Tree Genetics & Genomes (2011) 7:617–627
DOI 10.1007/s11295-010-0361-3

ORIGINAL PAPER

Identification of quantitative trait loci affecting ectomycorrhizal symbiosis in an interspecific F_1 poplar cross and differential expression of genes in ectomycorrhizas of the two parents: *Populus deltoides* and *Populus trichocarpa*

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Received: 24 August 2010 / Revised: 17 November 2010 / Accepted: 10 December 2010 / Published online: 11 January 2011

Abstract A *Populus deltoides*×*Populus trichocarpa* F_1 pedigree was analyzed for quantitative trait loci (QTLs) affecting ectomycorrhizal development and for microarray characterization of gene networks involved in this symbiosis. A 300 genotype progeny set was evaluated for its ability to form ectomycorrhiza with the basidiomycete *Laccaria bicolor*. The percentage of mycorrhizal root tips was determined on the root systems of all 300 progeny and their two parents. QTL analysis identified four significant QTLs, one on the *P. deltoides* and three on the *P. trichocarpa* genetic maps. These QTLs were aligned to the *P. trichocarpa* genome and each contained several megabases and encompass numerous genes. NimbleGen whole-genome microarray, using cDNA from RNA extracts of ectomycorrhizal root tips from the parental genotypes *P. trichocarpa* and *P. deltoides*, was used to narrow the candidate gene list. Among the 1,543 differentially expressed genes (p value≤0.05; ≥5.0-fold change in transcript level) having different transcript levels in mycorrhiza of the two parents, 41 transcripts were located in the QTL intervals: 20 in Myc_d1, 14 in Myc_t1, and seven in Myc_t2, while no significant differences among transcripts were found in Myc_t3. Among these 41 transcripts, 25 were overrepresented in *P. deltoides* relative to *P. trichocarpa*; 16 were overrepresented in *P. trichocarpa*. The transcript showing the highest overrepresentation in *P. trichocarpa* mycorrhiza libraries compared to *P. deltoides* mycorrhiza codes for an ethylene-sensitive EREBP-4 protein which may repress defense mechanisms in *P. trichocarpa* while the highest overrepresented transcripts in *P. deltoides* code for proteins/genes typically associated with pathogen resistance.

Keywords Quantitative trait loci · Poplar · Symbiosis · Ectomycorrhiza · *Laccaria*

Communicated by W. Boerjan

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Introduction

The majority of terrestrial plants live in association with symbiotic fungi which facilitate their access to soil nutrients. The ectomycorrhizal (ECM) symbiosis is the most common association in forest under boreal and temperate climates. ECM symbioses represent complex biological systems involving numerous multifaceted interactions between the two partners (Martin et al. 2003; Martin and Nehls 2009; Nehls et al. 2001; Peterson and Bonfante 1994). Ectomycorrhizal establishment also depends on edaphic and climatic conditions (Smith and Reads 1997), as well as genetic traits of the partners (Rosado et al. 1994; Tagu et al. 2001).

Poplars are pioneer trees living in riparian ecosystems. They are associated with a great variety of arbuscular mycorrhizal fungi and ectomycorrhizal fungi without which they could not colonized these disturbed habitats. Selected poplar exhibiting a large susceptibility to ectomycorrhizal colonization could have an advantage when planting on to disturbed sites. But the absence of ectomycorrhizal inoculum in these types of sites could be a disadvantage, requiring artificial inoculation. In richer soils, like previously cleared agricultural sites, the selection of clones less dependent on ectomycorrhizal colonization could be another strategy.

