Relation Between Protein Markers and Quantitative Traits in Maritime Pine (*Pinus pinaster* AIT.)

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Summary

Eighteen maritime pines (*Pinus pinaster* AIT.) were genotyped at 84 loci using 2-dimensional electrophoresis of total protein contained in their haploid megagametophyte. These trees were also characterised for megagametophyte weight and for 49 traits related to young and adult growth of their progenies. Three loci were associated to megagametophyte weight. The genotype of the trees at 17 loci was significantly associated to different growth traits. Among these 17 loci, there were more loci responsible for quantitative variation of polypeptide spots than what would have been expected by chance only. The significance of these associations is discussed and the relevance of 2-dimensional electrophoresis of protein for the study of quantitative traits genetics is outlined.

Key words: pine, 2-dimensional electrophoresis, QTL, growth, seed weight, megagemetophyte.

FDC: 165.3; 181.6; 232.19; 232.312.31; 537; 174.7 *Pinus pinaster*.

Introduction

The use of marker assisted selection (MAS) in breeding programs relies on the presence of linkage disequilibrium between marker loci and quantitative trait loci (QTL). Because linkage disequilibrium decreases every generation, the efficiency of marker assisted selection will quickly declines unless markers are found that are tightly linked to the QTLs, in the extreme, being the QTLs themselves (ZHANG and SMITH, 1992). Markers based on protein polymorphism may in this respect be useful, since proteins act directly on biochemical processes, and thus must be closer to the "build-up" of the phenotype (GALLAIS, 1990) than DNA. Protein variation could therefore be more informative about variability expressed at the organism level than DNA variation, and provide markers directly affecting quantitative traits.

In forest trees, several traits selected for have a low heritability, and the number of individuals on which the traits can be measured is limited. In both situations MAS is more efficient than direct selection (LANDE, 1992). Furthermore, for most forest trees, a reduction in the length of selection cycles should lead to a sharp increase in genetic gain per time unit. However, early selection has so far been hampered by the lack of reliable early selection criteria. In the case of maritime pine (*Pinus pinaster* AIT.), total height was shown to be a good predictor of the volume, given that the trees are more than 12 years old. Before that age genetic gains would be negligible because genetic correlations between height at ages 10 and 50 can be negative or nil (KREMER, 1992; DANJON, 1994). This lack of correlation is probably due to the existence of different growth phases during the lifetime of woody plants (POETHIG, 1990; LASCOUX, 1992; LAWSON and POETHIG, 1995). Both phases have been morphologically and phenologically characterised under a variety of environmental conditions (LASCOUX, 1992) but genetic differences between the phases have not been studied. 2-dimensional electrophoresis of proteins proved to be both a relevant source of monogenic and codominant markers, and an interesting technique for the development of expressed genes maps (DE VIENNE et al., 1996). In the present study, 18 maritime pine trees with contrasting growth patterns and growth abilities at mature age were studied. The trees were genotyped at 84 loci using two-dimensional electrophoresis of total proteins extracted from the megagametophytes (GERBER et al., 1993). Offspring of these trees were raised under both growth accelerating conditions and water stress. Growth related traits were assessed in all environments (LASCOUX, 1992). We propose to use this dataset to detect quantitative trait loci for seed weight and to elucidate the relationships that may exist between the protein markers genotype and the juvenile and mature growth traits.

Material and Methods

Material

Based on their growth performances and general combining abilities in progeny tests, 18 trees were chosen among the first generation of the maritime pine breeding program. They are part of the 550 "plus" trees selected for their superior shape and volume in the Landes area between 1960 and 1974. These trees were vegetatively propagated and installed in a clonal seed orchard. The 18 trees were sampled to represent the range of variability observed for growth pattern (polycyclic versus monocyclic) and growth capacity (below average, average and above average). A growth cycle is a morphological unit defined as the sequence of a sterile zone, a zone with secondary needles and a zone with long shoots and cones (DEBAZAC, 1963). Trees that regularly produce more than 1 cycle per growing season are called polycyclic. Conversely, trees that seldom or never produce more than 1 cycle are called monocyclic.

