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Genetic analysis of phenotypic variation for ectomycorrhiza formation in an interspecific F1 poplar full-sib family

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Abstract A plant's capability to develop ectomycorrhizal symbiosis is under the control of both genetic and environmental factors. In order to determine the roles played by these different factors, we have performed a quantitative genetic analysis of the ability of poplar trees to form ectomycorrhizas. Quantitative genetics were applied to an interspecific family of poplar for which the two parental genetic maps had already been described, and for which data analyses concerning fungal aggressors were obtained. Quantitative trait loci (QTL) related to ectomycorrhiza formation were identified and located in the genetic maps of the two parents. One QTL was located at a linkage group of the genetic map of *Populus trichocarpa* showing a high concentration of several QTL involved in the pathogenic interaction with the fungus *Melampsora larici-populina*, the causal agent of leaf rust.

Keywords Mycorrhiza · Poplar · Quantitative trait loci · Rust · Symbiosis

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Introduction

Within forest soils, ectomycorrhizal fungi are almost ubiquitous (Smith and Read 1997). The mutualistic ectomycorrhizal symbiosis allows trees to grow efficiently in suboptimal environments in boreal, temperate and some subtropical forest ecosystems. The formation and functioning of the symbiosis involve major changes in cellular and tissue morphology (Peterson and Bonfante 1994), as well as in the biochemistry and physiology of the partners (Nehls et al. 2001; Martin et al. 2003). Central to the success of the ectomycorrhizal associations is the exchange of nutrients between the symbionts (Nehls et al. 2001; Tagu et al. 2002). The ecological performance of ectomycorrhizal fungi is thus a complex phenotype affected by many different genetic traits and by biotic and abiotic environmental factors. In order to gain a predictive understanding of the complex biological systems that evolve from ectomycorrhizal interactions, a wave of studies based on functional genomics (gene discovery, cDNA array analysis of gene expression, proteomics) have allowed an assessment of the development and functioning of ectomycorrhizal symbioses on a larger scale (Martin 2001). These techniques have enabled researchers to rapidly identify genes and to perform large-scale functional analyses of thousands of them (Voiblet et al. 2001; Podila et al. 2002; Peter et al. 2003).

Genetic mapping can also provide a robust means to shed light on the genetics of ectomycorrhiza development. Genetic factors, i.e. quantitative trait loci (QTLs), affecting a trait can be identified on the chromosomes of the organisms with the aid of linked molecular markers. These factors can be further studied in terms of the magnitude of their effects on the phenotype, the mode of their gene action, the parental origins of the favourable QTL alleles, and the relationships between QTLs underlying different physiological processes. Molecular information on the inheritance and transmission of ectomycorrhiza-specific traits will permit the production of even better improved phenotypes through more effective breeding and parental selection.

Populus, an ectomycorrhizal genus, has been advanced over the last decade as a model woody plant (Bradshaw et al. 2000) because of its relatively modest genome size, extensive genetic resources, rapid early growth, ease of clonal propagation, and routine transformation protocols (Cervera et al. 1996; Bradshaw et al. 2000; Lefèvre et al. 1998; Cervera et al. 2001; Wullschleger et al. 2002). The availability of up to 200,000 expressed sequence tags (Sterky et al. 1998, Kohler et al. 2003) and the sequencing of the *P. trichocarpa* genome to approximately 7.5× depth add to a long list of important factors for research. Furthermore, QTL on genetic maps of poplar corresponding to different pathogen-resistance traits have already been localized (Cervera et al. 1996; Villar et al. 1996; Newcombe 1998; Zhang et al. 2001).

In this paper, we identify QTLs that affect the development of the symbiosis by relating the *Populus* genome map to the formation of ectomycorrhizal root tips. Co-localisation of these QTLs with those controlling incompatibility and partial resistance to the foliar rust are then discussed.

