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Genetic Analysis of Needle Proteins in Maritime Pine

1. Mapping Dominant and Codominant Protein Markers Assayed on Diploid Tissue, in a Haploid-Based Genetic Map

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Abstract

A genetic analysis of proteins was performed in a 3-generation inbred pedigree of maritime pine (*Pinus pinaster* AIT.). Proteins were extracted from needles (2n) and revealed by 2-dimensional gel electrophoresis. A total of 17 qualitative variants (presence/absence variations and position shifts) were observed and conformed to Mendelian inheritance patterns. These markers were localized in a previously reported genetic map based on RAPD markers assayed on megagametophytes (1n). To achieve this integration, evenly spaced RAPD markers were genotyped on diploid tissue. The internal amino acid sequences of 3 proteins were determined, and 2 of them

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corresponded to genes of known function. We discuss the use of proteins separated in 2-dimensional gels for constructing a genetic map of expressed genes.

Key words: *Pinus pinaster*, 2-dimensional electrophoresis, needle proteins, RAPD, linkage map, microsequence.

FDC: 165.3./4; 160.201; 174.7 *Pinus pinaster*.

Introduction

The choice of genetic markers and pedigrees for genetic analysis (linkage and QTL studies) largely depends upon the objectives and the accumulated results of previous genetic studies. In agricultural crops or experimental plants, genetic mapping and quantitative trait dissection analysis has usually been based on RFLP (Restriction Fragment Length Polymorphism) markers in crosses among inbred lines (reviewed by TANKSLEY, 1993). Forest trees are typically outcrossing organisms and inbreeding has been considered impractical because tree generation times are long and most tree species exhibit severe inbreeding depression probably due to deleterious recessive alleles (e.g. SORENSEN and MILES, 1982).

The recent advance of arbitrarily-primed PCR-based molecular marker methods (*e.g.* RAPD, random amplified polymorphic DNA, WILLIAMS et al., 1990), combined with genetic analysis in 2-generation pedigrees has greatly changed the prospects for application of molecular markers in forest trees (O'MALLEY, 1996; GRATTAPAGLIA and SEDEROFF, 1994). The major advantage of RAPD markers is that they do not depend on prior knowledge of DNA sequence and constitute a powerful tool for constructing single tree maps in a very short period of time (SOBRAL and ONEYCUTT, 1993). In conifer species, the haploid megagametophyte tissue constitutes an ideal plant material for genetic analysis with dominant RAPD markers. In particular, it has been used to construct relatively dense linkage maps in several coniferous species (reviewed by NEALE et al., 1994). The DNA extraction from megagametophytes yields enough DNA to perform hundreds of PCR reactions and the high level of heterozygosity in most conifer species allows production of hundreds of polymorphisms.

The major limitation of the "megagametophyte approach" is that it requires the development of specific populations and is not applicable to the analysis of QTL (Quantitative Trait Loci) for economically important traits in existing plantations. Indeed, the megagametophyte is a temporary tissue that can only be collected from seedlings during germination of the embryo. Conversely, conifer trees can be grafted and propagated by cuttings, and diploid progenies constitute perpetual populations, analogous to recombinant inbred lines in crop plants (BUR et al., 1988). In addition, the use of clones of individual genotypes increases the precision of quantitative measurements for QTL analysis (BRADSHAW and FOSTER, 1992). Therefore, it appears essential to use the sporophytic tissue of coniferous species because it is unlimited and will make it possible to gather new molecular markers (e.g., RFLPs, microsatellites, proteins) and map them. Screening RAPD primers for informative markers which segregate 1:1 in diploid tissues of angiosperms or gymnosperms hybrid progeny (CARLSON et al., 1991) has already proved to be a very powerfull strategy for establishing single tree maps with diploid tissue (GRATTAPAGLIA and SEDEROFF, 1994; KUBISIAK et al., 1995).

