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Genetic dissection of height in maritime pine seedlings raised under accelerated growth conditions

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Abstract Random Amplified Polymorphic DNAs (RAPDs) were used to investigate quantitative trait loci (QTL) for traits related to height growth on 126 F_2 seedlings of maritime pine (*Pinus pinaster* Ait). The haploid megagametophyte was used to determine the maternal genotype of each F_2 individual. The seedlings were raised for 2 years in a greenhouse under accelerated growth conditions consisting of intense fertilization combined with continuous light treatments. Total height was measured at different developmental stages, and height growth components were measured after the second growth period. QTLs were identified for each trait. For total height, QTLs of different developmental stages were located on distinct linkage groups. However, rather than a complete temporal change in QTL expression, our results showed that maturation may induce a progressive shift of the genetic control of height growth. This may provide an explanation for a low juvenile-mature phenotypic correlation previously reported for height. Height growth components related to the initiation (controlled by the apical meristem) and elongation of shoot cycles (controlled by the subapical meristem) were mapped to different chromosomes, suggesting that the activity of these meristems is controlled by separate genetic mechanisms.

Key words *Pinus pinaster* · Maturation · Height · RAPD · QTL · Megagametophyte

Introduction

Perennial organisms such as forest trees pass through a series of developmental phases during their life (Sussex 1976; Poethig 1990). These phase changes are related to profound morphological, biochemical and physiological changes, mostly associated with maturation of the plants (reviewed by Haffner et al. 1991). In addition to the obvious transition from vegetative to reproductive structures, progressive changes in vegetative characteristics occur during the lifetime (Hackett and Murray 1993). In *Pinus* species, seedlings produce two kinds of stem units during the first 2 years of growth. In the first season, elongation of the stem occurs in the free growth phase, which ends when a terminal bud is produced during the second season. In the following seasons, the plant produces one or two shoot cycles. The transition between free growth and fixed growth is an important change in *Pinus* species. Acceleration of this phase change and hastened maturation under optimal environments has been used to identify early selection criteria of adult performance in the first season of growth (Bongarten and Hanover 1985; Greenwood 1987; Lascoux et al. 1993a). Lascoux et al. (1992, 1993b) and N'Guyen et al. (1995) investigated the ontogenetical aging of maritime pine seedlings exposed to continuous light. Their results showed that first-season seedlings raised under 18 weeks of continuous light moved from a free to a cyclic growth and exhibited morphological traits characteristic of mature trees. In addition, they showed that seedlings demonstrated a degree of maturation proportional to the number of shoot cycles achieved during this period.

Although morphological changes have been well described (e.g. Riding 1972; Romberger and Gregory 1977), the physiological, biochemical and genetic basis of maturation processes remain elusive (Sussex 1976; Hackett 1985; Greenwood et al. 1989; Poethig 1990). Investigations have been carried out to identify differential expression of the genome at distinct developmental stages, and gene expression was assessed using various techniques such as DNA methylation (Wescott 1987; Greenwood et al. 1989), isolation of mRNA (Hutchison et al. 1990; Hutchison and Greenwood 1991), isolation of rRNA (Zimmermann et al. 1985) or two-dimensional gel electrophoresis of proteins (Stabel et al. 1990). A differential expression of the same genes was usually observed in the juve-

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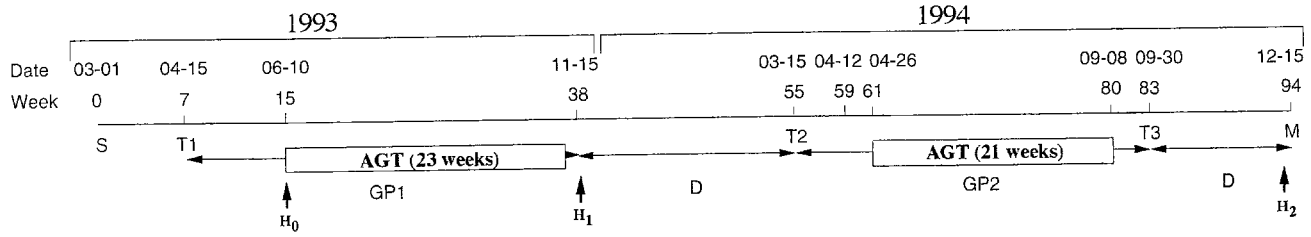

Fig. 1 Description of the experiment for the F_2 family Measurements of total height at different periods are indicated by H_0, H_1 and H_2. *S* Sowing date in the growth chamber, *T1* last transplantation date from germination box to greenhouse. *GP1* first growth period, *AGT* application of the accelerated growth treatments (continuous light and intense fertilization), *D* dormancy induction and dormancy period under natural condition, *T2* transplantation to bigger pots, *GP2* second growth period, *T3* transplantation date from greenhouse to nursery, *M* measurement of height growth components

nile and mature phases, with few or no genes being found that were expressed exclusively at a single stage.

In the investigation described in this paper, we used genetic mapping and quantitative trait dissection analysis (Tanksley 1993) to localize the genetic factors (quantitative trait loci, QTLs) that control the variation of traits related to height growth during the maturation of maritime pine seedlings. The first objective was to study the stability of QTLs for total height at different developmental stages: the juvenile stage (before applying an accelerating growth treatment) and more mature stages (after one and two periods of growth under continuous light). Shoot growth is a complex integrated trait that could be affected by different genes in different years. Because of the low correlation between young and mature plants for total height (e.g. Lambeth et al. 1983; Kremer et al. 1991; Danjon 1994) and the decrease in correlations between annual height increments when the lag between corresponding growing seasons increases (e.g. Kremer 1992; Hodge and White 1992), it has been suggested that the genetic control of height growth could be controlled by different sets of genes at different ages (Kremer et al. 1991; Hodge and White 1992; Kremer 1992). Here we present some of the first mapping data showing evidence for a change in the genetic control of early growth in forest trees. Another objective was to study the genetic architecture of height growth components (number of stem units and mean stem unit length) and to determine whether the same or different QTLs control these traits under accelerated growth conditions.