Materials and methods

Materials

The plant material consisted of cloned F1 individuals from one interspecific controlled cross (family 54B) (Lefèvre et al. 1998) between *Populus deltoides* (female clone from Illinois, no. 73028–62) and *Populus trichocarpa* (male clone from Washington, no. 101–74) (Lefèvre et al. 1998; Tagu et al. 2001). The two parents and 146 progeny were tested for ectomycorrhiza formation after inoculation with the basidiomycete *Laccaria bicolor* S238 N (Di Battista et al. 1996). The 146 F1 clones were chosen without any a priori from the entire pedigree of 342 genotypes but included the 91 genotypes used for the construction of the two parental genetic maps (Faivre-Rampant et al. 1999; Cervera et al. 2001). The *L. bicolor* S238 N fungal strain (USA origin) was maintained on Pachlewski's medium (Pachlewski and Pachlewska 1974) in the INRA-Nancy collection of ectomycorrhizal fungi, and was chosen for its ability to form ectomycorrhizas with poplar (Tagu et al. 2001). For inoculum production, the mycelium was grown aseptically in a peat-vermiculite nutrient mixture (Duponnois and Garbaye 1991) in glass jars for 2 months in the dark at 25°C and kept at 4°C before use (Tagu et al. 2001).

Inoculation

Inoculation was performed as described in Tagu et al. (2001). Briefly, micro-cuttings of one single internode of each of the 146 poplar F1 clones and the two parents were rooted for 1 month, prior to fungal inoculation of 25 micro-cuttings per clone. After 1 month, a visual selection for each clone of the 20 best rooted plantlets was performed. The 2,960 selected plantlets were individually inoculated with the *L. bicolor* fungal inoculum. Plantlets were watered for 2 months, and then with a nutrient solution applied weekly (Frey-Klett et al. 1997). Then, six to seven plants per genotype were randomly distributed in each of the three independent complete blocks of the experimental design. Each block contained six or seven single tree plots of each 146 genotypes. Blocks were designed in order to control environmental heterogeneity of the greenhouse.

Inoculation with the pathogenic fungus *Melampsora larici-populina* Kleb., and measurements of epidemiological components, were performed on excised leaf-discs in independent experiments published elsewhere (Lefèvre et al. 1998), with five different strains, 93ID6, 93CV1, 93JE3, 98AG69 and 98AR1, provided by Jean Pinon (INRA-Nancy, Pathologie Forestière). Non-immune response or compatibility to strains 93ID6, 93JE3 and 98AG69 presented a 1:1 segregation in family 54B. As no complete resistance for strains 93CV1 and 98AR1 was detected in the entire pedigree, genetic variation for partial resistance was evaluated according to these three factors: (1) latent period of spore germination, (2) number of fungal uredia, and (3) mean size of fungal uredia.

Measurements

At the end of the experiment, shoots were cut and kept for dry weight determination. Pots were stored at 4°C to stop the process of ectomycorrhiza formation. The proportion of mycorrhizal root tips was determined as described in Tagu et al. (2001). For each root system, a total of 100 apices were randomly examined and assessed as mycorrhizal or non-mycorrhizal. For each plantlet, the dry weights of the shoots and of the roots were determined.

Map and molecular data

The "pseudo-testcross strategy" (Carlson et al. 1991; Grattapaglia and Sederoff 1994) takes advantage of the high level of heterozygosity of most forest tree species. It is based on the linkage analysis of dominant markers that are heterozygous in one parent and null in the other and vice versa. Consequently, two linkage maps are generated, one for each parent (19 chromosomes as haploid stock). The mapping population consisted of 91 F1 individuals derived from the same interspecific cross (family 54B). Genotyping data based on 725 molecular markers were grouped with MAPMAKER/EXP (Lincoln et al. 1992) by two-point linkage analysis using a log of odds likelihood ratio (LOD) score of 4.0 and a recombination fraction of 0.3. The non-saturated maternal *P. deltoides* map had a total of 356 markers (158 AFLPs, 135 RAPDs, 1STS, 1SCAR, 40 RFLPs, 21 SSRs) distributed in 30 linkage groups of more than three markers. The non-saturated paternal *P. trichocarpa* map had a total of 366 markers (164 AFLPs, 139 RAPDs, 1 STS, 35 RFLPs, and 27 SSRs) placed in 27 linkage groups of more than three markers (Cervera et al. 2001). For QTL detection, genotyping data included those of the 146 F1 individuals used in this study especially for AFLP, SCAR and SSR markers. Only partial correspondences are available between linkage groups of the different maps developed on *P. deltoides* and *P. trichocarpa*. We will choose group classification proposed by Bradshaw et al. (1994).

