Theor Appl Genet (1990) 79:119–124

Chloroplast DNA in *Pinus monticola*

1. Physical map

E. E. White

Pacific Forestry Centre, 506 West Burnside Road, Victoria, British Columbia, V8Z 1M5, Canada

Received July 12, 1989; Accepted July 31, 1989
Communicated by P. M. A. Tigerstedt

Summary. Restriction sites on the chloroplast genome of *Pinus monticola* have been mapped, and the gene for the large subunit of ribulose bisphosphate carboxylase/oxygenase, the genes for the photosystem II polypeptides psbA, psbD and psbC, and the 16S and 23S ribosomal RNA genes have been located. The genome lacks the large inverted repeat characteristic of most angiosperms. The gene order is similar to that found in *P. radiata*. The presence of dispersed repeated sequences is likely. Two structural features, lack of a large inverted repeat and the presence of dispersed repeats, may confer a degree of variability on the genome which will prove useful in studies of population structure.

Key words: Chloroplast DNA – Physical map – Western white pine – *Pinus monticola* – Chloroplast genes

Introduction

A program to establish western white pine (*Pinus monticola* Dougl. ex D. Don) seed orchards of trees with increased resistance to white pine blister rust (*Cronartium ribicola* J. C. Fisch) has recently been established in western Canada (Hunt et al. 1987). Information about the population structure of western white pine was required when setting up this program. Indirect indications of the degree and pattern of population genetic variation can be obtained by examining the variation of biochemical characteristics under strong genetic control. Studies of isozyme (Steinhoff et al. 1983) and terpene (Hunt and von Rudloff 1977) variation indicated these were highly variable within populations of western white pine.

The DNA variation revealed by comparing the size of fragments produced by digesting DNA with restriction enzymes (restriction fragment length polymorphisms, or RFLPs) has been proposed as another biochemical characteristic for assessing genetic variability (Beckman and Soller 1983; Helentjaris et al. 1985). Chloroplast genomes have been reported to be evolving slowly relative to nuclear and mitochondrial DNAs (Palmer 1987). Chloroplast DNA (cpDNA) variation might reflect patterns of population differentiation obscured by within-population variation in isozyme and terpene data in highly variable species such as white pine.

Evaluating the functional and phylogenetic significance of an RFLP requires knowledge of the physical structure of the genome. This study was made to map restriction sites on the cpDNA of *P. monticola* as a basis for examining RFLP variation in the species.

Materials and methods

Biological material

Open-pollinated seed collected from several wild trees in one stand (Harbour Lakes, 51°30′N, 119°10′W) was stratified, germinated and grown for two seasons before needles were sampled.

cpDNA cloning

Chloroplast DNA was extracted as described previously (White 1986). HindIII and EcoRI digests of cpDNA were ligated into pUC9 and pTZ18R (Maniatis et al. 1982 and suppliers' protocols). Competent *E. coli* cells (strain MC1029 for pUC9 and NM522 for pTZ) were transformed and grown overnight on L agar plates with IPTG, X-gal and 25 μg/ml ampicillin. Recombinant colonies were grown in LB broth and glycerized for storage at −70 °C (Maniatis et al. 1982). Plasmid DNA was extracted from 2-ml shake cultures in LB broth by the method of Birnboim and Doly (1979).

Molecular hybridization

Restriction digests of cpDNA were prepared using BamHI, EcoRI, and HindIII, plus the same three enzymes in combina-