Genotyping of the 18 trees proceeded from analysing about 12 haploid megagametophytes per tree using 2-dimensional electrophoresis of the total protein extracted from this organ by denaturing conditions. A total of 84 loci, responsible for presence/absence, position shift or quantity modifications of polypeptide spots, were detected (GERBER et al., 1993).

Quantitative traits

Quantitative traits were assessed in various experiments (see *Table 1*) at both mature and juvenile stages.

Mature age

Firstly, among the 18 trees of the sample, 15 were present in a progeny test (test A, *Table 1*) planted in 1975. The following traits were measured: total height at 9 years (in 1983, noted H83) and at 13 years (in 1987, H87), circumference at breast height at 9 and 13 years (C83, C87), butt stem angle of lean at 9 years (BSA83). General combining abilities (GCA) of the mother trees for the 5 traits were estimated according to the least square method generalised to non orthogonal designs

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Table 1. – Description of the experimental tests.

Tests	Type of experiment	Age of assessments	Experimental design	Family size	Y_{ij}
A	Progeny test	9 and 13	Incomplete blocks	36 to 47	GCA*
B	Clonal test	9 to 14	Randomisation	3 to 19 (mean 10)	Clonal value
C	Progeny test	3	Randomisation	6 to 12 (mean 8)	GCA
D	Progeny test	1.2	Split-plot	50	GCA

*) General Combining Ability

(SEARLE, 1971). Secondly, an average of 10 vegetative copies of 16 of these trees were installed in a clonal seed orchard from 1962 to 1966 (test B, *Table 1*). The sum of the primary shoots (SSI) and the sum of the secondary shoots (SSII) were recorded during 4 years (1971 to 1975).

Juvenile stage

In a first experiment, open pollinated progeny of 16 of the 18 trees (test C, *Table 1*) were subjected to a water stress during the development of the bud in the second growth period. At the end of the third year, the mean height per family of the first cycle (HFC) was measured in normal and stressed conditions. In a second experiment, open pollinated progeny of 11 of the 18 trees were cultivated under growth accelerating conditions at the Stockholm Phytotron (test D, *Table 1*). During the first growth period, about 50 seedlings per family were subjected to continuous light during 9 weeks. They were then exposed to a dormancy inducing period of 13 weeks, with an 8-h photoperiod and low temperatures. The second growth period consisted of 9 weeks with a 16-h photoperiod, followed by a dormancy induction period (LASCOUX, 1992).

At the end of the first growth period, morphological traits and dry weights were measured. More specifically, the total length of the seedling (TL), the length of the longest branch (LLB), the number of branches (NB), the number of secondary needles (NNII), the number of primary needles, corresponding to the total number of internodes (NSU, number of stem unit), the number of internodes in the rosette (ROSET) and the mean length of the internodes (MSUL, mean stem unit length = TL/NSU), branch weight (BW), primary needle weight (NWI), secondary needle weight (NWII), rosette weight (RW) and stem weight (SW) were assessed. A stem unit is defined as a node plus its internode (DOAK, 1935). At the end of the second growth period all seedlings were polycyclic. Three successive cycles could be distinguished. For the i^{th} cycle, the following morphological traits and dry weights were recorded: number of branches (NBi), length of the seedling (TLi), number of internodes (NSUi), number of secondary needles (NNIIi), mean length of internodes (MSULi = TLi/NSUi), branch weight (BWi), primary needle weight (NWIi), secondary needle weight (NWIIi), stem weight (SW).

General combining abilities of the 11 mother trees for every trait were estimated with the GLM procedure of the SAS software (LASCOUX, 1992). Germination rate and 100 seed weight were measured as well for the 18 trees. Finally, the family effect, tested by an analysis of variance, was significant for all traits. Overall, 49 traits were measured on all or part of the 18 families.

Methods

Detecting Quantitative Trait Loci (QTL) implies the simultaneous study of the segregation of marker loci and of the expression of a given quantitative trait, both on the same set of individuals. In our case, the segregation of 84 loci was observed on haploid megagametophytes whereas most of the quantitative traits were measured on diploid trees. Hence, even if we still are not in a classical situation since the 18 trees of our sample were not related, QTL can be detected for the megagametophyte weight. In relation to growth and morphological traits, the effect of the diploid genotype of the trees, locus per locus, can be studied.