Materials and methods

Plant material and accelerated growth conditions

Selfed seeds were obtained from a single hybrid tree (accession H12) derived from a cross between trees from Corsican (accession C10) and Landes (accession L146) provenances of maritime pine. Compared with other *Pinus* species, maritime pine is less affected by inbreeding, especially for the percentage of filled seeds and general vigor (Durel et al. 1996). In addition, a comparative mapping analysis between two genetic maps constructed with megagametophytes of selfed and open-pollinated seeds did not reveal any genetic load in the hybrid tree (Plomion et al. 1995a). Therefore, this F_2 progeny constituted a unique opportunity for quantitative trait dissection studies in forest trees. The seeds were sown in 67-well format Hiko plates (10 cm deep) containing a 1:2 ratio of vermiculite: peat potting mix. They were treated weekly with a fungicide and watered biweekly. Each plate was placed in a germination box located in a growth chamber with a 18 h/6 h photoperiod and 20°C day/10°C night temperature regime. At 4 weeks post-germination, seedlings were transplanted into individual pots (1.5 l) containing a mixture of white sand, peat and bark (1/1/1), and placed in a greenhouse. The temperature in the greenhouse was maintained near 25°C. The pots were randomly installed on a surface of 6 m^2 and 10 m^2 during the first and second growth period, GP1 and GP2, respectively (Fig. 1). The experiment was surrounded with two border lines and was set up in the middle of the greenhouse in order to decrease the longitudinal heterogeneity due to ventilation on one side of the greenhouse and cooling pad on the other side.

A continuous light treatment, i.e. natural light during the day and artificial light furnished by Sodium lamps (SGR 200, Philips) during the night (average photon flux density of 150 µmol m^{-2} s^{-1} at seedling level) was applied 8 weeks after the start of GP1 (Fig. 1). The plants received daily individual automatic watering combined with a high level of fertilization (0.2 g/l of Hakaphos: N=2.14 m*M*/l, P=0.65 m*M*/l, K=0.77 m*M*/l, enriched in MgO and micronutrients) A higher concentration (0.36 g/l of Hakaphos: N=3.42 m*M*/l, P=1.03 m*M*/l, K=1.23 m*M*/l) was used from weeks 26 to 38. After a period of 23 weeks under continuous light, plants were placed under natural conditions (short day) to induce dormancy. Just before the second growth period (GP2), the plants were transplanted to bigger pots (4 l) filled with a 2:1 ratio of bark:peat. They received 0.25 g/l of Hakaphos (N=2.67 m*M*/l, P=0.80 m*M*/l, K=0.96 m*M*/l, enriched in MgO and micronutrients). Continuous light treatment was applied again for 21 weeks during GP2 (Fig. 1). Seedlings of the same progeny were also raised under natural conditions and served as a non-accelerated control. These individuals were not genotyped.

Quantitative traits measured

Total height was first measured on week 15 (H_0), i.e. before the first accelerated growth treatment was applied (Fig. 1), on week 38 (H_1) and on week 92 (H_2), i.e. after one and two periods of growth under continuous light, respectively. The following height increments were computed from these data: $\Delta H_{0,1}=H_1-H_0$; $\Delta H_{1,2}=H_2-H_1$. From weeks 92–94, the plants were uprooted and height growth components measured for each shoot cycle of GP2. A shoot cycle or shoot growth is the elongation that occurs from a terminal bud break to terminal bud formation. Whorls of branching are initiated when buds are formed. Each cycle C_j was divided into a fertile (LF_j) and a sterile zone (LC_j-LF_j), as defined by Debazac (1963). The sterile zone was situated at the begining of the shoot cycle and defined as the section that bears only scales or primary needles. The fertile zone was located above the sterile zone and was defined as the section of the shoot between the first secondary needle and the top of the cycle; this region only bore secondary and primary needles. Components measured were: cycle length (LC_j, measured from the base of the whorl of secondary shoot j up to the whorl j+1), fertile zone length (LF_j), number of stem units in the fertile zone (NSU_j, i.e. number of primary and secondary needles in the fertile zone) and mean stem unit length ($MSUL_j$=

LF_j/NSU_j). Additional seedling traits were measured: germination date (GERMD, from week 0–7), hypocotyl length (Lhypo, week 7) and megagametophyte weight (MW, week 0).

Data analysis

Analysis of variance (ANOVA) and covariance (ANCOVA) were performed using the SAS GLM procedure type III sum of squares (SAS Institute). The following ANOVA model was used:

$$Y_{ij}=\mu+I_i+C_j+\varepsilon_{ij},$$

where Y_{ij} is a trait measured on the j^{th} cycle of the i^{th} individual, μ is the overall mean, I_i is the individual effect, C_j is the cycle effect and ε_{ij} the error term. Autocorrelation of the residual effects between adjacent cycles, $r(\varepsilon_{i,j},\varepsilon_{i,j+1})$, were generally not significant for all the components except for LF. LC and NSU between cycle 3 and 4.

The ANCOVA model used for each cycle j was:

$$Y_{ij}=\mu+\beta_j{}^*Z_{ij}+\varepsilon'_{ij},$$

where Z_{ij} is the covariate of Y_{ij} and ε'_{ij} the error term. This ANCOVA model allow us to adjust the total length (LC_j) and the length of the fertile zone (LF_j) to the number of stem units (NSU_j) in order to evaluate pure growth independently from NSU_j (initiation-related trait). Adjusted variables LC_j^{ad}, LF_j^{ad}, were derived from this analysis.

