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Genetic Analysis of Needle Proteins in Maritime Pine

2. Variation of Protein Accumulation

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Abstract

Experimental variation and genetic background effects in protein accumulation were studied in a three-generation F_2 inbred pedigree of maritime pine *(Pinus pinaster* AIT.). Proteins extracted from needles were revealed by high-resolution silver-stained two-dimensional polyacrylamide gel electrophoresis and analysed with a computer-assisted system for single spot quantification. The integrated intensity of 77% of the studied spots showed a linear relationship with the total amount of protein loaded into the gel. A significant difference of integrated intensity was found among both parents and their hybrid for 31% of the studied proteins, from which 78% followed a non-additive mode of inheritance. The extent of the observed non-additivity is discussed and compared with results found in similar experiments in pea, maize and wheat. Finally, QTL mapping allowed the detection of PQL (Protein Quantity Loci) that explained part of the quantitative variation of protein accumulation.

Key words: Pinus pinaster, two-dimensional electrophoresis, proteins, inheritance, mapping, QTL, additivity.

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Introduction

Qualitative variations of proteins revealed by two-dimensional polyacrylamide gel electrophoresis (2-DE) (O'FARRELL, 1975) were used for genetic studies in maritime pine *(Pinus pinaster* AIT.). Protein markers have been used both in genetic mapping (BAHRMAN and DAMERVAL, 1989; GERBER et al., 1993; PLOMION et al., 1995, 1997) and in population genetic studies (BAHRMAN et al., 1994; PETIT et al., 1995).

To analyse the genetic basis of quantitative variation of proteins separated by 2-DE, DAMERVAL et al. (1994) measured the quantity of each protein in a F_2 progeny of maize. The protein quantity was assessed through integrated optical density of each single spot using an automatic image-analysis system. Their study was based on QTL (Quantitative Trait Loci) mapping procedure (reviewed by TANKSLEY, 1993; KEARSEY, 1998). They used a linkage map to locate the regulatory factors or "PQL" (Protein Quantity Loci) that would explain part of the spot intensity variation. They concluded that multifactorial control of protein quantity variation was a general feature of genome expression. Recently, DE VIENNE et al. (1999) combined the PQL methodology with a traditional QTL mapping experiment, to characterise QTLs of economically important traits. They showed that three PQLs controlling the quantity of a single leaf protein and three QTLs of height growth in maize were co-located.

Given the large genome size of conifers (OHRI and KOSHOO, 1986; WAKAMIYA et al., 1993), the PQL approach seems to be a

more realistic alternative to map-based cloning (ROMMENS et al., 1989) in order to define the biological meaning of QTLs in these species. The more general goal of this study is to investigate the use of protein quantity as a new trait in genetic studies in maritime pine. The specific objectives are the following: (i) to study the relationship between spot integrated intensity and protein amount over a range of protein concentrations, (ii) to study the genetic inheritance of protein amount (quantified through the variation in spot integrated intensity) from parental individuals to their F_1 hybrid, and (iii) to investigate the possibility of detecting PQLs controlling protein quantity variation.

Materials and Methods

Genetic material

The experimental material was developed by crossing maritime pine individuals from the Landes (accession 'L146') and Corsican (accession 'C10') origin. From this cross, one hybrid individual (accession 'H12') was selfed and 192 F_2 plants were produced. A genetic map of 'H12', was constructed using 436 RAPD and 27 protein markers segregating 1:1 in haploid F_2 megagametophytes (PLOMION et al., 1995). Recently, seventeen needle protein loci were added to this map (PLOMION et al., 1997).

Two-dimensional gel electrophoresis and automatic 2-DE gels analysis

Total protein extraction and two-dimensional gel electrophoresis (isoelectric focusing and SDS-PAGE dimensions) were performed as described in BAHRMAN et al. (1997). The gels were silver-stained according to DAMERVAL et al. (1987). The 2-D gels were scanned and analysed with the Bio Image 2-D Analyzer, version 6.0.3 (BioImage Products, Millipore/Biosearch, Ann Arbor, MI). An average of 1000 spots were detected per gel. The analysis was focused over a few dozen of spots distributed throughout the gels. Their integrated intensity was estimated by summing the density of each pixel within the boundaries of the spot. For the comparison of protein accumulation among F_2 genotypes, the integrated intensity of each spot was corrected for the gel staining effect according to the linear scaling method described by BURSTIN et al. (1993).