QTL for megagametophyte weight

For each locus and each tree heterozygous at this locus, the relationship between the megagametophyte weight and the alleles present at the locus was studied in 2 steps. In a first analysis we removed the "tree" effect and in a second analysis we took into account the fact that the presence of the same allele in 2 different trees does not correspond to the same genetic situation, the 2 trees being unrelated. The tree effect was removed by using the model:

$$P_i = m + l_{j(i)} + e_i$$

where P_i is the weight of the i^{th} megagametophyte, m stands for the mean of the trait, $l_{j(i)}$ stands for the effect of the j^{th} allele at the locus (j being a function of i), and e_i is the residue. These analyses, as well as subsequent analyses of variance, were made with procedure GLM of the SAS software (SAS Institute, 1990) using Type III sum of squares because the design was unbalanced. The homogeneity of the residual variances corresponding to the different heterozygous trees was tested using a BARTLETT test. Then, if the null hypothesis was not rejected, all trees heterozygous at the locus were considered simultaneously. Let SSA_k stand for the sums of squares of the "allele" factor (1 degree of freedom) and let SSA_k stand for the residue (n_k-2 degrees of freedom, where n_k stands for the number of megagametophytes studied for the tree k), both obtained with the previous analyses of variance. Let H be the number of heterozygous trees at the locus. The sum of squares $SSA = \sum_{k=1}^{H} SSA_k$ has H degrees of freedom and $SSA = \sum_{k=1}^{H} SSA_k$ has $N = \sum_k (n_k - 2)$ degrees of freedom. Consequently, if the locus studied and the megagametophyte weight are independent, the quotient:

$$F = \frac{SSA/H}{SSE/N}$$

follows a $F_{[H, N]}$ distribution, allowing the detection of a relation between these 2 characters. The BARTLETT test and the final analyses of variance were programmed with the SPLUS software (BECKER et al., 1988).

Relation between genotype and quantitative traits

Analysis of variance with 1 factor were carried out for each trait. The value of the j^{th} tree having the i^{th} genotype at a given locus can be written as follows:

$$Y_{ij} = m + g_i + e_{ij}$$

where m is the mean of the trait, g_i stands for the effect of the i^{th} genotype at the locus and e_{ij} is the residue. A significant effect of the locus on the trait is detected by an F test. The means corresponding to the different genotypes at the locus were compared with a TUKEY test.

False positive

When a large number of statistical tests are carried out, Type I errors are likely to occur. Let T be the total number of tests computed, and α the significance level. The expected number of test corresponding to Type I errors follows a binomial law with parameters T and α. Consequently, the probability P(K) of finding, by chance only, at least K tests significant at the α level among the T tests can be written:

$$P(K) = 1 - \sum_{i=0}^{K-1} \binom{T}{i} \alpha^i (1-\alpha)^{T-i}$$

A SPLUS (BECKER et al., 1988) program was written to calculate these probabilities.

Results

Megagametophyte weight

Altogether, 84 tests, i. e. 1 test per locus, were carried out. Three loci showed a significant effect at the 1% level, and the corresponding BARTLETT tests were non significant (*Table 2*). The effect of each locus was tested on a minimum of 7 heterozygous trees, that is on more than 70 megagametophytes. The probability to find at least 3 significant tests at the 1% level is 5% in this case. These 3 loci, No. *4209*, *3204* and *2323* belong to 3 different linkage groups and are responsible for position shift of polypeptide spots (see *Figures 2* and *3* in GERBER et al., 1993).

Table 2. – The 3 loci having a significant effect on the megagametophyte weight at the 5% level.

Locus*	Linkage group*	Number of heterozygous trees	Significance levels	
			F	Bartlett
4209	14	15	0.016	0.11
3204	1	7	0.010	0.08
2323	9	10	0.008	0.39

*) see GERBER et al., 1993

Growth traits

For each of the 49 traits, the effect of each of the 84 loci was tested; thus a total of 49 x 84 = 4116 tests were performed. The probability of observing by chance only a similar or greater number of significant tests than what was actually observed is given in *Table 3* for different significance levels. We decided to consider the tests with a significance level smaller than 0.4%, because these tests would have been significant by chance with a probability of only 0.3% (*Table 3*). We discarded the extreme cases where a genotype was only represented by a single tree significantly different of all other genotypes. The 22 final tests obtained are given in *table 4.* Thirteen traits and 17 loci are involved.