Statistical analysis

The S-Plus 2000 package was used for statistical analysis. The percentages of mycorrhizal root tips were transformed by arcsin $\sqrt{X/100}$ function prior to analysis to achieve the hypothesis of a linear model. The following mixed linear model was applied on an individual basis to test the presence of significant differences among clones for the percentage of mycorrhizal root tips, root and shoot dry weights:

$$Y_{ijk} = \mu + B_j + G_i + (\mathrm{B} \times \mathrm{G})_{ij} + E_{ijk} \quad (1)$$

where Y_{ijk} is the individual observation on the k th plant of the i th genotype (F1 clone or parent) in the j th block, μ is the overall mean, B_j is the fixed block effect, G_i is the random contribution for the i th genotype with variance σ^2_{G}, $(\mathrm{B}\times\mathrm{G})_{ij}$ is the Block×Genotype interaction effect with variance $\sigma^2_{\mathrm{B}\times\mathrm{G}}$ and E_{ijk} is the random residual error with variance σ^2_{W}.

The estimates of variance components for the residual error (σ^2_{w}), B×G interaction effect ($\sigma^2_{\mathrm{B}\times\mathrm{G}}$), and genotype effect ($\sigma^2_{\mathrm{G}}$) were obtained by the restricted maximum likelihood method

restricted to the 146 F1 clones. For each trait, individual broad-sense heritability (h^2) was estimated as follows:

$$h^2 = \sigma^2_G / \left(\sigma^2_G + \sigma^2_{B\times G} + \sigma^2_W\right) \quad (2)$$

and SEs of heritability were estimated according to Falconer (1989). The genetic coefficient of variation (CV_G) was used (Cornelius 1994) to compare the relative amounts of genetic variation of traits with different means:

$$CV_G = \sqrt{\sigma^2_G / \mu} \quad (3)$$

Phenotypic relationships between traits were analysed by Pearson linear correlations.

QTL detection

The association between markers and phenotypic traits was assessed using a one-way ANOVA to take in account the numerous unlinked markers. The significance threshold was assigned a value of 0.001 in order to limit false-positive detection. When more than one marker was significant in the same linkage group, the interval mapping method was used to assess the position of the QTLs (computer program QTL Cartographer 1.13 1999; C. J. Basten, B. S. Weir, Z. B. Zeng, Department of Statistics, University of North Carolina, Raleigh, N.C.). In this preliminary analysis, a LOD threshold of 2 (that a QTL is present vs. absent), corresponding approximately to an overall α-type error risk of 10% (Lander and Bostein 1989) was selected to identify putative QTLs associated with mycorrhizal phenotype. QTLs associated with components of rust resistance have been identified elsewhere on 325 genotypes of the same pedigree (Faivre-Rampant et al. 1999; Dowkiw et al. 2003).