The development and functioning of ectomycorrhizas involve the expression of numerous genes encoding for developmental proteins and/or regulatory enzymes both in the fungi and their partners (Martin et al. 2007). The genetic predisposition and molecular adaptation needed for mycorrhizal establishment most likely involve the combined action of numerous gene networks (Martin and Nehls 2009). One quantitative trait loci (QTL) related to ectomycorrhizal formation was located on *Populus trichocarpa* linkage group IIIa also containing a QTL involved in the pathogenic interaction with the fungus *Melampsora larici-populina*, the causal agent of leaf rust (Tagu et al. 2005). The recent release of genomic resources, such as the *Laccaria bicolor* genome (Martin et al. 2008) and the *P. trichocarpa* genome (Tuskan et al. 2006), will facilitate the identification of genetic factors involved in these complex interactions.

Here, we (1) confirmed and extended the previous QTL analysis (Tagu et al. 2005) by positioning QTLs involved in root colonization by *L. bicolor* on two improved *Populus* genetic maps, using an extended progeny set (300 F_1 individuals), (2) anchored the QTL markers on the *P. trichocarpa* genomic sequence, and (3) created a candidate gene list from these QTL intervals. Using a whole-genome oligoarray of *P. trichocarpa* (Tuskan et al. 2006), we also compared the differential gene expression between *P. trichocarpa* and *Populus deltoides* ECMs. Finally, we determined whether genes showing a differential expression between the parents exhibiting a low ectomycorrhizal colonization (*P. deltoides*) and the parent exhibiting a high ectomycorrhizal colonization (*P. trichocarpa*) were located in QTL intervals related to ectomycorrhizal colonization.

Material and methods

Biological materials

Plant material consisted of 300 clonally replicated F_1 progeny from an interspecific *P. deltoides* (female parent from Illinois, no. 73028-62) by *P. trichocarpa* (male parent from Washington, no. 101-74) control-cross family (Family 54B, Pop2; Lefèvre et al. 1998; Tagu et al. 2001; Tagu et al. 2005). Ectomycorrhizal formation was evaluated by inoculating the two parents and the 300 progeny with *L. bicolor* S238N (Di Battista et al. 1996; Tagu et al. 2001). The 300 F_1 genotypes were chosen at random among the 336 genotypes used for the construction of the genetic maps (Jorge et al. 2005). Moreover, these 300 genotypes included the 90 genotypes used for the construction of the initial Pop2 genetic map (Faivre-Rampant et al. 1999). The *L. bicolor* S238N fungal strain, issued from the INRA-Nancy collection of ectomycorrhizal fungi, was maintained on Pachlewski's medium (Pachlewski and Pchlevska 1974). This model fungal strain was chosen for its ability to form ectomycorrhizas with *Populus* and for the availability of its genome and transcriptome (Martin et al. 2008; Peter et al. 2003; Tagu et al. 2001). Mycelium was produced on a peat–vermiculite nutrient mixture and grown in glass jars for 2 months in the dark at 25°C and kept at 4°C before use (Duponnois and Garbaye 1991).

Inoculation

Internode cuttings of each progeny and the two parents were rooted and individually inoculated at the same time in 1–l pots containing a mixture of fungal inoculum (1:9; *v*/*v*) and calcinated attapulgite (Oil Dri US Special, Damolin, Denmark; http://www.damolin.dk). Inoculated cuttings were grown for three-and-a-half months in a greenhouse at 15–28°C, photoperiod 12 h at INRA-Nancy (48°41′37″ N, 6°11′05″ E) during spring. Cuttings were watered twice daily and received a weekly nutrient application as described in Frey-Klett et al. (1997). In order to control environmental heterogeneity of the greenhouse, eight clonal replicates for each genotype were randomly distributed in eight blocks. Each block contained one rooted cutting of each of the 300 progeny and the two parents.

Measurements

The percentage of mycorrhizal colonization, as described by Tagu et al. (2001; 2005) was determined three-and-a-half months after inoculation by eight observers. All the observers observed randomly the plants within eight blocks and block after block. Each root system was rinsed with tap water, cut in 1-cm pieces and analyzed under a dissecting microscope. For each root system, 100 apices were randomly examined and assessed as mycorrhizal or non-mycorrhizal. Approximately 25,000 colonized root tips were examined.