In order to know whether these 17 loci could be considered as a random sample of the 84 loci or whether they were preferentially drawn from 1 of the 3 groups to which the 84 loci belonged (position shift, quantity variation or presence/absence of polypeptide spots) we calculated the number of loci we would have theoretically obtained had the 17 loci been chosen at random (*Table 5*). The G test (SOKAL and ROHLF, 1981) with 2 degrees of freedom, corresponds to a significance level of 5%. The 17 loci were thus not the result of random sampling. Among them, there was an excess of loci responsible for quantity variation and, consequently, a deficit of loci corresponding to position shift.

Table 3. – Probabilities of Type I errors ($P(K)$) in the detection of loci related to quantitative traits.

Significance level retained (α)	Number of significant tests observed	Probability of Type I error, $P(K)$
0.005	33	0.007
0.004	29	0.003
0.003	24	0.002
0.002	17	0.005
0.001	8	0.058

Discussion

Megagametophyte weight and markers

Surprisingly, given the genetic structure of the data, we detected 3 loci related to seed weight. This result can be explained by the presence of QTL linked to the markers or by the involvement of the markers themselves in the trait, a strong hypothesis which has to be suggested with caution. The 18 trees of our sample were selected in different parts of the Landes area, and are therefore probably unrelated. Because the Landes area can be considered as a panmictic population the first possibility, which supposes a large amount of linkage disequilibrium in our sample seems unlikely, unless (i) natural selection acted strongly on seed weight, and (ii) phenotypic selection of the trees makes our sample non representative. Evidence for a selection acting on seed weight is weak. In maritime pine, the megagametophyte represents 37%, the embryo 7% and the seed coat 56% of the total weight of the seed. Maternal effects on the weight of the 3 parts of the seed were very important, but effect on subsequent growth vanished rapidly (GUIGNARD, 1983; KREMER et al., 1991). Phenotypic selection of "plus" trees is unlikely to have generated enough linkage disequilibrium to explain our results.

From an evolutionary point of view, because the increase of seed size implies a metabolic cost for the mother, but also constitutes a costless advantage for the father, the parents have conflicting interests. Alleles at loci controlling seed size will be selected for if they increase the fitness of the seedlings without diminishing too much the number of seeds produced by the mother (HAIG and WESTOBY, 1991). The absence of a strong selection towards an optimal seed size could be an explanation to the observed variability in seed size (TEMME, 1986), notably in conifers (SORENSEN and CAMPELL, 1985). Because the adaptative value of seed weight is not easy to define, the presence of a stable linkage disequilibrium between QTL and close genetic markers is unlikely to be the result of a "hitchhiking" effect (THOMSON, 1977, cited in MUONA, 1982). In any case, if any selection, it would act on the haploid level.

Consequently, complete linkage or identity of markers and QTLs appears to be the simplest interpretation of our observations. However, this hypothesis is strong and should be further verified. A pleiotropic locus acting on the polypeptides corresponding to the marker loci and on processes related to seed weight would permit to explain the observed effect. Alternatively, these polypeptides could also themselves be involved in seed weight acquisition, and in any case, are a fraction of this weight. Two of the 3 loci show a remarkably

Table 4. – Loci with significant effects on different traits.