Table 1. Sal I fragments

Probe	Major hybridizations to membrane bound fragments (kb)								
	Eco	Eco/Sal	Bam	Bam/Sal	Hind	Hind/Sal	Pst	Pst/Sal	Sal
Sal 1	6.5	6.5	5.0	5.0	14.1	12.7	28	17.6	37.7
(37.7 kb)	5.8	5.8	4.7	4.7	11.3	11.3	15.3	10.9	
	4.8	4.8	3.9	3.9	6.9	6.9	10.9	9.2	
	4.3	4.3	2.7	2.7	5.9	3.7			
	1.3	1.3	2.4	2.4					
			1.7	1.7					
Sal 2	21.6	21.6	7.4	7.4	12.2	8.6	28	18.4	34.4
(34.4 kb)	5.5	5.5	6.4	6.4	8.6	6.1	20.2	16.0	
	3.9	3.5	4.7	4.7	5.8	4.5			
			3.9	3.9	4.5	3.8			
					1.7	1.7			
Sal 3	6.3	6.3	6.9	6.9	4.6	4.6	28	13.5	21.1
(21.1 kb)	5.9	5.9	6.0	5.0	4.3	4.3	13.5	6.8	
	4.0	3.9	5.0		4.0	4.0		1.3	
	3.9	3.1							
Sal 4	3.9	3.9	3.6	3.3	4.0	4.0	28	11.8	17.4
(17.4 kb)	2.7	2.7	2.0	2.0	3.0	3..0			
	1.6	1.6	1.1	1.1	2.5	2.5			
Sal 5	3.5	3.5	7.4	3.1	–	–	–	4.6	4.6
(4.6 kb)									
Sal 6	3.9	1.6	6.0	3.7	12.2	4.3	–	–	4.3
(4.3 kb)									
			4.4	1.0					
Sal 7	4.1	3.7	1.9	1.5	5.9	3.8	28	3.9	3.9
(3.9 kb)									

tion with either PstI or SalI. Single digests with PstI and SalI, and double digests of the two together were prepared. Digested fragments were separated with single and double digests in adjacent lanes on 0.6% agarose. Electrophoresis was carried out in TAE buffer (Maniatis et al. 1982) at 0.5 V/cm, gels were stained in 700 ng/ml ethidium bromide and photographed. Sizes of fragments were measured on enlarged photographs using an IBI gel reader interfaced to an Olivetti M24 personal computer. Standard deviation of repeated measurements of the same fragment was ± 0.5 kb for fragments greater than 20 kb, ± 0.2 kb for fragments 10–20 kb and ± 0.1 kb or less for fragments less than 10 kb. Southern blots of gels were prepared on nylon membranes by the method of Smith and Summers (1980).

Blots were probed with each of the PstI and SalI fragments, with the inserts of recombinant plasmids from shotgun-cloned cpDNA and with inserts of cloned heterologous cp genes. Photosystem gene probes were a 1.6-kb HindIII insert in pUC18 containing about 560 bp of the 3′ end of barley psbA, a 1.2-kb EcoRI insert in pUC13 with 933 bp of the 5′ end of barley psbD, and a 1.1-kb EcoRI/BamHI insert in pUC13 with 987 bp of the 5′ end of barley psbC, as well as 129 bp of the 3′ end of psbD, which overlap in barley (Neumann and Berry-Lowe 1987; Neumann 1988). Ribosomal RNA probes were a 1.3-kb HindIII/SacI insert in pUC19 internal to the 16S rRNA gene of *Anacystis nidulans*, and a 870-bp HindIII fragment in pUC19 internal to Anacystis 23S rRNA (Lidholm et al. 1988a). The rubisco large subunit probe was a 0.9-kb HindIII fragment from the coding region of the *Chlamydomonas reinhardii* large subunit gene (Dron et al. 1982).

Probes were radiolabeled with ^{35}S using the supplier's modification of the method of Feinberg and Vogelstein (1984). Hybridization was carried out in 3 × SSC/2.5 × Denhardt's/0.25% SDS/50% formamide with 500 μg heterologous DNA overnight at 42 °C. Blots were washed twice in 2 × SSC for 30 min and once in 0.1 × SSC for 10 min at 65 °C. Autoradiography was carried out using Kodak X-Omat AR film at −70 °C (Maniatis et al. 1982).

Results

Tables 1 and 2 give the results of hybridization of white pine cpDNA PstI and SalI fragments to single and double digests of cpDNA. The results of hybridization of cloned white pine cpDNA inserts are given in Table 3, and Table 4 gives the results of hybridization of heterologous cp genes.