QTL mapping models are purely based on statistical association between traits and molecular markers. QTLs have been identified that account for variation in many phenotypic traits, yet there has been little analysis of the relationship of known genes with those QTLs (e.g. TOUZET et al., 1995a). The accessibility of the biological meaning of a QTL can proceed from co-location between QTL and qualitative mutations (BEAVIS et al., 1991; DOEBLEY and STEC, 1991, 1993), between QTL and known-function genes (GOLDMAN et al., 1994), or between QTL of both agronomic and biochemical traits (CAUSSE et al., 1995). In maritime pine, we already demonstrated (PLOMION et al., 1995a) that most RAPD markers amplify from highly repetitive chromosomal regions (i.e., mostly non coding DNA). This is likely to be true for any conifer species characterized by a large genome size (WAKAMIYA et al., 1993; OHRI and KHOSHOO, 1986). Therefore, RAPD markers appear to have limited value for defining the relationship between phenotypic variation and genes. An alternative approach with a greater probability for defining such relationships uses markers based upon sequences (e.g., anonymous structural proteins revealed by 2-D PAGE, or cDNA clones). Marker-trait associations are performed, sequences of protein or DNA markers that fall within a QTL of interest are determined, and possible functions are identified based upon similarities to sequences in public database. Such an approach should allow for the testing of the relationships between the genetic variation of quantitative traits and known-function genes.

With the limits of the "RAPD-megagametophyte" approach in mind, our objectives were 3 fold: (i) determine the patterns of inheritance of proteins presenting qualitative variations (presence/absence, position shifts) in a population of segregating progeny, (ii) localize these protein in an existing genetic map constructed with RAPD markers assayed on megagametophytes, and (iii) determined the function of some proteins by microsequence analysis. The availability of an "ideal F2" population within the maritime pine breeding program provided a unique apportunity for genetic analysis of protein markers in this species.

Materials and Methods

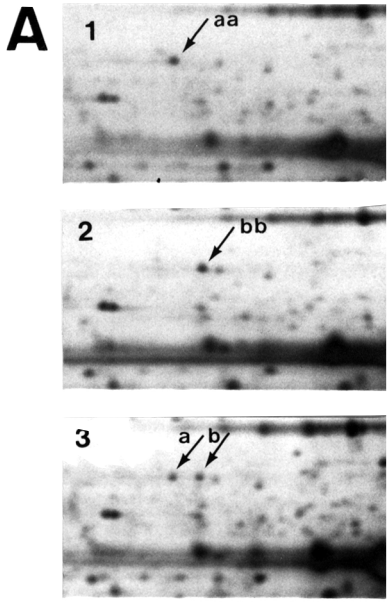
Genetic material and marker analysis

The experimental material was developed by crossing one individual of the Landes race (accession 'L146') with one individual of the Corsican race (accession 'C10'). From this cross, one hybrid individual (accession 'H12') was selfed and 192 F2 plants were produced. The megagametophyte of each F2 individual was collected from germinated seeds. A genetic map of 'H12' was constructed with RAPD markers segregating 1:1 in haploid megagametophyte of the F2 seedlings as has been previously reported (PLOMION et al., 1995a and b, 1996). This "haploid map" consisted of 436 RAPD markers from which 100%, 46% and 28% were assayed on 62, 94 and 126 megagametophytes, respectively. In this study, a total of 150 RAPD markers distributed over the "haploid map" (data not shown) was amplified from diploid tissue (needle) of 130 to 192 F2 seedlings. From these 192 plants, 68 individuals that were genotypes with RAPD markers in both haploid and diploid material were also genotyped with protein markers. DNA extraction from needles and RAPD analysis are described elsewhere (PLOMION et al., 1995a and b). Total protein extraction from needles tissue and 2-dimensional gel electrophoresis (isoelectric focusing and SDS-PAGE dimensions) were performed as described in BAHRMAN and PETIT (1995). The gels were silver stained according to DAMERVAL et al (1987) and dried. Qualitative variation (presence/absence denoted as P and position shift denoted as S) were observed by superimposition of gels of both grand-parents, the hybrid parent and the F2 progeny, upon an illuminated box. Molecular weight standards for SDS-PAGE were purchased from Pharmacia and consisted of

phosphorylase b (*Mr* 94,000), bovine serum albumin (*Mr* 67,000), ovalbumin (*Mr* 43,000), carbonic anhydrase (*Mr* 30,000), soybean trypsin inhibitor (*Mr* 20,100) and α-lactalbumin (*Mr* 14,400).