Tests (see Table 1)	Trait (Number of families)	Locus[15]	Linkage group[15]	P[16]	Differing genotypic classes[17]
A	H83[3] (15)	*4406*	6	0.0006	$q_2q_2+q_2a<q_1q_2+q_1q_1$
	H87[3] (15)	*4406*	6	0.0017	$q_2q_2+q_2a<q_1q_2+q_1q_1$
		4305	15	0.0016	aa+pp<pa
		4202	15	0.0016	$q_2q_2+q_1q_1<q_1q_2$
	BSA83[4] (15)	*3206*	3	0.0022	$q_1q_2<q_2q_2$
B	SSII[5] (16)	*3111*	10	0.0015	pa<pp
C	HFC[6] (16)	*2428*	*	0.0003	$v_1v_2<v_2v_2$
D[1]	NB[7] (9)	*2322*	12	0.0028	$q_2q_2<q_1q_2$
		3227	*	0.0028	aa<pa
	BW[8] (9)	*2421*	*	0.0014	$q_2q_2<q_1q_2$
		3418	6	0.0006	pa<pp+aa
D[2]	NB1[9] (11)	*1215*	*	0.0023	$q_2q_2+q_1q_1<q_1q_2$
	NNII2[10] (11)	*4101*	*	0.0008	$v_1v_2<v_1v_1$
	MSUL3[11] (10)	*1410*	*	0.0032	$q_1q_1<q_1q_2$
		2210	11	0.0032	$q_1q_1<q_1q_2$
		4211	5	0.0031	pp<pa
	NWII1[12] (11)	*2322*	12	0.0012	$q_2q_2<q_1q_2$
		3227	*	0.0012	aa<pa
		2421	*	0.0005	$q_2q_2<q_1q_2$
		3418	6	0.0033	pa+pp<aa
	NWI2[13] (11)	*3309*	17	0.0014	pp<pa
	BW3[14] (11)	*2215*	*	0.0001	pp<aa<pa

[1]) First growth duration
[2]) Second growth duration
[3]) Heights at 9 and 13 years
[4]) Butt stem angle of lean at 9 years
[5]) Sum of secondary shoots
[6]) Height measured after a water stress
[7]) Number of branches
[8]) Weight of branches
[9]) Number of branches on the first cycle
[10]) Number of secondary needles on the second cycle
[11]) Mean lenght of the internodes on the third cycle
[12]) Weight of the secondary needles of the first cycle
[13]) Weight of the primary needles of the second cycle
[14]) Weight of the branches of the third cycle
[15]) Loci and linkage groups are given in GERBER et al. (1993)
[16]) F test level
[17]) TUKEY test, 5% p and a alleles: presence/absence of the polypeptide spot
v_1/v_2 alleles: first/second position of the spot
q_1/q_2 alleles: small/larger quantity of the spot
*) Unmapped locus

large amount of polymorphism (loci No. *4209* and *2323*, with 4 and 6 alleles, see GERBER et al., 1993). As for soybean (DIERS et al., 1992), QTL for protein or lipid rates or any other seed trait could be easily found in gymnosperms.

Growth traits

In gymnosperms, the development of megagametophytes is completed before fertilisation, and this organ has therefore a

Table 5. – Comparision of the distribution of loci according to their type.

Samples	Number of loci according to their type:		
	Position	Quantity	Presence/absence
84 loci	30	22	32
17 loci	2	8	7
17 loci at random	6.07	4.45	6.48

storage function during embryo development, germination and seedling emergence (MISRA and GREEN, 1991). Not surprisingly, since the genome present in the megagametophyte is exactly identical to "half" the genome of the embryo in the same seed, many proteins present in the embryo are also present in the megagametophyte (MISRA and GREEN, 1990; HAKMAN et al., 1990; GIFFORD et al., 1991). During the first days of germination, the embryo is independent and uses its own reserves, but the embryo will consume sugars and amino-acids of the megagametophyte later on (GROOME et al., 1991). The megagametophyte proteins have thus a direct influence on seedling development. However, the relationship between the diploid genotype of the mother tree, and the quantitative traits measured on its half-sib progenies appears less obvious.

It would be interesting to understand how a particular allele influences the expression of a quantitative trait. The 18 trees of our sample come from an allogamous, panmictic population in which linkage disequilibrium is likely to be weak (MUONA, 1982). Neither natural selection nor the mass selection performed on growth traits in the first breeding generation are likely to have generated a detectable amount of linkage disequilibrium in such a limited sample. There could be tightly linked loci in the genome that would maintain a relationship between a marker and a quantitative trait, but this kind of relationship can be preserved in an allogamous population only with strong epistasis or tight linkage (HASTINGS, 1989).