In the text significance levels of phenotypic correlation coefficients (ρ) are indicated by ***, ** and * for $P<0.001$. $P<0.01$ and $P<0.05$, respectively.

Randomly amplified polymorphic DNA (RAPD) analysis and map construction

The limitation imposed by the dominant mode of inheritance of RAPD markers (Williams et al. 1990) for genetic mapping in an F_2 family was resolved by using the megagametophyte (haploid tissue in seeds that is derived from the same megaspore that gives rise to the maternal gametes) of each F_2 individual. Each megagametophyte was collected from each seedling 2 weeks after germination, just before the seed coat would be cast off. Sixty-two of them had been previously genotyped with 436 RAPD markers and used for mapping the F1 hybrid tree (Plomion et al. 1995a,b). In the present study, the mapping sample was extended to 126 individuals. The additional 64 individuals were genotyped for a subset of 120 RAPD markers on the genetic map. DNA extraction and RAPD reactions were performed as described elsewhere (Plomion et al. 1995a,b). Linkage analysis with 126 informative gametes was performed using the MAPMAKER/EXP V3.0 software (Lander et al. 1987). Linkage groups were obtained by choosing 0.30 as the maximum recombination fraction and 4.0 as a minimal LOD score value. The marker order was estimated using an interval support > 3.0 (Keats et al. 1991). Recombination fractions were converted to map distances using the Haldane mapping function.

Marker-trait associations

MAPMAKER/QTL (Lincoln and Lander 1990) was used to estimate QTL parameters and test statistics at 2-cM intervals between every marker pair, and to determine the most probable location for the QTL. Approximate thresholds of interval mapping tests for QTL detection were computed for each linkage group with a Fortran program provided by A. Rebaï (Rebaï et al. 1994). For a per-linkage group type-I error of $P<0.01$. LOD significant thresholds varied between 2.2 and 2.4. The variations were due to the variable density of markers within each linkage group. With a probability of $P<0.05$ that a false positive is declared at the chromosome level, LOD score thresholds varied between 1.5 and 1.6 for individual tests. This more relaxed LOD threshold corresponded to the highest chance of reporting false positives but allowed us to reduce the Type-II error. Therefore, we chose the 5% probability level as an appropriate level for judging significance of the marker-trait association. For each LOD peak, we determined the LOD 1.0-support interval (that is, the region in which the LOD score remains within 1.0 unit of the peak). For all detected QTLs, the percentage of phenotypic variation explained and the shift in trait value in phenotypic standard deviations are reported. Multipoint estimates of the total variation explained by the mapped QTLs were obtained by interval mapping with MAPMAKER/QTL (MMQ) and by multiple regression using SAS GLM procedure (SAS Institute). Statistical tests for two-locus epistasis were performed using the SAS GLM procedure (SAS Institute) to test for the interaction term. A minimum P value of 0.001 was used to declare a significant interaction.

Results

Linkage map construction

A total of 120 markers were used to construct a linkage map. Twelve linkage groups corresponding to the haploid number of chromosomes of *Pinus pinaster*(2n=2x=24) were obtained using a LOD≥4.0 and recombination fraction ≤0.30 (Fig. 2). These markers were amplified from 62 RAPD primers. When genotyping with RAPD markers, Plomion et al. (1995b) observed that each primer amplified an average of three polymorphic markers. Thus, these primers were chosen to maximize the genome coverage with the minimal number of RAPD reactions. With 44% of the primers used to produced the first saturated maritime pine genetic map (i.e. 62 out of 142 arbitrary primers) we were able to cover 95% of the previous map (Plomion et al. 1995b). On the basis of the simulation study of Darvasi et al. (1993) the average density of the map, 1 marker every 17.6 cM, was considered sufficient for marker-trait association analysis. No distortions from the expected 1:1 Mendelian segregation ratio was reported ($\alpha=0.01$). The order of the selected markers was well-conserved when compared to our previous map of maritime pine.

QTL analysis

A normal distribution of phenotypes is an inherent assumption for interval mapping. Only two traits (GERMD and H2) presented a slight deviation from the normal distribution on the basis of the Shapiro-Wilk (1965) W statistics. The non-parametric interval mapping approach (Kruglyak and Lander 1995) was tested for these two traits, but it yielded identical results as the parametric method (Lander and Botstein 1989). Therefore, we only present statistical tests obtained with the parametric approach. Significant marker-trait associations are listed in Table 1. The proportion of phenotypic variation explained by each QTL (%VAR in Table 1) was calculated with MMQ and by ANOVA. We followed the ANOVA approach used by Edwards et al. (1987) involving comparison of the phenotypic means for the alternative genotype classes at individual marker loci. Higher values were always obtained by the interval mapping approach than by the linear regression method. The significant correlation ($\rho=0.70$**) between