Protein sequencing and similarity search

The internal amino-acid sequence was obtained by the Laboratoire de Microséquençage des Protéines, Institut Pasteur (Paris, France) as described by TOUZET et al. (1995b). The amino acid sequences were compared to the sequences of 4 databases of the National Center for Biotechnology Information (Dec., 20, 1996): (i) the Non-redondant Peptide Sequences Database (234,430 protein sequences), by the use of the BLASTt program; this database comprises: CDS translations from Genbank, Brookhaven Protein Data Bank (PDB), SwissProt and PIR, (ii) the Non-redondant Nucleotide Sequence Databases (280,350 nucleotide sequences), by the use of the tBLASTn program; this database comprises all non-redundant GenBank, EMBL, DDBJ and PDB sequences, and (iii) the Non-redundant Database of GenBank STS (44,563 sequences) and EST (761,858 sequences) Divisions.



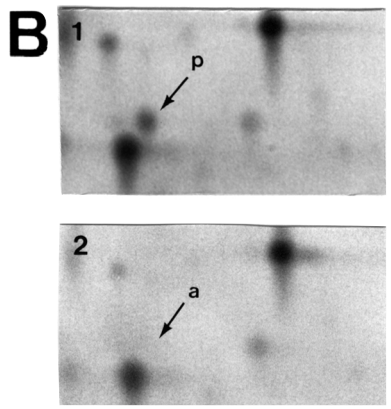


Fig. 1. – (A) Cuttings from 2D gels showing a position variation (allelism) in the needles of the Landes grandparent (accession L146, panel A1: homozygote « aa »), Corsican grandparent (accession C10, panel A2: homozygote « bb ») and the bybrid parent (accession H12, panel A3: heterozygote « ab »). (B) Cuttings from 2D gels showing a presence (p)/absence (a) variation in the needles of 2 F2 genotypes.

Linkage analysis

Significant linkage between RAPD and protein markers assayed on needles of the F2 progeny was determined using a minimal linkage LOD of 2.5 and a maximum recombination fraction θ of 0.35. The analysis was performed using the MAPMAKER program for the Macintosh (LANDER et al., 1987) under the F2 intercross model. Chi-square tests were performed to examine if the observed genotypic frequencies of the protein and RAPD loci deviated from the expected 1:2:1 and 3:1 MENDELian ratios, for co-dominant and dominant markers, respectively.

Results

Inheritance of qualitative protein variants

A total of 26 spots belonging to 13 polypeptides corresponded to allelic products of genes, varying in position, and segregated 1:2:1 in the F2 progeny ($P < 0.01$). Allelic variations were based on the following criteria: (i) loci encoding each spot of a pair originate from different grandparent; (ii) they are relatively close to each other on 2D pattern; i.e., they have similar pI and apparent molecular masses (*Fig. 1A*). Four spots concerned presence/absence variations and segregated 3:1 ($P < 0.01$) (*Fig. 1B*). Such variations were already found to be under monogenic control (BAHRMAN and DAMERVAL, 1989; GERBER et al., 1993).

Localization of dominant and codominant protein loci in the "haploid map"

An other important objective of our study was to assign the protein markers assayed on diploid tissue to their most probable location in the existing "haploid map". However, linkage analysis between markers segregating 1:1 in the haploid mapping sample and dominant or codominant markers segregating 3:1 or 1:2:1, respectively, in the F2 diploid mapping sample, is not efficient. The linkage information (measure of the precision of the estimated recombination frequency; ALLARD, 1956; RITTER et al., 1990) for those marker pair configurations is very low. The information functions, i_p, for the calculation of the standard error of the recombination frequency p are: i_p (1:1 by 1:2:1) = $1/2p(1-p)$ (this study), and i_p (1:1 by 3:1) = $(1+2p-2p2)/2p(1-p2)(2-p)$ (RITTER et al., 1990).