Statistical analysis

Relationship between spot integrated intensity and total protein amount

The effect of total protein amount on spot integrated intensity was tested by analysis of variance for 63 randomly spots distributed throughout 2D-gels of three genotypes: L146, C10 and H12. Four protein-UKS (urea-potassium carbonate-SDS solution) mixture loads (5 µl, 10 µl, 15 µl and 20 µl) corresponding to a range of 15 µg to 60 µg of total protein per gel were used. Five to six replicated gels were available for each loading, resulting in a total of 22 gels.

Mode of inheritance of protein amount

Analysis of variance was used to identify protein accumulation differences among the three genotypes, L146, C10, and H12, for a set of 100 spots. Four replicated gels were available per individual. A 20 µl protein load was used for this experiment. Whenever F-tests were significant, the mode of inheritance of each protein in the F_1 hybrid was analysed by comparing spot intensities between each one of the parents and the hybrid. A t-test was used to distinguish additive inheritance (i.e., the spot intensity of hybrid is not significantly different from the mean spot intensity of both parents) from non-additive inheritance (i.e., the spot intensity of the hybrid is significantly different from the mean spot intensity of both parents). In this second case, we tested if the spot intensity of the hybrid was not significantly different from the spot intensity of one of the two parents, i.e. "dominance"; or if the spot intensity of the hybrid was outside the parental range: (more intense than the highest parental spot intensity or less intense than the lowest parental spot intensity), i.e. "overdominance".

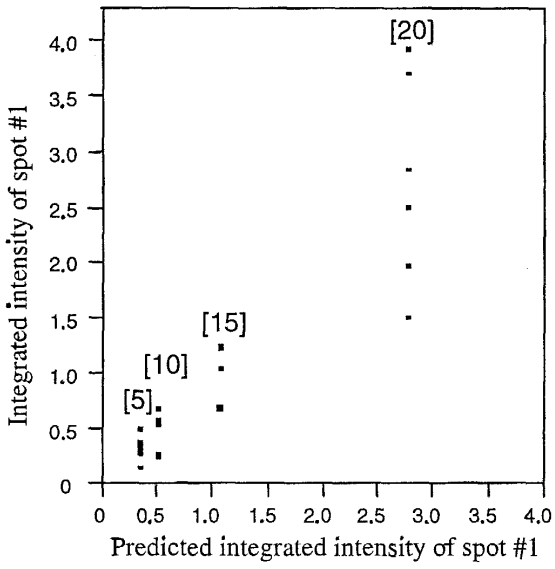
PQL detection

The detection of PQL (Protein Quantity Loci, DAMERVAL et al., 1994) was performed on the basis of 68 F_2 seedlings according to the method of interval mapping (PATERSON et al., 1988) using the QGENE software (NELSON, 1997). Considering the small number of F_2 individuals, a type I error of 0.005 was used for claiming the presence of PQL. In addition, only one replicated gel was available per F_2 genotype. Only ten proteins were analysed to illustrate the possibility of detecting PQL. The methodology used in this preliminary study is about to be extended to 163 proteins, using twice as much F_2 individuals (COSTA et al., in preparation).

Results and Discussion

Linearity of spot integrated intensity in relation to total protein amount

Protein accumulation was quantified through spot staining intensity (i.e. integrated optical density) estimated by an automatic system of image analysis. For studying variation in protein accumulation, an assumption is made that protein spot intensity is linearly related to the total amount of protein loaded. In this study, the effect of protein load on spot integrated intensity was tested over 63 spots and, for 77 % of them, the result was significant (P<5 %). A linear response was observed for these spots, as shown in *figure 1.* This result clearly indicates the reliability of silver-staining for polypeptide quantification.



Source	DF	SS	MS	F Ratio	Prob>F
Model	3	21.6	7.20	24.7	0.0000
Error	17	4.9	0.29		
Total	20	26.6			

Fig. 1. – Analysis of variance for spot #1 among four protein loads (5 µl, 10 µl, 15 µl and 20 µl).

Scaling procedures

In maize, BURSTIN et al. (1993) showed that the major source of experimental spot intensity variation was due to the silver staining. They observed that the volume of a given spot could be linearly described by a variable that account for global gel intensity: e.g., the mean intensity (MI) of several dozen of spots. In this study, data also confirm the hypothesis that the spot integrated intensity can be well described by the mean intensity of the studied spots. The relationship between the intensity Y_{ij} of spot i in gel j and MI_j (mean intensity of the 63 spots in each gel j) was found to be linear for 89% of the spots ($P<0.01$) *(Fig. 2)*. The linear scaling factor, K_j, used afterwards to minimize the global between-gel experimental variation, was computed as follows:

$K_j = MI_{..}/MI_{.j}$,

where $MI_{.j}$ and $MI_{..}$ are the mean intensity value of the studied spots in each gel j and the mean intensity value of the same spots for all the gels, respectively.