Results

Ectomycorrhiza formation

Progenies and parental clones could be grouped following their degree of total mycorrhizal infection (Fig. 1). *Thelephora terrestris* fruitbodies and ectomycorrhizas revealed the presence of this frequent greenhouse contaminant in some pots, but this contamination did not influence the general level of infection of the plantlets; for one of the three blocks, a separate determination and counting of *L. bicolor* and *T. terrestris* ectomycorrhizas were performed, and no difference in the total number of mycorrhizal root tips was detected (data not shown). The two parents differed in their response to *L. bicolor* colonization (Fig. 1). The percentage of colonization of the different genotypes varied from 10% (female parent *P. deltoides*) to 61% (hybrid clone 54B092), with an average of 35%. The approximately normal distribution of the classes indicated that the ability to form ectomycorrhiza is a quantitative trait under polygenic control. The genetic variation in the interspecific family was assessed by a linear mixed model (Table 1). The percentage of mycorrhizal roots, and the root and shoot dry weights were clone-dependent. However, no linear correlation between the number of mycorrhizal roots and the root or shoot dry weights was noted (r=0.045 and r=0.091 respectively). This indicates that under our experimental conditions, for one given plant, its growth parameters were independent of its ability to form ectomycorrhizas. The broad sense heritability value for the percentage of mycorrhizal roots was 0.09 (CV_G=16.26%), indicating a high involvement of environmental factors in the success of ectomycorrhiza development. A significant block effect was detected for the percentage of mycorrhizal roots, and the root and shoot dry weights (Table 1).

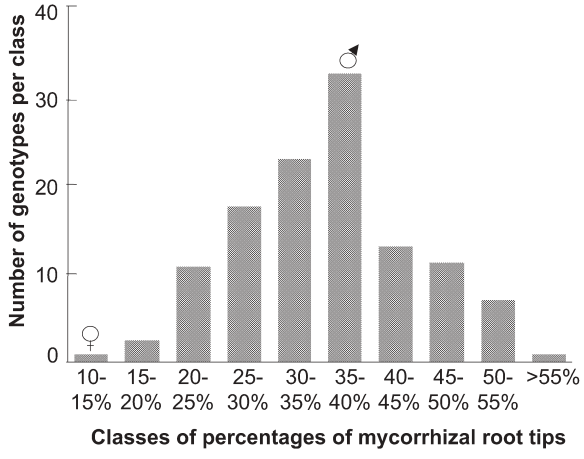

Fig. 1 Distribution of *Populus deltoides* (female) and *P. trichocarpa* (male) and their F1 progeny following their root colonization by *Laccaria bicolor*. The bars represent the number of genotypes distributed in each class according to their percentages of mycorrhizal root tips

Table 1 ANOVA for the percentage of mycorrhizal roots (*% Myc*) and dry weights of shoots and roots of F1 progeny from a cross between *Populus deltoides* and *Populus trichocarpa*. *n.a.* Not applicable

		df	*F* value
% Myc	Clone	147	2.99*
	Block	2	14.7*
	Clone/Block	291	1.32*
	Residuals	2,253	n.a.
Dry weight shoots	Clone	147	3.35*
	Block	2	118.87*
	Clone/Block	291	0.99*
	Residuals	2,253	n.a.
Dry weight roots	Clone	147	3.35*
	Block	2	118.87*
	Clone/Block	291	0.99
	Residuals	2,253	n.a.

* P <0.05

QTL determination

Genomic regions controlling the ability to form ectomycorrhizas were localized on the linkage maps of the two parents by using 146 progeny. One single putative QTL which explained 8.4% of the phenotypic variation of the ectomycorrhizal trait was detected on a linkage group E of a *P. deltoides* parent (LOD score=4.35). Another single QTL which explained 5.4 % of the phenotypic variation was detected with a LOD score of <2.2 on the linkage group M of a *P. trichocarpa* parent (Fig. 2). This putative

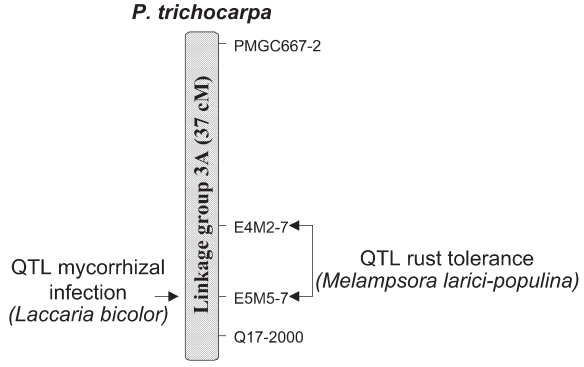

Fig. 2 Location on the *Populus trichocarpa* linkage map of the putative quantitative trait loci (*QTL*s) corresponding to the degree of root colonization by *L. bicolor* S238 N and different components of partial resistance to *Melampsora larici-populina*. *PMGC667–2* microsatellite marker, *E4M2–7* amplified fragment length polymorphism (AFLP) marker, *E5M5–7* AFLP marker, *Q17–2000* random amplification polymorphic DNA marker