The maximum information is obtain for a pair of markers segregating 1:1 and 1:2:1 but it still remains under the information for a pair of dominant markers linked in coupling. The information for a pair of markers segregating 1:1 and 3:1 is about 2 times less than the information for the 1:1 and 1:2:1 configuration. However, the information for a pair of markers segregating 3:1 and 1:2:1 is approximately 2 to 3 times greater than for the 2 previous configurations. Thus, the strategy that was adopted to locate protein markers in the existing "haploid map" consisted on genotyping F2 diploid individuals, with a subset of RAPD markers of known position in the "haploid map", and performing cosegregation analysis between these RAPDs and the proteins. We previously used a rigorous strategy to choose RAPD markers that should be highly repeatable and easily scored from both haploid and diploid materials (PLOMION et al., 1995b).

A total of 150 RAPD markers spanning the whole genome (data not shown) and segregating 3:1 ($P < 0.01$) were assayed on 130 to 192 F2 plants. Out of these 192 individuals, 68 were also genotyped with proteins revealed on 2-D gels as described in the materials and methods. Linkages between RAPD markers segregating 3:1 and protein markers segregating 3:1 or 1:2:1 were established with LOD scores ranged from 2.6 to 17.2 (*Table 1*). Proteins were then assigned in the "haploid map" to a relative location based on the closest linkage to the RAPD locus in the F2 diploid mapping sample (*Table 1*). Their position on the 2D pattern as well as their map location are being made available through the following internet address: http ://www.pierroton.inra.fr/genetics/2D/mapped.html

Table 1. – List of the protein markers revealed by 2-dimensional electrophoresis and linkage relationships with the nearest RAPD marker assayed on the diploid progeny. The nomenclature of the RAPD markers follows PLOMION et al. (1995b). The type of variant is indicated: S for position shift and P for presence/absence variation.

Protein Marker	Molecular weight (kDa)	Type of variation	map distance in Kosambi cM from the nearest RAPD locus	LOD score	Linkage Group*
1271-72	> 94	S	7 (E8_1023/+)	10.4	Group 1
2205-87	40	S	0 (K20_540/+)	17.2	Group 2
2227-06	51	S	19 (P14_1409/-)	2.6	
1361-62	71	S	7 (P14_280/-)	4.9	Group 3
1306-44	71	S	2 (P14_280/-)	7.0	
1205-06	80	S	5 (I11_566/+)	12.4	Group 4
3235-20	30	S	9 (N10_599/+)	6.5	Group 7
3207	33	P	13 (P11_344/+)	6.5	
1270-73	76	S	13 (A7_1418/-)	4.4	
1275-76	76	S	9 (A7_1418/-)	5.3	
2229-74	43	S	9 (F3_852/-)	5.3	Group 8
3002	> 94	P	12 (J16_965/-)	5.4	Group 9
4219-20	24	S	8 (I11_287/+)	8.5	Group 10
1247-48	61	S	3 (Z20_939/-)	12.2	
2431-32	50	S	13 (B12_1396/+)	7.0	
1346	76	P	2 (B12_1396/+)	14.1	
4206-17	21	S	9 (I4_682/-)	8.8	Group 11

S: position shift (1:2:1 segregation ratio)
P: presence/absence variation (3:1 segregation ratio)
*: see PLOMION et al. (1995b)

Protein sequences similarity

Two of the sequenced proteins showed a high similarity with proteins encoded by genes of known function. The amino-acid microsequences of spot #S1205-06 in linkage group 4 (XGESWETPETGDEVE) and spot #S2205-87 in linkage group 2 (DVNWPLGWPVGGYPG) were 78.5% and 100% identical to peptidylproyl cis-trans-isomerase (EC 5.2.1.8) and glutamine synthase (EC 6.3.1.2), respectively. For the third microsequence (spot #S1247-48 in linkage group 10: HEEQITQPSATNDEA), 75% similarity was found with a STS sequence of the eubacteria *Chlamidya trachomatis* (accession G12495).