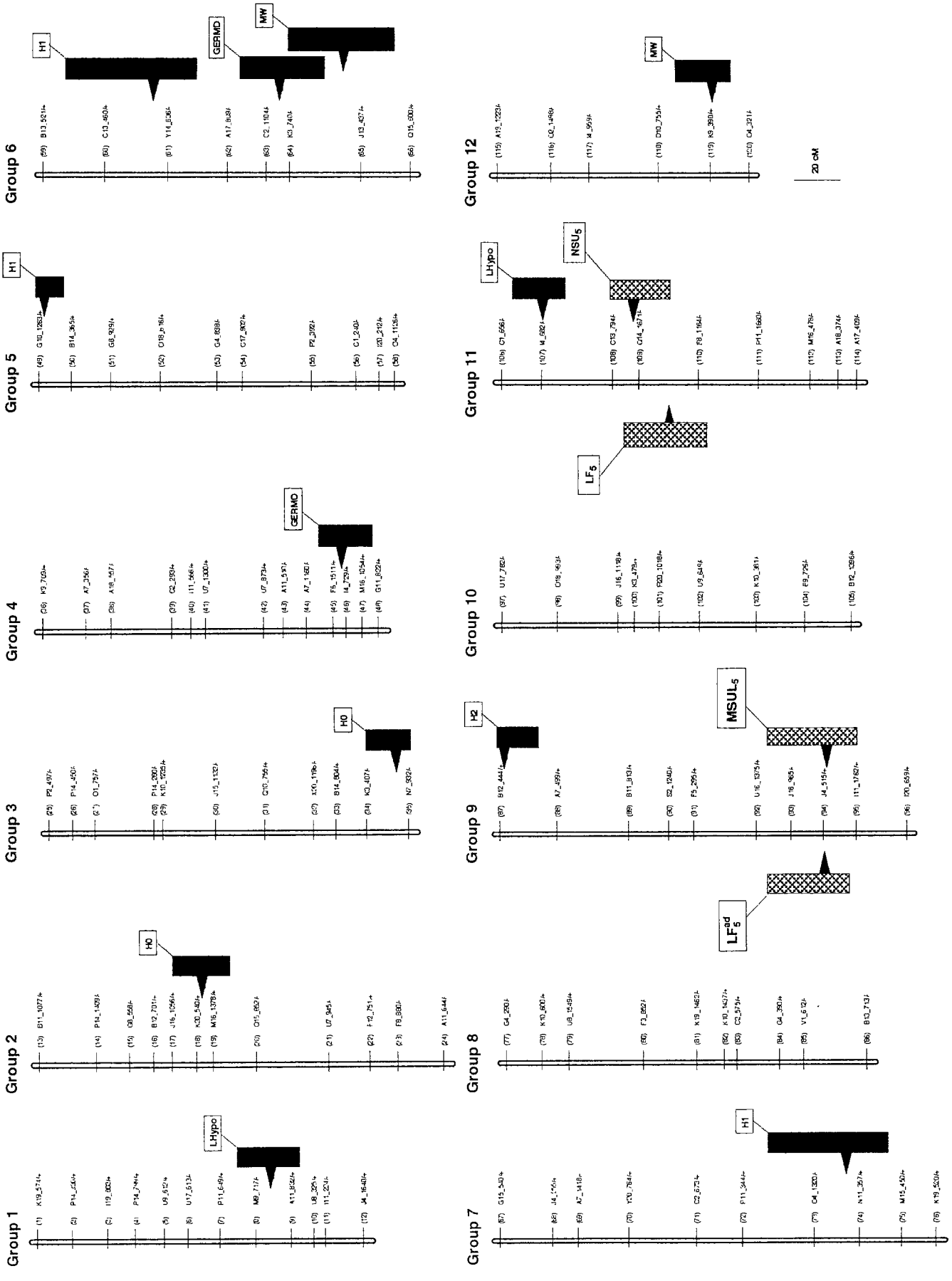
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Group 7
Group 8
Group 9
Group 10
Group 11
Group 12
LHypo
H0
H0
GERMD
H1
H1
GERMD
MW
LHypo
NSU5
LF5
H2
MSUL5
LF5ad
MW
20 cM

Table 1 QTL summary for seedling traits and height growth components of cycle 5 Listed are the LOD peak values, the location and percentage of phenotypic variation explained by each QTL and the source of the beneficial allele. Linkage group refers to the map in Fig. 2

Traits[a]	Linkage group	Marker interval	LOD peak	QTL position (cM)[b]	LOD 1.0 support interval (cM)[c]	% VAR MMQ[d]	R^2_mMMQ[e]	% VAR GLM[f]	R^2_mGLM[g]	D[h]
Seedling traits										
MW	6	64–65	2.0	26.9	0/16	8.6		6.8		0 50C
	12	119–120	1.6	0.2	14/10	6.0	12.6	5.5	10.4	0.46L
GERMD	4	45–46	4.2	4.0	6/12	15.0		13.4		0.70C
	6	63–64	4.4	8.0	12/16	17.0	30.0	13.6	24 0	0.80C
LHypo	1	8–9	2.4	6.0	10/4	9.5		7.3		0.53L
	11	107–108	3.7	0.0	14/–24	12.7	20.9	12.4	17.2	0.70L
H_0	2	18–19	1.8	2.4	12/8	7.0		6.5		0.58C
	3	34–35	2.9	14.5	0/OFF END	12.0	17.0	10.8	15.0	0.67C
H_1	5	49–50	1.7	0.1	OFF END/–6	6.2		6.2		0 48L
	6	60–61	2.7	14.0	18/14	11.5		8.0		0.62L
	7	73–74	1.6	16.2	24/16	6 5	21.0	5.5	17.5	0.50L
H_2	9	87–88	2.6	0.4	OFF END/–10	10.0	10.0	9.5	9.7	0.63L
Height growth components of cycle 5										
NSU_5	11	108–109	4.0	10.0	10/16	20.4		16.0		0.82C
LF_5	11	109–110	3.6	13.8	8/12	19.6		14.5		0.79C
$MSUL_5$	9	94–95	1.6	0.1	26/14	6.0		5.9		0.51L
LF_5^{ad}	9	93–94	2.6	10.0	10/12	11.8		9.9		0.63L

[a] MW, Megagametophyte weight; GERMD, germination date: LHypo, hypocotyle length; H_0, H_1 and H_2, see definition in Materials and methods; NSU_5, number of stem units; LF_5, length of the fertile zone; $MSUL_5$, mean stem unit length; LF_5^{ad}, length of the fertile zone adjusted to NSU_5