A physical map consistent with this data is given in Fig. 1.

As well as the major hybridizations listed in Tables 1–4, both native cpDNA fragments and cloned white pine cpDNA fragments in some cases showed reproducible weaker hybridizations to double digest fragments which hybridized more strongly to other probes. In par-

Table 2. Pst fragments

Probe	Major hybridizations to membrane-bound fragments (kb)								
	Eco	Eco/Pst	Bam	Bam/Pst	Hind	Hind/Pst	Pst	Pst/Sal	Sal
Pst 1	21.6	10.9	6.9	6.2	8.6	8.6	28	17.6	38
(27.8 kb)	5.9	4.8	6.4	4.7	6.9	6.9		16.0	34
	4.8	4.1	4.7	4.4	5.9	5.9		11.8	21.1
	4.1	3.9	3.6	3.6	5.8	5.8		6.3	17.4
	3.9	2.7	3.3	3.1	4.6	4.6		3.9	3.9
	2.7	1.3	2.0	2.0	4.3	4.3			
	1.3		1.9	1.9	4.0	4.0			
Pst 2	21.6	10.7	7.4	7.4	12.2	9.2	20.2	18.4	34
(20.2 kb)	5.5	5.5	4.4	3.9	4.5	3.9		2.0	
			3.9		1.7	1.7		1.8	
Pst 3	6.5	6.5	7.4	2.4	14.1	10.6	15.3	9.2	38
(15.3 kb)	4.3	3.5	2.4	1.8					
	3.5	2.5	1.8	1.6					
Pst 4	6.3	6.3	6.9	5.0	4.6	4.6	13.5	13.5	21.1
(13.5 kb)	3.9	3.9	6.0	3.6	4.0	3.6			
			5.0	2.5					
Pst 5	4.3	1.8	5.0	3.9	14.1	7.6	10.9	10.9	38
(10.9 kb)	1.8		3.9	1.6	11.3	3.3			
			2.7						
Pst 6	3.9	1.6	6.0	1.5	12.2	2.0	2.0	2.0	4.3
(2.0 kb)	1.8		4.4						
Pst 7	3.9	1.8	6.0	1.8	12.2	0.9	–	–	15.3
(1.8 kb)									

Table 3. Cloned HindIII fragments

Probe	Major hybridizations to membrane-bound fragments (kb)											
	E[a]	E/P	E/S	B	B/P	B/S	H	H/P	H/S	P	P/S	S
5.9 kb	4.8	4.8	4.8	3.3	3.3	1.7	5.9	5.9	3.8	28	17.6	38
	4.1	4.1	3.7	1.7	1.7				2.1			
4.6 kb	6.3	6.3	6.3	5.0	5.0	5.0	4.6	4.6	4.6	13.5	13.5	21.1
4.5 kb	21.6	10.7	21.6	3.9	3.9	3.9	4.5	3.9	4.5	20.2	18.4	34
4.3 kb	5.9	4.8	5.9	6.9	4.4	6.9	4.3	4.3	4.3	28	6.3	21.1
4.0 kb	3.9	3.9	3.9	3.6	3.3	3.6	4.0	4.0	4.0	28	11.8	17.4
	2.7	2.7	2.7	2.0	2.0	2.0						
	1.6	1.6	1.6	1.1	1.1	1.1						
1.7 kb	21.6	10.7	21.6	7.4	7.4	7.4	1.7	1.7	1.7	20.2	18.4	34

[a] Abbreviations: E – EcoRI, P – PstI, S – SalI, B –BamHI, H – HindIII

ticular, the Sal 21.1-kb fragment, when used as a probe, hybridized to the two larger Sal fragments as well as to the 21.1-kb fragment, to Pst/Sal double digest fragments totalling 73 kb and to double digest fragments produced with Sal and other enzymes totalling more than 21.1 kb. Similarly, the Pst 15.3-kb fragment, when used as a probe, hybridized both to the 15.3-kb and other Pst fragments, and to double digest fragments totalling more than 15.3 kb. The cloned 4.5-kb Hind fragment which mapped at the junction of the 27.7-kb and 20.2-kb Pst fragments showed weak hybridization to fragments mapping to the Sal 21.1-kb fragment. Another (2.6-kb Eco) cloned fragment hybridized to the 15.3-kb Pst fragment as well as to the 28-kb and 20.2-kb Pst fragments, to three Sal fragments and to several Bam and Hind fragments. These hybridization signals were reproducibly obtained