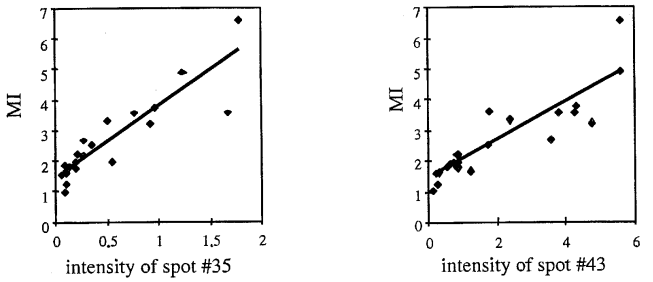


Fig. 2. – Intensity of spot #35 and #43 plotted on the mean intensity (MI) of the studied spots. The regression coefficients (r = 0.89) are significant at the 1% level.

The corrected intensity of spot i in gel j was expressed as follows:

$Y_{ijcorr} = Y_{ij} * K_j$

If it is assumed that total protein amount (i.e. the sum of the spot intensities) is the same in the different genotypes, this scaling factor is independent from a genetic effect.

Mode of inheritance of protein accumulation in the hybrid individual

Thirty one percent of the studied spots showed significant quantitative variation between the three genotypes (C10, L146 and H12). Results indicated that 7 and 24 of them have additive and non-additive mode of inheritance, respectively *(Fig. 3)*. The other studied proteins showed no significant difference of intensity in the three genotypes. For 50% of the polypeptides with non-additive inheritance (i.e. 12 spots), the hybrid spot exhibited an intensity similar either to the lowest (6 spots) or to the highest (6 spots) intensity of one of the parental spot. For the other 50% of protein, the hybrid spot showed a greater intensity than the most intense parental spot, in 10 cases and a lower intensity (than the less intense parental spot) in 2 cases.

Possible causes of a reduced protein level in the hybrid (i.e. "recessiveness" or "negative overdominance") have been discussed by LEONARDI et al. (1987) and DE VIENNE et al. (1988). This feature may be attributed to inherited regulatory factors that degrade the protein or reduce its stability. "Dominance" or "positive overdominance" inheritance suggests that structural genes and genes involved in regulating the expression of these proteins were inherited in the hybrid and did not interact in an inhibitory manner, since there were no alteration in the synthesis levels or their stability in the hybrid. Thus, "positive over-dominance" may reflect interacting complementary regulatory factors inherited from both parents, resulting in a higher F_1 hybrid protein synthesis level or increased stability.