Discussion

Inheritance of qualitative variants in the F2 progeny

Pattern of protein in both Landes and Corsican parents, the F1 hybrid and their F2 progeny suggested inheritance modes that conform to simple MENDELian assortment ratios (i.e. 3:1 and 1:2:1 segregation ratios for dominant and codominant markers, respectively). Other studies have reported MENDELian inheritance for proteins (e.g. DAMERVAL et al., 1994; BURSTIN et al., 1994). Out of 14 position variants 13 corresponded to horizontal shifts (i.e. an isoelectric point modification of the protein due to amino-acid substitutions) whereas only one corresponded to a vertical shift (i.e. molecular weight change). Such observation has already been reported (ANDERSON et al., 1985; BAHRMAN and PETIT, 1995; BAHRMAN et al., 1996). Indeed, there should be greater selective constraints against the mutations affecting the size of the polypeptides than those affecting the charge. TOUZET et al. (1995b) demonstrated that these electrophoretic variations corresponded either to allelic differences in the primary structure of a protein, or monogenic co- or post-translational modifications of a protein. Sixty-five % of the polymorphic protein spots had a molecular weight greater than 50 kDa. More variation for the larger than for the smaller proteins agrees with variation in the structure of the proteins and translate the relation between the length of the polypeptide and the substitution rates (NEI, 1987). This result obtained in a single segregating progeny confirmed the observation of BAHRMAN and PETIT (1995) from 18 unrelated individuals of the maritime pine breeding population.

Toward the mapping of the expressed genome in maritime pine

A saturated genetic map of maritime pine was constructed with RAPD markers segregating in the 1:1 MENDELian ratio in megagametophytes of F2 seeds. A total of 27 proteins assayed on the same megagametophytes were already localized (PLOMION et al., 1995a). In this paper, we show that an additional set of 17 protein markers assayed on needles, could be easily incorporated in the same genetic map. This total of 44 proteins are well distributed throughout the map but still do not provide complete coverage of the genome. The number of such markers corresponding to translated chromosomal regions could be substantially increased by analyzing of some physiologically contrasted organs of the same individuals, until full coverage of the genome could be obtained. This was proposed by DE VIENNE et al. (1996) as a means of obtaining a map of expressed sequences in maize. We expect that the development of this type of marker that exclusively represent expressed genes will provide a powerful tool toward the dissection of quantitative traits in maritime pine. Partial amino-acid sequence of proteins showing co-location with QTLs of interest should be a productive way to determining the genes involved in particular physiological processes. Indeed some of these proteins may be recognizable by sequence similarity to others proteins published in sequence databases (e.g. TOUZET et al., 1996). As a demonstration for maritime pine, we determined the microsequences of 3 needle proteins and identified the function of 2 of them. Such analysis of gene expression has been developed by sequencing cDNA in crop plants (UCHIMIYA, et al., 1992; HÖFTE et al., 1993; KEITH et al., 1993; PARK et al., 1993; SASAKI et al., 1994) as well as in forest trees (KINLAW et al., 1996). However, in many plant species and particularly in conifer species, cDNA generally reveal several unlinked loci (DEVEY et al., 1994), making it difficult to identify the gene(s) that is actually expressed. Therefore, 2-D PAGE may prove to be a complementary marker technique for constructing maps of expressed genes. In maritime pine, proteins were successfully extracted and separated on 2D PAGE for different tissues including bud, megagametophyte, phoem, pollen, root, xylem. Gel pictures are accessible via internet (http://www.pierroton.inra.fr/genetics/2D).

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