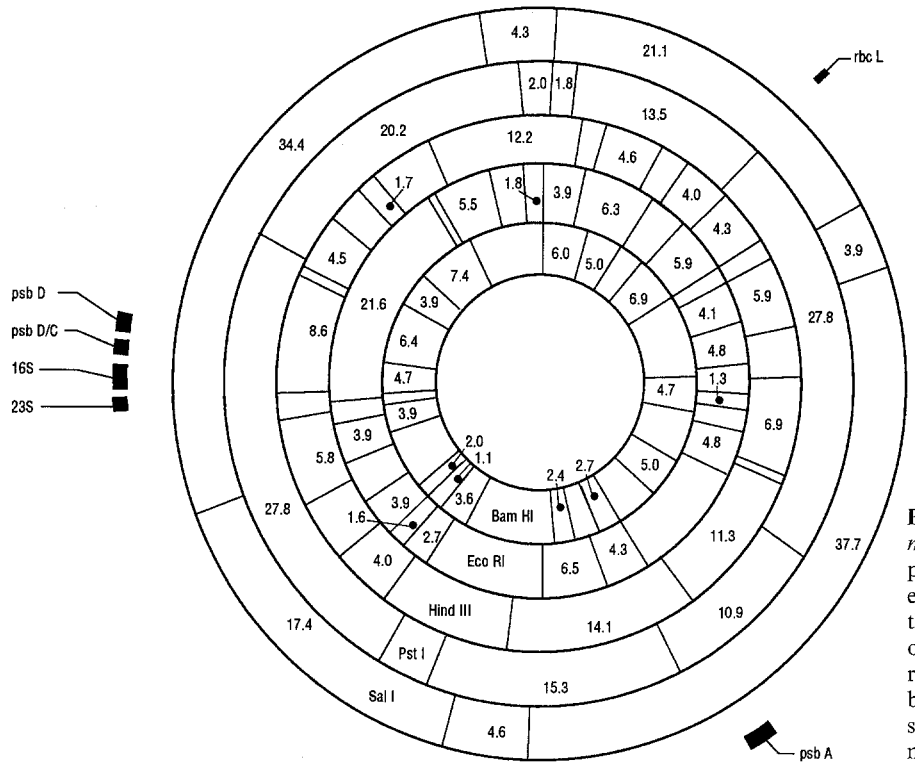

Fig. 1. Restriction site map of *Pinus monticola* chloroplast DNA, showing positions of hybridizations with heterologous gene probes. *Bars* marking the positions of genes indicate the size of the probe used. The order of the 16S rRNA, psbD and psbC genes could not be determined from the data; the order shown is that found in tobacco. Fragment sizes are given in kb

Table 4. Heterologous cp genes

Probe	Major hybridizations to membrane-bound fragments (kb)											
	E [a]	E/P	E/S	B	B/P	B/S	H	H/P	H/S	P	P/S	S
psb A	4.3	1.8	4.3	2.7	1.6	–	14.1	3.3	12.7	10.9	10.9	38
psb D, 5′ end	21.6	10.9	21.6	6.4	6.2	6.4	8.6	8.6	8.6	28	16.0	34
psb D, 3′+psb C, 5′ end	21.6	10.9	21.6	6.4	6.2	6.4	8.6	8.6	8.6	28	16.0	34
16S rRNA	21.6	10.9	21.6	6.4	6.2	6.4	8.6	8.6	8.6	28	16.0	34
23S rRNA	21.6	10.9	21.6	4.7	4.7	4.7	1.0	1.0	1.0	28	16.0	34
rubisco i.s.u.	5.9			6.9			4.0			13.5	13.5	21.1

[a] Abbreviations: E – EcoRI, P – PstI, S – SalI, B – BamHI, H – HindIII

under standard hybridization and washing conditions. The fragments to which they hybridized were among those routinely observed on ethidium bromide-stained gels.