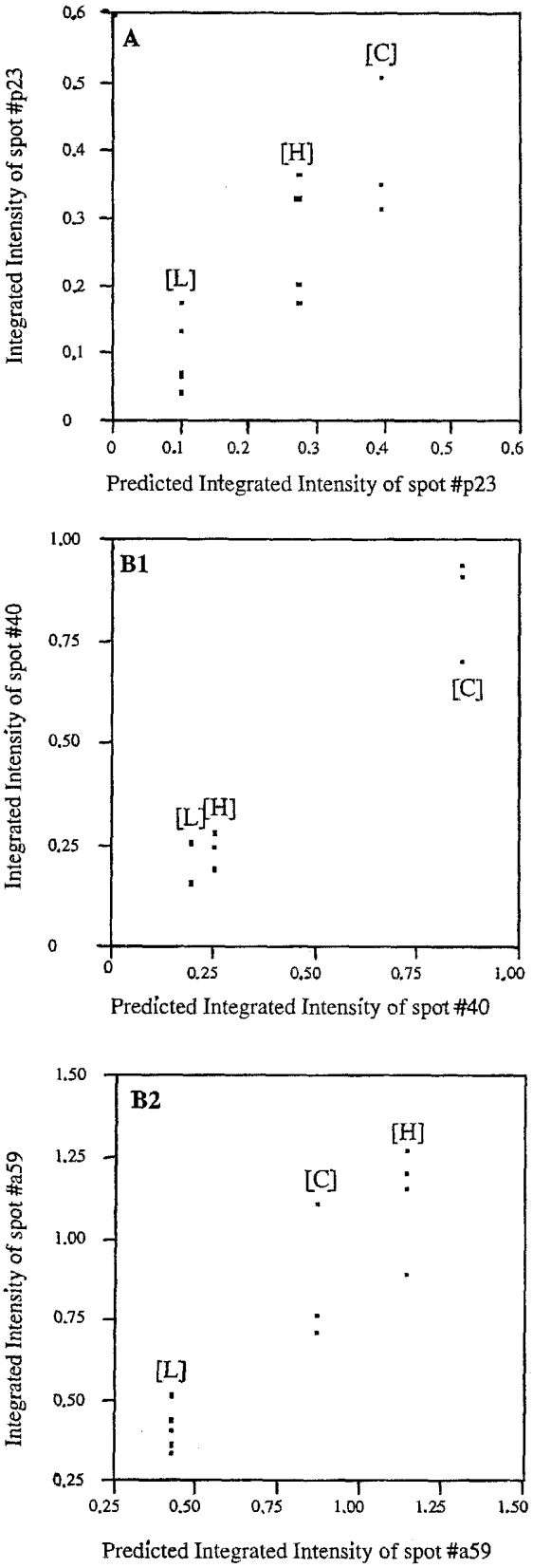


Fig. 3. – Examples of inheritance of protein amounts. Additivity (A) and non-additivity: dominance (B1) and overdominance (B2). Abbreviations C, L and H are for accessions C10, L146 and H12 respectively.

The quite low level of additivity in maritime pine (22% of the spots) contrasted with the extent of additivity observed for 2D-PAGE proteins in pea (96%, DE VIENNE et al., 1988), maize (89%, LEONARDI et al., 1987) and wheat (M. ZIVY, personal communication). Reviewing the literature about inheritance of enzyme activity and/or quantity, LEONARDI et al. (1987) pointed out that non-additivity could result from the action of distant trans-acting regulatory elements controlling protein quantity.

However, other hypothesis could be suggested: (i) the high genetic load, characteristic of allogamous forest tree species, could favour the expression of non additivity (the genetic load is most likely to be purged in domesticated species where inbred lines are usually used); (ii) because of a higher polymorphism in the pine trees compared to the strains of the crop plants, opportunity for epistasis between regulatory elements and the gene encoding the structural protein are greater in pine and may also explain the extent of non-additivity. Moreover, maritime pine accessions "C10" and "L146" originated from different geographical areas in France: the Corsica island and the coastal Landes, respectively. Provenances of these two geographical areas were been described as quite different at morphological (HARFOUCHE et al., 1995a) and biochemical levels (BAHRMAN et al., 1994; PETIT et al., 1995), suggesting genetic divergence between them. These two trees as well as their hybrid progeny where planted in the early 70's in the same field trial of the Landes region. Regarding their genetic divergence, environmental effects on protein expression could be different in both parents. The combination of the "Landes" and "Corsican" genomes in the hybrid and their interaction (epistasis, dominance) could also induce a complex regulation of protein accumulation, resulting in a high level of non-additivity. These results should be interpreted with caution since only a single hybrid was analysed. However, it is important to note that significant non-additive effects (heterosis) were observed for growth traits at a young stage in a hybridization experiment of maritime pine (HARFOUCHE et al., 1995b).

Alternatively, additivity could be the rule, but could not be detected because of the high level of heterozygosity of the parents: the phenotypes of the parents may already be the result of additivity (e.g., $[a_1+a_2]/2$ and $[a_3+a_4]/2$ for the female and male parents) and segregation of alleles into the hybrid progeny could give a pseudo-non-additivity because the phenotype of the segregating alleles is unknown.

PQL detection

PQLs controlling the accumulation of individual proteins were detected for half of the studied spots and located by interval mapping on the genetic map of maritime pine. A total of 1 to 4 unlinked QTLs were identified *(Figure 4)*. For each protein, these genomic regions explain between 9.2% to 16.5% of the observed spot intensity variation. This evidence confirms the result of DAMERVAL et al. (1994) who first reported that protein accumulation was controlled by numerous genetic factors dispersed throughout the genome. They showed that at least 70 genetic factors controlled the quantity of 42 maize proteins.

