Sci USA 82:1015–1019

Felsenstein J (1993) PHYLIP (phylogeny inference package) version 3.5c. Distributed by the author, Department of Genetics, University of Washington, Seattle

Goldman IL (1996) A list of germplasm releases from the University of Wisconsin carrot breeding program, 1964–1994. HortScience 31:882–883

Ichikawa H, TannoSuenaga L, Imamura J (1989) Mitochondrial genome diversity among cultivars of *Daucus carota* (ssp. *sativus*) and their wild relatives. Theor Appl Genet 77:39–43

Kitagawa J, Posluszny U, Gerrath JM, Wolyn DJ (1994) Developmental and morphological analyses of homeotic cytoplasmic male sterile and -fertile carrot flowers. Sex Plant Reprod 7:41–50

Makaroff CA, Apel IJ, Palmer JD (1990) Characterization of radish mitochondrial atpA: influence of nuclear background on transcription of atpA-associated sequences and relationship with male sterility. Plant Mol Biol 15:735–746

McCollum GD (1966) Occurrence of petaloid stamens in wild carrot (*Daucus carota*) from Sweeden. Swed Econ Bot 2:361–367

Miller JC (1991) RESTSITE: A phylogenetic program that sorts raw restriction data. J Hered 82:262–263

Morelock TE, Simon PW, Peterson CE (1996) Wisconsin Wild: another petaloid male-sterile cytoplasm for carrot. HortScience 31:887–888

Nei M, Li W-H (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76:5269–5273

Palmer JD (1992) Mitochondrial DNA in plant systematics: applications and limitations. In: Soltis PS, Soltis DE, Doyle JJ (eds)

Molecular systematics of plants. Chapman and Hall, London, pp 36–49

Pingitore M, Matthews B, Bottino PJ (1989) Analysis of the mitochondrial genome of *Daucus carota* with male-sterile and male-fertile cytoplasm. J Hered 80 : 143–145

Ronfort J, Saumitou-Laprade P, Cuguen J, Couvet D (1995) Mitochondrial DNA diversity and male sterility in natural populations of *Daucus carota* ssp. *carota*. Theor Appl Genet 91 : 150–159

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Steinborn R, Weihe A, Boerner T (1992) Mitochondrial genome diversity within a cultivar of *Daucus carota* (ssp. *sativus*) revealed by restriction fragment analysis of single plants. Plant Breed 109 : 75–77

Thompson DJ (1961) Studies on the inheritance of male sterility in the carrot, *Daucus carota* L. var. sativa. Proc Am Soc Hortic Sci 72 : 332–338

Vedel F, Pla M, Vitart V, Gutierres S, Chetrit P, De Paepe R (1994) Molecular basis of nuclear and cytoplasmic male sterility in higher plants. Plant Physiol Biochem 32 : 601–618

Ward BL, Anderson RS, Bendich AJ (1981) The size of the mitochondrial genome is large and variable in a family of plants (Curcurbitacae). Cell 25 : 793–803

Wolfe KH, Li W-H, Sharp PM (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84 : 9054–9058

Wolyn DJ, Chahal A (1998) Nuclear-cytoplasmic interactions for petaloid male-sterile accessions of wild carrot, *Daucus carota* L. J Am Soc Hortic Sci (in press)