Discussion

The chloroplast genome of white pine lacks the inverted repeat characteristic of most angiosperms (Palmer 1987). It has single copies of the 16S and 23S rRNA genes, which in angiosperms characteristically occur within the repeat, the total genome size is about 120 kb, and regions of symmetrically opposed restriction sites are not apparent (Fig. 1). The absence of an inverted repeat is characteristic of most of the other gymnosperms for which data are available (Strauss et al. 1988; Lidholm et al. 1988a; Palmer and Stein 1986).

The genes for the 16S and 23S ribosomal RNAs are located near the photosystem II protein coding genes psbD and psbC, while the genes for the large subunit of ribulose bisphosphate carboxylase and the 32-K photosystem II protein coding gene psbA are each located about 40 kb distant and about 30 kb from each other. This gene arrangement is similar to that in radiata pine (*Pinus radiata* D. Don), and unlike the arrangement in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco] (Strauss et al. 1988) or in pea (*Pisum sativum* L.) (Palmer

et al. 1985) or broad bean (*Vicia faba* L.) (Shinozaki et al. 1984), angiosperms which are unusual in not having the inverted repeat.

There are several possible explanations for the very reproducible hybridization of some fragments to more than one probe. In some cases (e.g. the 4.3-kb Eco fragment which gave a medium signal when the 38-kb Sal fragment was used as a probe and a stronger one with the 21.1-kb Sal fragment), the reason was simply that the membrane-bound fragments were heterologous doublets. For other hybridizations, this was unlikely. The sum of all fragments giving a signal with these probes was greater than the size of the probe (e.g. the 21.1-kb Sal fragment, the 15.3-kb Pst fragment and the 4.5-kb Hind fragment; see above), and the intensity of staining with ethidium bromide did not indicate that the membrane-bound fragments were doublets. Homology between different parts of the genome is a more likely explanation in these cases. Homology between different chloroplast-encoded genes occurs in barley (Neumann 1988), repeated tRNA genes or pseudogenes have been reported in pea and broad bean chloroplast genomes (Crouse et al. 1986) and duplication of the psbA gene has been reported in *Pinus contorta* (Lidholm et al. 1988b). The presence of several dispersed repetitive sequence families has been reported in *Pseudotsuga menziesii* (Tsai and Strauss 1988). The results of this study indicate that dispersed repeated sequences occur in *Pinus monticola* also. The 21.1-kb Sal fragment probably contains sequences repeated at least three times, since it hybridized to the two larger Sal fragments as well as to the 21.1-kb fragment and to Sal/Pst fragments totalling more than three times its size. The same is true of the 15.3-kb Pst fragment. The 2.6-kb cloned Eco fragment appears to contain sequences repeated at least three times and present in the 15.3-kb and larger Pst fragments. The additional hybridizations of the cloned 4.5-kb Hind fragment indicate sequences near the psbD/C gene complex are repeated near the gene for the large subunit of ribulose bisphosphate carboxylase.

The lack of a large inverted repeat and the presence of dispersed repeats may confer greater diversity on the chloroplast genome of *P. monticola* than occurs in angiosperms. Rearrangement, likely as a result of recombination between repeats, is observed more frequently in chloroplast genomes lacking a large inverted repeat (Palmer and Thompson 1982; Day and Ellis 1984). The chloroplast genomes of *P. monticola* and other conifers may be evolving through rearrangement more rapidly than most angiosperms (Strauss et al. 1988). In addition, sequence divergence, as evidenced by restriction site differences, was observed within *P. monticola* (White 1989). This relative instability may be a basis for conifer cpDNA variation useful in studies of population structure.