Statistical analysis

The percentage of mycorrhizal colonization was transformed using an arcsine $\sqrt{X}/100$ function prior to variance

analysis (ANOVA). Clonal variation was tested by a two-factor ANOVA according to $yij = \mu + gi + bj + eij$, where *yij* is the observation value of the *i*th clone in the *j*th block, μ is the overall mean, *gi* is the genetic effect of the *i*th clone, *bj* the block effect of the *j*th block, and *eij* is the error term (Table 1). The following mixed linear model was applied on an individual cutting basis to detect significant differences in mycorrhizal colonization among the clones:

$$Y_{ijk} = \mu + B_i + G_j + O_k + \varepsilon_{ijk}$$

where, μ is the overall mean, *B* is the block effect (fixed), *G* is the genotype effect (random), *O* is the observer effect (fixed), and ε is the random pooled residual error. Restricted maximum likelihood estimates of genetic, block, and residual variance components ($\sigma^2{}_G$, $\sigma^2{}_B$, and $\sigma_\varepsilon{}^2$) were computed, and for each trait, individual broad-sense heritability (h^2) was estimated as:

$$h^2 = \sigma_G^2/\left(\sigma_G^2 + \sigma_\varepsilon^2/n\right)$$

where, *n* is the average number of replicates per genotype. Standard deviations (SD) of h^2 were derived from classic estimation of SD for a ratio *x*/*y* where $x = \sigma^2{}_G$ and $y = \sigma_G^2 + \sigma_\varepsilon^2/n$. All analyses were performed with the statistical programs JMP 5.0 (SAS Institute Inc., Cary, NC, USA) and R version 2.9.1 (R. Development Core Team, 2003, www.R-project.org). The genetic coefficient of variation (CV_G) was used to compare the relative amounts of genetic variation of traits with different means (Cornelius 1994):

$$CV_G = \sqrt{\sigma_G^2}/\mu$$

Molecular data and QTL detection

Populus genotyping was conducted using RFLP, STS, RAPD, and microsatellite (SSR) markers in a subset of 90 genotypes. SSR and AFLP genotyping was then extended to 253 additional genotypes (Jorge et al. unpublished). RFLP and STS genotyping was conducted as described by Bradshaw and Stettler (1993). RFLP markers were coded by the letter "P" followed by a 3- to 4-digit number (e.g., P1273). RAPD genotyping was performed according to Villar et al. (1996). RAPD markers were coded by the Operon primer name followed by the molecular weight of the polymorphic band (e.g., M02-1150). AFLP genotyping was performed as described in Jorge et al. (2005). AFLP markers were named after the code of the EcoRI/MseI combination followed by the band ranking on the gel (e.g., E5M5-7). The SSR primers came from two different sources: (1) the International *Populus* Genome Consortium, SSR named "PMGC" (http://www.ornl.gov/sci/ipgc/ssr_resource.htm), "ORPM" (Tuskan et al. 2004), and "WPMS" (Van der Schoot et al. 2000) and (2) SSR named "ai," "bi," and "bu" (Jorge et al. unpublished) developed from public EST databases. Different labeling techniques were successively used for SSR genotyping: primers were labeled with γ-[^{33}P] ATP, with forward fluorochrome-labeled primer or the M13 tailing strategy (Schuelke 2000) as described by Jorge et al. (2005). PCR reactions were performed in a total volume of 10 µl containing 10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM each of dATP, dCTP, dGTP, and dTTP; 0.2 unit *Taq* Polymerase (Invitrogen); 5 pmoles reverse primer; 0.5 pmoles M13-tailed forward primer; 5 pmoles M13-labeled primer; and 40 ng DNA. PCR reactions were conducted with 42 cycles of a 30-s denaturation at 94°C, 30 s annealing at 55°C or 52°C, and 30 s extension at 72°C. Each forward primer was 5′-tailed with the M13 forward consensus sequence. The M13-tailed forward primers were then used in combination with a standard M13 primer labeled with fluorescent dye (6FAM, HEX, or NED) at its 5′-end. The amplicons for each SSR marker were separately produced, diluted, and pooled post-PCR by three-color multiplexes (6FAM, HEX, and NED) for polymorphism screening. SSR polymorphisms were visualized using an ABI PRISM 3,100 genetic analyzer (PE Applied Biosystems, Foster City, CA). SSR allele lengths were recorded by GeneScan and Genotyper or GeneMapper softwares.