As in the case of seed weight, but again, with caution, we could alternatively hypothesised that the loci have a direct effect on the traits. This is a strong and somewhat audacious hypothesis which should be checked in independent experiments. Among the 17 loci concerned by the associations, the proportion of loci responsible for quantity variation of polypeptide spots was higher than what would be obtained by chance (*Table 5*). Furthermore, the presence/absence variation could also express quantity modification if the lower quantity is under the level of detection. If this direct effect is possible, it has to be assumed that the variability observed in the megagametophyte is also present in the meristem, where growth processes are initiated. Likewise, the genetic factors must act additively in both a haploid (megagametophyte) or diploid (tree) context. In maize, few cases of non-additivity for protein quantities could be detected when comparing 2-dimensional electrophoresis of hybrids and parental lines (LEONARDI, 1989). However the comparison of profiles from different tissues of the same ploidy level revealed that 70% of the quantitative variation and non-additivity cases were organ specific (LEONARDI, 1989).

The comparison of electrophoretic profiles of vegetative buds and needles of the 18 pines of our sample revealed 82% of common spots but the quantitative variation of these spots across organs is important (BAHRMAN and PETIT, 1995). Moreover, as in maize (LEONARDI et al., 1988) or in mice (KLOSE, 1982) organ specific proteins seem to be more variable than non specific ones (BAHRMAN and PETIT, 1995). Nevertheless, regulation processes must play an important role during growth. The application of cytokinin, that stimulates bud formation, on petunia callus (RENAUDIN et al., 1991) or on spruce embryo (STABEL et al., 1990) strongly modifies the expression level observed on 2-dimensional electrophoresis protein profile. The study of maturation processes in larch similarly suggests that expression rates of specific protein are changing between the juvenile and mature stages (GREENWOOD et al., 1989; HUTCHISON et al., 1990). If relationships between variations observed in megagametophytes and quantitative traits were confirmed, it would be interesting to compare the expression of protein in these different organs, and particularly their regulation.

Conclusion

In conclusion, our results indicate that, even with small samples, it is possible to correlate the variability of protein expressed in haploid megagametophyte to growth related traits. Variation induced by regulators seems to be often implied in these effects.

Even though the use of MAS for organisms such as forest trees that are entering domestication may be questionable (STRAUSS et al., 1992), markers can nonetheless help in understanding the genetic basis of juvenile-mature correlations, which may well be a prerequisite to the establishment of reliable early selection procedures. To detect QTLs, DNA markers are certainly more suitable, since dense genomic maps were recently constructed for maritime pine (PLOMION et al., 1995). However 2-dimensional electrophoresis is a very promising technique if the aim is to understand the quantitative traits expression. For instance, quantifying the activity of regulator elements could be very useful to explain variability of quantitative traits. HEDRICK and MCDONALD (1980) supported this point of view when they distinguished between "producing" and "controlling" loci, the latter acting on the former. If "controlling" loci are acting early in processes, which is likely, a modification of their control should have an important effect on the resulting trait.

Our observations agree with previous results obtained on maize, showing that quantitative differences of proteins are correlated with differences in performances (DAMERVAL et al., 1987; LEONARDI et al., 1991). Pines could constitute an interesting material for this kind of studies. However, if many traits and many loci are available, false positive associations are very likely to be detected. It is therefore important to choose properly the traits to be studied. GOTTLIEB (1984) suggests that the analysis of growth can provide us with components controlled by one or few genes. The dissection of growth into simpler components, initiated by KREMER (1985) should be a good way to get closer to the basic plant functioning, and especially to the meristem. Apical meristem contains the information which allows all parts of a plant to develop and the study of genes expressed in this organ is currently under progress (MEDFORD et al., 1991).

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Micropropagation of *Cupressus sempervirens* L. and *Chamaecyparis lawsoniana* (A. MURR.) PAR.

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Abstract

Shoots from 18-month-old seedlings of *Cupressus sempervirens* and *Chamaecyparis lawsoniana* were established *in vitro* on modified MURASHIGE and SKOOG medium. Proliferation of axillary shoots occurred without addition of benzyladenine, although a significant increase in numbers of shoots resulted on addition of 0.001 mg.l^{-1} to 1.0 mg.l^{-1} benzyladenine. Following conditioning on a growth regulator-free medium for 28 days, 95% of *C. sempervirens* shoots rooted on ½ strength medium containing 1% sucrose and 0.5 mg.l^{-1} indole butyric acid. Similar levels of rooting were recorded with *Chamaecy-*

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