[b] Most probable QTL position corresponding to the LOD peak (in centiMorgans from the leftmost marker of interval)

[c] Interval over which the position of the QTL is at most ten times less likely than the peak position. From left to right: distance (in centiMorgans) from the left marker and distance from the right marker (a negative number is given for the extreme right limit within the marker interval)

[d] Percentage of the phenotypic variation explained by each peak with MAPMAKER/QTL

[e] Multipoint estimates of the percentage of phenotypic variation explained by the mapped QTL, obtained with MAPMAKER/QTL

[f] Percentage of the phenotypic variation explained by the closest marker using PROC GLM (SAS)

[g] Multipoint estimates of the percentage of phenotypic variation explained by the closest RAPD marker of the marker interval, obtained using PROC GLM (SAS)

[h] Difference between the two QTL allele effects at the closest RAPD marker of the marker interval, expressed in phenotypic standard deviations. L (Landes) and C (Corsican) indicate the source of favorable allele causing an increase in the phenotypic mean

the map distances from the marker used in the ANOVA and the difference of %VAR values of both methods clearly indicated a higher efficiency of the interval mapping approach. For the same reason, simultaneous multilocus estimates of the proportion of phenotypic variation explained by the joint action of the QTLs obtained by multipoint interval mapping with MMQ (R^2_mMMQ in Table 1) were higher than those obtained by multiple linear regression using PROC GLM (R^2_mGLM in Table 1). In the multiple linear regression procedure, observations with missing genotypic data were discarded from the analysis, resulting in smaller sample sizes. This could also explain the differences in results obtained with both methods. Hence, when comparing individual and combined effects of the QTLs, we only considered the values obtained with MMQ.

Seedling traits

Associations were found between RAPD markers and all seedling traits (Table 1). One to 3 QTLs were mapped per trait (Fig. 2). For H_0 (total height before applying the accelerated growth treatment), 2 QTLs were found in groups 2 and 3, and they accounted for 7% and 12% of the phenotypic variation of the trait, respectively. A multilocus model explained 17% of the variation. For H_1 (total height measured after one period of growth under continuous light) 3 QTLs were found in groups 5, 6 and 7. They accounted for 6.2%, 11.5% and 6.5% of the phenotypic variation of the trait, respectively, and a multilocus model

Fig. 2 Genomic linkage map of maritime pine (12 linkage groups established with a LOD≥4.0 and θ≤0.30) and location of putative QTLs. RAPD fragments are identified by a sequential code (1–120) followed by the OPERON primer name. RAPD fragments within a particular primer are named according to the estimated size in base pairs. The name includes the grandparental origin (+ denotes markers inherited from the Corsican grandparent, - denotes markers inherited from the Landes grandparent). *Boxes* to the *left* or *right* of linkage groups represent the LOD 1.0-interval support. *Filled boxes* indicate putative QTLs for seedling traits, and *hatched boxes* indicate putative QTL for height growth components of cycle 5. For the definition of the traits, see Materials and methods

explained 21% of the variation. For H_2 (total height measured after two periods of growth under continuous light) 1 QTL that accounted for 10% of the phenotypic variation was detected on linkage group 9. Significant correlations were observed between H_0 and H_1 (ρ=0.30**) and between H_1 and H_2 (ρ=0.49***). Less correlated variables ($-0.01<\rho<0.21$*) were derived from these traits: height increments ($\Delta H_{0.1}$ and $\Delta H_{1,2}$) and adjusted data H_1^{ad} (residual of H_1 adjusted to H_0, following the model: $H_{1i}=\mu_1+\beta_1 \cdot H_{0i}+\varepsilon_i$) and H_2^{ad} (residuals of H_2 adjusted to H_1, following the model $H_{2i}=\mu_{2i}+\beta_2 \cdot H_{1i}+\varepsilon'_i$). The same QTLs were mapped for H_1, $\Delta H_{0.1}$ and H_1^{ad} and for H_2, $\Delta H_{1,2}$ and H_2^{ad}.

Two QTLs were mapped for the other seedling traits (Fig. 2): in groups 6 and 12 for the megagametophyte weight (MW), in groups 4 and 6 for the germination date (GERMD) and in group 1 and 11 for the hypocotyl length (Lhypo). Estimates of the proportion of phenotypic variation explained by each QTL varied from 6% to 17%, and accounted together for 12.6–30% of the variation of the traits (Table 1). MW and GERMD apparently shared a QTL on group 6: the LOD 1.0 support interval of these traits overlapped (Fig. 2). In such a QTL mapping experiment, we can not distinguish between pleiotropy and tight linkage of genes. Indeed, because of the low-density map and the large base pair to centiMorgan ratio in maritime pine (an average of 13 Mbp/cM, Plomion et al. 1995b), a 20-cM interval containing an average of 260 Mbp could contain many loci. However, differences in emergence rates are often cited as the primary mechanism by which seed size operates (Dunlap and Barnett 1983). Thus, it was reasonable to suggest a common control of both traits, even though the correlation between MW and GERMD was not very high (ρ=0.19*).

For the seedling traits, differences between alternative QTL alleles expressed in phenotypic standard deviation ranged from 0.46 σ to 0.80 σ (Table 1). The source of the allele (i.e., Landes or Corsican) causing an increase in the phenotypic mean is indicated (Table 1).