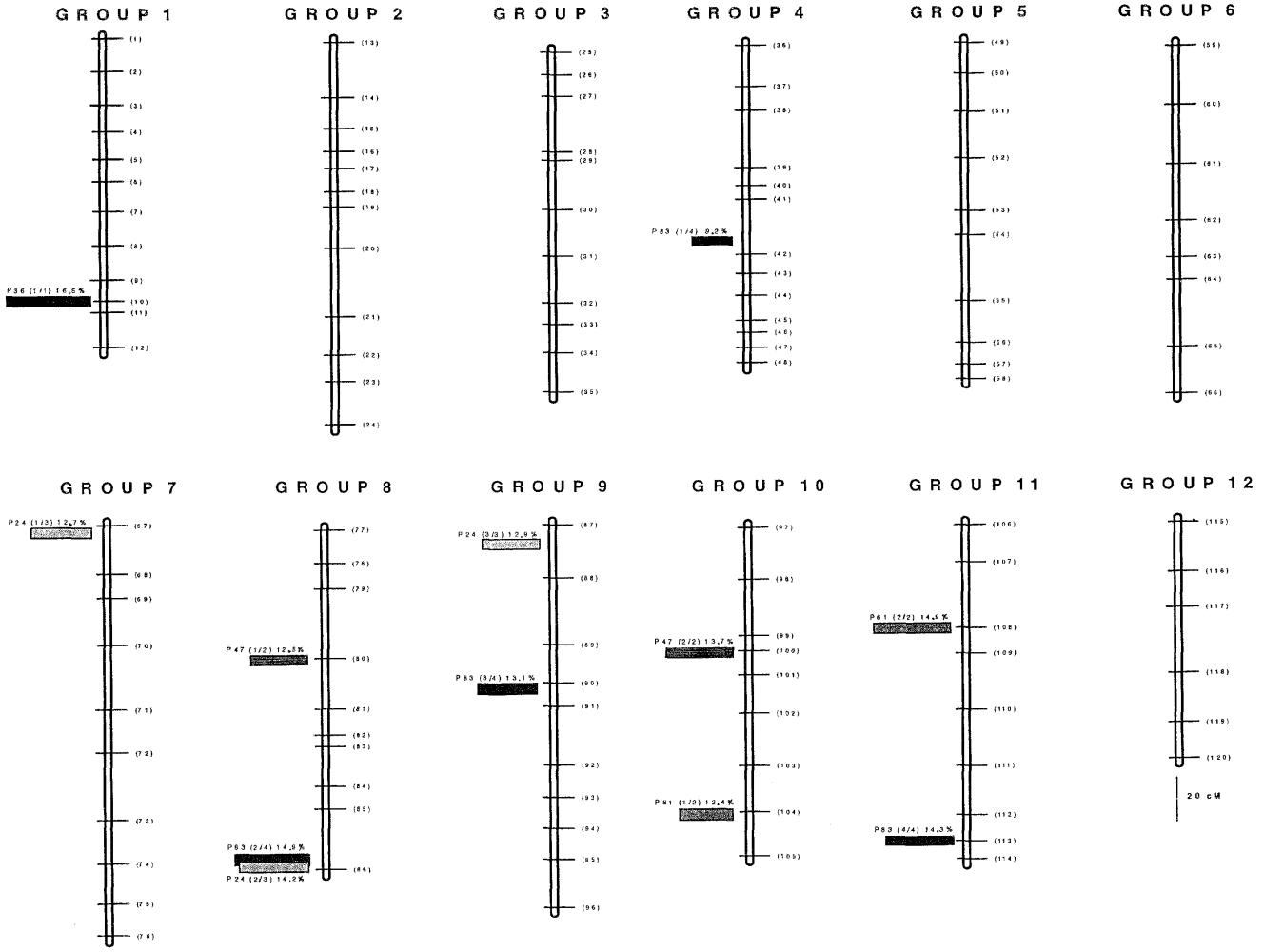


Fig. 4. – Genetic map of *Pinus pinaster* (from PLOMION et al., 1996). PQL for 5 protein spots are shown with vertical boxes, proportional to the phenotypic variations explained by each PQL.

Perspectives

Variations in protein accumulation are related to polymorphisms in gene expression. They represent an integrated level of genetic variation compared with the DNA and protein structure variation level (monogenic mode of inheritance) and may constitute an intermediary step between the gene and the variation obtained at the phenotypic level. The question arises now as to whether or not the variability of genetic expression and its consequences in terms of protein quantity variation, may also play a role in the phenotypic variability. Indeed, correlation between protein quantity and hybrid performances in maize have been reported, which might suggest that PQL could correspond to QTL of agronomical traits (LEONARDI et al., 1991). We are investigating such a "candidate protein" approach in maritime pine, for physiological traits (carbon isotope discrimination, predawn water potential, osmotic potential, stomatal conductance) responding to drought stress and PQL controlling the quantity of drought inducible proteins (COSTA et al., 1998, 1999).

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