QTL coincided with the major genomic region previously revealed for influencing uredia size observed for different strains of *M. larici-populina* and other epidemiological components of partial resistance to different strains of *M. larici-populina*.

Discussion

Owing to the availability of several genetic maps (Bradshaw et al. 1994; Cervera et al. 2001) and QTLs for plant-pathogen interactions (Newcombe and Bradshaw 1996; Cervera et al. 1996; Villar et al. 1996; Zhang et al. 2001), a *Populus* hybrid was selected as the host plant for identifying ectomycorrhiza-related QTLs. Here, we confirmed that the ability of poplar trees to form ectomycorrhizas is under genetic control. Heritability of the ectomycorrhizal trait was investigated in *Pinus elliottii* (Rosado et al. 1994) and a strong narrow sense heritability was found (0.81) for the percentage of ectomycorrhizas formed. A similar experiment was performed on poplar (Tagu et al. 2001) and a heritability value of 0.49 was found. This indicates that: (1) in tightly controlled conditions, genetic bases are involved in the ectomycorrhizal phenotype; and (2) genetic variability exists between half-sib progenies. The broad sense heritability found in the present study (i.e. 0.09) was low. Several biological traits can explain the variability observed in our study, such as the physiological status of the different 2,920 micro-cuttings, the developmental kinetics, and/or competition for light between plants. Such variations have been detected by significant differences according to block. Efficiency of QTL identification for mycorrhiza formation will likely be improved by analysing the symbiosis formation in in vitro systems with limited micro-climatic heterogeneity. Another aspect which could partially explain the variability we obtained is the nature of the measured parameters. The capacity to form an ectomycorrhiza was estimated in our study as related to the percentage of mycorrhizal roots. This is the regular parameter which is used in greenhouse, nursery and field experiments to score colonization status of a root system. However, the number of mycorrhizas does not totally reflect the efficiency of the symbiosis. Direct measurement of nutrient uptake and/or fluxes between the partners would better reflect the physiological activities of symbiotic tissues. However, these techniques used to measure metabolites are difficult to adapt to several thousand samples. Furthermore, a determination of the number of mycorrhiza could give information on the early steps of this fungal/plant interaction, whereas nutrient uptake would more reflect the functioning of the symbiosis, i.e. they are two complementary aspects.

One putative QTL was positioned on the genetic map of each parental genotype, *P. trichocarpa* and *P. deltoides*. The QTL on the male *P. trichocarpa* genetic map was localised close to a linkage group previously shown to be involved in the interaction between *Populus* and the foliar rust fungus *Melampsora larici-populina* (Faivre-Rampant et al. 1999; Dowkiw et al. 2003). However, this preliminary result needs further demonstration. These are the first putative QTLs found for the ectomycorrhizal association.

Our data provide a genetic indication that the ectomycorrhizal trait could be associated with loci and genes that might be common to the interaction with mutualistic root and pathogenic leaf fungi. Similar indications were already provided for arbuscular endomycorrhizas (Ruiz-Lozano et al. 1999) and it is well known that in different symbiotic relationships (root nodules and arbuscular endomycorrhizas for instance) both bacterial and fungal partners are able to trigger a similar plant host genetic program involved in symbiotic tissue morphogenesis (Gianinazzi-Pearson and Denarié 1997; Kistner and Parniske 2002). A search for *Populus* genes regulated after the early colonization of roots by *L. bicolor* and leaves by *M. larici-populina* is underway by comparing transcriptome of infected leaf and colonized root.

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