Height growth components

With the accelerated growth conditions used for 2 years (continuous light combined with high fertilizer level), two to three growth increments were expressed during the first season while six to eight increments were expressed during the second season. F_2 seedlings were harvested on weeks 92–94 (Fig. 1), and growth components (NSU, MSUL, LF and LC) were only measured for each cycle of GP2. An analysis of variance was performed to test individual and cycle differences in order to see which effects made a significant contribution to the variation of each component. Individual effect was not significant for LC and LF, and was low but significant for NSU and MSUL. In addition, serial phenotypic correlations at lag 1 (i.e., correlations between traits expressed between adjacent cycles) were generally not significant or low for all of the components. These results suggested that a large proportion of the observed variation is phenotypic. This indication of a low individual control on height growth components could be attributed to physiological differences between cycles that could be associated with maturation. In contrast, cycle effects were highly significant for all of the traits. The components varied according to the shoot cycle and followed a trend parallel to the growth curve (data not shown) with maximum values in cycle 3 for all the components. Based on the highly significant pairwise correlations between (NSU, LC) and between (NSU, LF) for each cycle ($0.76^{***}<\rho<0.97^{***}$), we adjusted LC and LF to the covariate NSU. The resulting residuals, LC^{ad} and LF^{ad}, showed a low but significant individual effect. LC and LC^{ad} were further discarded from the analysis to avoid redundancy of information (phenotypic correlations between LC and other components ranged from 0.26*** to 0.97***). The biological meaning of such transformed variables is not straightforward. Height growth of a shoot is the result of the initiation and elongation of internodes. NSU is the result of the initiation of foliar primordia in the bud. Adjusted data of LF to NSU represent the growth potential of the shoot in our experimental condition, initiation being removed. Higher correlation coefficients were obtained between MSUL and LF^{ad} (0.85*** on average) than between MSUL and LF (0.50*** on average), which was expected by construction. On the other hand, correlations obtained between LF and NSU were higher (0.82*** on average) than those between LF and LF^{ad} (0.54*** on average). Two types of variables to be used for a quantitative trait dissection study could be selected from this preliminary analysis of the data: components more involved in the initiation (NSU and LF) and components more involved in the elongation of the shoot (MSUL and LF^{ad}). For the four selected traits (NSU, LF, MSUL, LF^{ad}), permutation tests for association (Dietz 1983) showed that correlation matrices did not significantly differ between cycles of GP2 (data not shown). Thus, it was decided to present the QTL results for the most demonstrative cycle: cycle 5. The QTLs detected for the other cycles will be discussed at another time.

One QTL effect was found for each growth component of cycle 5 (Table 1). QTLs for variables NSU_5 and LF_5 were mapped in group 11 (Fig. 2) and accounted for 20.4% and 19.6% of the phenotypic variation, respectively. QTLs for variables $MSUL_5$ and $LF^{ad}{}_5$ were mapped in group 9 and accounted for 6.0% and 11.8% of the phenotypic variation, respectively. Two putative linked QTLs with no overlapping LOD 1.0-support intervals were obtained for LF_5. The procedure suggested by Lander and Botstein (1989) was used to test for linked QTL effects. The position of 1 QTL was fixed and the chromosome scanned again to detect further regions with a significant contribution to the expression of the trait. An additional region was considered to contribute significantly to the trait variance when the LOD score of the scan exceeded by 2 units the score of the previous scan. An increase of only 1.20 LOD units was observed for both linked QTLs. This result suggested 1 QTL in this chromosomal region for this trait which is indicated in Fig. 2.

Table 2 Number of tests, K, for two-locus epistasis significant at the P=0.001 level compared to the number expected by chance. The probability $P(K)$ of observing at least K significant tests by chance is determined by $P(K)=1-\sum_{i=0}^{K-1}\binom{i}{T}*\alpha^{i}*(1-\alpha)^{T-i}$, where α is the probability threshold and T the number of tests. Tests were performed on all pairs of markers and on pairs involving one QTL-marker. Significant values (P<0 05) are underlined

Probability threshold (α)	Significant tests involving	Number of test expected by chance		Height components (cycle 5)				Seedlings traits					
				NSU_5	LF_5	$MSUL_5$	LF_5^{ad}	MW	GERMD	LHYPO	H_0	H_1	H_2
P=0.001	All marker pairs	7.14	K	8	3	6	6	13	8	8	9	7	8
			P(K)	0.42	0 97	0.72	0 72	0.03	0.42	0.42	0.29	0 57	0.42
	1QTL×1 marker	0.119	K	0	2	0	2	3	2	1	3	5	0
			P(K)	/	0.01	/	0.01	0	0.01	0.18	0	0	/

Epistasis

Epistasis was tested by two-way ANOVA with interaction for all pairs of markers (Table 2). For height growth components, the number of significant interactions were not different from what was expected by chance. For seedling traits, interactions was significantly more frequent than expected by chance for MW and H_1. These numbers significantly increased for most of the traits when the tests were performed for pairs of markers with one having a significant principal effect on the trait. This indicates a possible role of significant interaction enhanced when at least one marker is involved in a putative QTL. A similar result has already been reported in maize (Causse et al. 1995).

Discussion

QTL mapping with haploid megagametophytes

Dominant RAPD markers are not efficient in quantitative trait dissection analysis in an F_2 progeny because additive and dominant effects cannot be tested separately. The genotyping of both haploid and diploid tissues for each RAPD marker would not help to determine the complete diploid RAPD genotype in the mapping progeny because F_2 genotypes could not be distinguished unambiguously from RAPD fragments present in both haploid and diploid tissues. Actually, only pairs of *trans*-dominant linked markers would provide linkage information equivalent to codominant markers in a F_2 progeny. In our experiment, only the maternal contribution (megagametophyte) was determined. Hence, only the additive effect of individual QTL could be estimated, whereas dominance deviation could not be determined. If we consider a dominant marker completely linked with a QTL, only one contrast can be tested, which is: $\mu_M-\mu_m=0$, where μ_M and μ_m are the mean trait values for the dominant and recessive marker alleles, M and m, respectively. The expectation of this contrast is $\mu_M-\mu_m=0.5(\mu_{QQ}+\mu_{Qq})-0.5(\mu_{qq}+\mu_{Qq})$ with $\mu_{QQ}=\mu+a$, $\mu_{qq}=\mu-a$, $\mu_{Qq}=\mu+d$, where μ denotes the family mean, and a and d the additive and dominance effects of the QTL, respectively. This contrast becomes $\mu_M-\mu_m=a$.