Acknowledgements. I am grateful to Dr. E. Neumann, Texas A & M University, for providing the cloned photosystem genes, and to Drs. R. Kalla and P. Gustafsson, Umeå University, for the rubisco and rRNA genes. I thank B. Foord for skillful technical assistance.

References

Beckman JS, Soller M (1983) Restriction fragment length polymorphisms in genetic improvement. Theor Appl Genet 67:35–43

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Crouse EJ, Mubumbila M, Stummann BM, Bookjans G, Michalowski C, Bohnert HJ, Weil J-H, Henningsen KW (1986) Divergence of chloroplast gene organization in three legumes. Plant Mol Biol 7:143–149

Day A, Ellis THN (1984) Chloroplast DNA deletions associated with wheat plants regenerated from pollen: possible basis for maternal inheritance of chloroplasts. Cell 39:359–368

Dron M, Rahire M, Rochaix J-D (1982) Sequence of the chloroplast DNA region of *Chlamydomonas reinhardii* containing the gene of the large subunit of ribulose bisphosphate carboxylase and part of flanking genes. J Mol Biol 162:775–793

Feinberg AP, Vogelstein B (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 137:266–267

Helentjaris T, King G, Slocum M, Siedenstrang C, Wegman S (1985) Restriction fragment polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Plant Mol Biol 5:109–118

Hunt RS, Rudloff E von (1977) Leaf-oil-terpene variation in western white pine populations of the Pacific Northwest. For Sci 23:507–516

Hunt RS, Meagher MD, White EE, Mitchell LA (1987) The white pine tree improvement program in British Columbia. Proc 16th Congr Pacific Sci Assn, Seoul

Lidholm J, Szmidt AE, Hallgren J-E, Gustafsson P (1988a) The chloroplast genomes of conifers lack one of the rRNA-encoding inverted repeats. Mol Gen Genet 212:6–10

Lidholm J, Jansson S, Szmidt A, Gustafsson P (1988b) Structure and regulation of photosynthesis genes in *Pinus sylvestris* and *Pinus contorta*. Proc Frans Kempe Symp Mol Genet For Trees, Umea, pp 115–126

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Neumann E (1988) Primary structure of barley genes encoding quinone and chlorophyll a binding proteins of photosystem II. Carlsberg Res Commun 53:259–275

Neumann E, Berry-Lowe S (1987) The genes encoding the quinone and herbicide binding proteins of barley thylakoids. J Cell Biochem Suppl 11B:60

Palmer JD (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am Nat 130:S6–S29

Palmer JD, Stein DB (1986) Conservation of chloroplast genome structure among vascular plants. Curr Genet 11:823–834

Palmer JD, Thompson WF (1982) Chloroplast DNA rearrangements are more frequent when a large inverted repeat sequence is lost. Cell 29:537–550

Palmer JD, Jorgensen RA, Thompson WF (1985) Chloroplast DNA variation and evolution in *Pisum*: patterns of change and phylogenetic analysis. Genetics 109:195–213

Shinozaki K, Sun C-R, Sugiura M (1984) Gene organization of chloroplast DNA from the broad bean *Vicia faba*. Mol Gen Genet 197:363–367

Smith GS, Summers MD (1980) The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal Biochem 109:123–129

Steinhoff RJ, Joyce DG, Fins L (1983) Isozyme variation in *Pinus monticola*. Can J For Res 13:1122–1132

Strauss SH, Palmer JD, Howe GT, Doerksen AH (1988) Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. Proc Natl Acad Sci USA 85:3898–3902

Tsai C-H, Strauss SH (1988) Dispersed repetitive sequences in the chloroplast genome of Douglas-fir. Proc Western For Genet Assn, Davis

White EE (1986) A method for extraction of chloroplast DNA from conifers. Plant Mol Biol Rep 4:98–101

White EE (1989) Chloroplast DNA in *Pinus monticola*. 2. Survey of within-species variability and detection of heteroplasmic individuals. Theor Appl Genet (in press)