With an average of 10 loci per chromosome and a marker spacing of about 17.6 cM, the LOD thresholds used corresponded to a per-marker type-I error rate of 0.0045 and 0.00082 for LOD=1.6 and LOD=2.4, respectively (computed with the program provided by A. Rebaï, Rebaï et al. 1994). Thus, with P=0.0045 and 120 markers, 0.5-false positive QTL on average was expected to be found by chance for a given trait. As the markers are actually not independent, this number is overestimated. Although the stringency adopted to declare a QTL in our experiment seemed satisfactory, the power for QTL detection was limited. The detection of QTLs increases with the size of the segregating population, and the heritability of the trait, and depends on the probability criteria used for declaring a QTL effect significant (type-I error). No estimates of the heritability of the traits studied under accelerated growth conditions were available. Seedlings were raised in pots with individual watering. Therefore, the genetic individual effect (I_i) contained a random environmental factor that could have affected phenotype expression. This "micro-environmental noise" was not controlled and should have introduced a certain level of imprecision in the estimation of QTL effect and consequently have reduced the power of QTL detection. The low power could also be due to the sample size available in our experiment: 126 individuals. Darvasi et al. (1993) pointed out that 500 individuals would be needed to achieve a power between 0.58 and 0.68 (depending on the relative QTL position in an interval) for detecting a QTL in a 20-cM map with a standardized gene substitution effect of 0.25s. The proportion of total phenotypic variation explained by individual QTLs ranged from 6.0% to 20.4%. However, standardized effects were comprised between 0.46σ and 0.82σ phenotypic standard deviations. Thus, this experiment did not allow QTLs of low effect to be detected. For all these reasons, the number of QTLs found must be considered to be a minimal estimate.

In most QTL mapping experiments contrasting parents are chosen with respect to the QTLs affecting the traits of interest (e.g. Bradshaw and Stettler 1995). Our pedigree

corresponded to a more realistic situation, since advanced breeding programs are often based on elite individuals of equivalent performance. In maritime pine, our hybrid breeding program is focused on the two complementary Landes and Corsican races. The hybrid shows the good stem straightness and good branching habit of the Corsican grandparent and the vigor and frost resistance of the Landes grandparent. Although the Landes race is known to be more vigorous than the Corsican race (Harfouche et al. 1995; Harfouche and Kremer unpublished results), height at 13 years measured in a polycross test suggested that of the grandparents used in our experiment had a similar growth potential (data not shown). Thus, our pedigree could also be less favorable for the detection of very large QTL effects.

The LOD 1.0 support intervals (i.e. the interval over which the position of the QTL is at most ten times less likely than the most probable position) were rather large and varied between 15 and 50 cM (Fig. 2). As pointed out by Mangin et al. (1994) this confidence interval is biased because it depends on the value of the QTL effect. However, for breeding applications such as marker-assisted selection, the exact position of a QTL is not crucial and the LOD 1.0 support interval can be considered as a good indicator of the length of marker-intervals that should be used to ensure selection of favorable alleles at the QTL.

Height growth QTLs stability over developmental stages

The first apical budset was observed about 50 days after application of the continuous light treatment (i.e. 22 weeks after the sowing date, Fig. 1). Total height of the plants was measured before this first phase change (H_0 on week 15, Fig. 1). This first measurement corresponded to a juvenile stage. The second measurement was completed after the first "light" treatment (H_1 on week 38, Fig. 1). The plants presented an average of two shoot cycles interrupted by a nonresting terminal bud. Finally, the seedlings were measured after a second "light" treatment (H_2 on week 92, Fig. 1) and presented an average of eight cycles. Thus, H_0, H_1 and H_2 correspond to three distinct stages of development. Non-accelerated control seedlings presented the same characteristics at H_0, whereas and they were on a free growth and measured 105±22 mm at H_1; presented one to two shoot cycles and measured 575±70 mm at H2.

Differential QTL expression during development was observed for total height. None of the QTL significant at one stage (either H_0, H_1 or H_2) was also significant at another stage, with the type-I error rate used (Table 1). This result supports the idea that different loci are likely to be involved in the genetic control of height growth at different ages (Kremer et al. 1991). In poplar, Bradshaw and Stettler (1995) reported comparable observations in a study of height growth over two seasons. However, in our experiment, two kinds of QTLs could be observed: genomic regions showing a progressive expression during the plant growth (intervals 18–19, 73–74 and 87–88, Fig. 3) and genomic regions strongly expressed only at one of the three stages of development (intervals 34–35, 49–50 and 60–61,

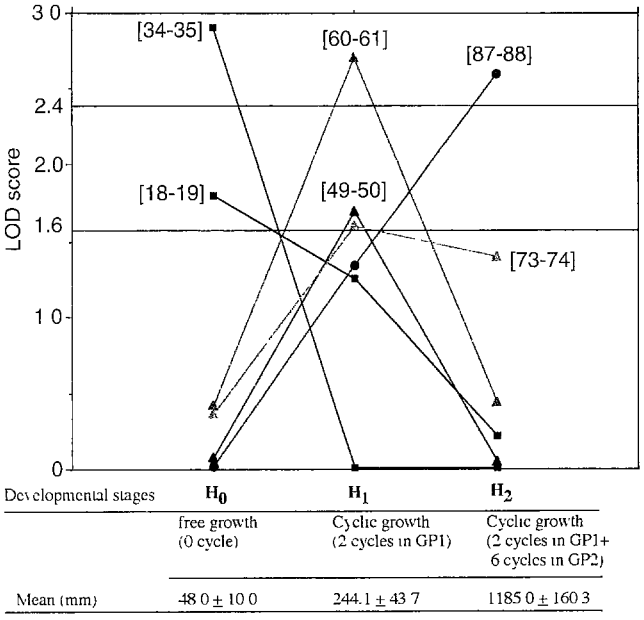

Fig. 3 Evolution of the LOD score values across developmental stages for the three height measurements (H_0, H_1, H_2). Segments [x,y] are indicated in Fig. 2 and Table 1

Fig. 3). Thus, rather than a complete change in QTL expression, maturation could also induce a progressive shift of the genetic control of height growth. A similar result has been reported in maize (Edwards et al. 1992). In larch, Greenwood et al. (1989) and Hutchison et al. (1990) showed that changes in morphological and physiological phenotypes due to maturation are also associated with changes in gene expression in developing foliage. However, cross hybridization of mRNA suggested that either uniquely expressed transcripts must be present at a very low levels or that maturation did not arise from the induction of new maturation specific genes or repression of juvenile-specific genes (Greenwood et al. 1989; Hutchison et al. 1990). Genes that are differentially expressed in juvenile and mature plants could be associated with a different "power" of QTL detection.

Seed-weight effect on seedling growth is usually strong during the first season and declines thereafter (Toon et al. 1991; Surles et al. 1993). In our case, the correlation between megagametophyte weight (MW) and growth potential at a young stage (H_0) was not significant (ρ=0.01). These traits shared no QTL, and seed effect on early growth could be discarded for the family studied.

Finally, if the same metabolic pathways are assumed to be responsible for height growth in both juvenile and more mature developmental stages, our results suggest that different regulatory genes or differential expressions of the same set of regulatory genes are involved at different stages of maturation. This epigenetic variation (i.e. expression of phase-dependent genetic factors within an individual) provides an explanation for the low or complete absence of a juvenile-mature correlation for growth performance in maritime pine (Kremer et al. 1991) and other tree species (reviewed by Greenwood and Volkaert 1992).

Height growth components QTLs

For cycle 5, QTLs affecting initiation (NSU and LF) or elongation (MSUL and LF^{ad}) were mapped on different chromosomes (Fig. 2). For the other five cycles, QTL analysis was also performed for variables NSU, LF, MSUL and LF^{ad}. A total of 21 significant QTLs (1.6<LOD<3.6) were identified. As expected, the clustering of QTLs for traits mostly involved in initiation or elongation were consistent with the high correlations among the traits. These ranged from 0.78*** to 0.90*** between MSUL and LF^{ad} and from 0.76*** to 0.92*** between NSU and LF. The mapping results concerning traits related to both phenomena agreed with those of Lascoux et al. (1993b, 1994). These authors found low phenotypic correlations and variable family mean correlations between NSU and MSUL of different cycles. In a few cases, genomic regions apparently affected the same traits across shoot cycles. Common QTLs were identified for the elongation-related traits in segment [93–96] for cycles 2, 3 and 5 and in segment [59–61] for cycles 1 and 4, while other QTLs were cycle-specific. For the initiation-related traits only one region showed up in more than one cycle: in segment [8–12] for cycles 1 and 3. These results could indicate a partly common control of the components across cycles. Leaf initiation (NSUL) is a function of apical meristem activity, whereas internode length (MSUL) is function of subapical meristem activity (Lanner 1976; Kremer 1984). The different QTL locations of NSU, LF (initiation-related traits) on the one hand and MSUL, LF^{ad} (elongation-related traits) on the other hand for each shoot cycle suggests the occurrence of specific genetic factors controlling the activity of both meristems under accelerated growth conditions.

At this point it is difficult to distinguish whether the differential expression of QTLs controlling (1) variation in total height or (2) variation in the same components across shoot cycles are related to maturation effects, environmental changes or a combination of both. Indeed, environmental conditions have changed during the 2 years of growth and could have potentially created QTL-by-environment interactions (natural/artificial light ratio and temperature being progressively different for each cycle). However, for maritime pine seedlings raised under accelerated growth conditions in a phytotron, Lascoux et al. (1992; 1993a, b) also observed the appearance of morphological traits characteristic of mature trees. Thus, it is probable that maturation effects were predominant in our experiment. In addition, the first apical budset was observed at very similar dates in our experiment and in previous studies.

Perspectives

The phenomenon of maturation is a complex developmental problem and little is known about its physiological or biochemical basis. An understanding of maturation and its manipulation is important because shortening the juvenile phase could be used for accelerating breeding and because rejuvenation would be of particular interest for the clonal propagation of selected trees. The phenotypic approach that was developed here could be complemented by a biochemical approach aimed at studying the expression of proteins at different maturation stages. Quantitative and qualitative differences in proteins during maturation of *Hedera helix* were observed by Wareing and Frydman (1976). Quantitative trait loci underlying gene product variation (e.g. proteins revealed by 2-D PAGE, Damerval et al. 1994) could be localized on the genetic map of maritime pine in order to analyze the regulation of genome expression during the developmental phases. Modification of the protein content between mature and rejuvenated tissue of *Sequoiadendron giganteum* has been reported by Bon and Monteuuis (1991). In addition, the localization of QTL controlling the level of gibberellic and abscissic acids (hormones involved in maturation, reviewed by Hackett 1985), could help in providing an understanding of the physiological causes responsible for differential gene expression of height growth in forest trees.

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