Markers significantly deviating from Mendelian segregation ratios [1:1] were eliminated from the linkage analysis. Linkage maps were constructed using Mapmaker version 3.0b (Lander and Botstein 1989) and were based on the pseudo-testcross strategy which resulted in the construction of two parental maps (Grattapaglia and Sederoff 1994). Steps for map construction were the same as in Jorge et al. (2005) except that the "Error detection" option was enabled.

QTL analysis

QTLs were determined with means corrected for observer effects as input data and using MultiQTL 2.4 (http://www.multiqtl.com/; Haïfa, Israel). Observer effects for data used in the QTL analysis were corrected using a logistic

Table 1 ANOVA for the percentage of mycorrhizal colonization (% Myc) in the interspecific family (54B) issued from a cross between *Populus deltoides* and *Populus trichocarpa*

Trait	Effect	*df*	*F* value[a]	*p*
% Myc	Genotype	300	7.47	0.0001
	Block	8	0.2726	0.2732
	Observer	8	5.38	0.0001

[a] Pooled error was used as the denominator in all *F* tests

regression. The option "marker restoration" was used to reduce the effect of missing data. The Kosambi mapping function was chosen for recalculation of maps on genotypic data. Single trait analysis was performed using a combination of interval mapping and multiple interval mapping. That is, the entire genome was first scanned using the one QTL model then a two-linked QTL model was used. Permutation tests (1,000 runs), comparing hypotheses H_1—there is one QTL on the chromosome vs. H_0—no QTL on the chromosome, were run to obtain chromosome-wise statistical significance. For chromosomes for which a single QTL was detected, permutation tests (1,000 runs) were run to compare the hypotheses H_2—two-linked QTLs in the chromosome vs. H_1. Subsequently, when $p_{(H2vsH1)} \leq 0.05$, permutations were run to compare H_2 vs. H_0. A two-linked QTL model was only accepted, if the two intervals were not adjacent to each other. To reduce calculation time, full permutations for the two-linked QTL models were only conducted with 1,000 runs when the p value was ≤ 0.1 after 100 runs. In a last step, multiple interval mapping was performed including all the significant intervals mapping QTLs (single and two-linked QTLs). A QTL was declared significant when $p \leq 0.05$. For the remaining significant QTLs, permutations were run per chromosome, using p value thresholds of 0.05 per chromosome. Bootstrap analysis was performed to estimate the 95% confidence interval. QTL analysis was performed with the genetic maps of *P. deltoides* and *P. trichocarpa* (Jorge et al. 2005).

RNA extraction and cDNA synthesis

Ectomycorrhizal root tips from *P. trichocarpa* and *P. deltoides* inoculated with *L. bicolor* S238N were harvested and instantly frozen in liquid nitrogen. Total RNA was extracted from three biological replicates (3×2 ECMs) of each genotype using the RNeasy micro kit (ref.74004, QIAGEN Courtaboeuf, France) following manufacturer's instructions. An in-column digestion step with DNAse I (ref.79254, QIAGEN Courtaboeuf, France) was included in the RNA purification. RNA quality was verified using Experion High sens Capillary gels (BIO-RAD, France). The cDNAs for NimbleGen microarrays were synthesized using the Super Smart cDNA Synthesis kit (ref.635000, Clontech-Takara Bio Saint-Germain-en-Laye, France).

NimbleGen microarrays

The *Populus* whole-genome expression array version 2.0 (DiFazio et al. unpublished data) manufactured by NimbleGen Systems Limited (Madison, WI), containing duplicate sets of three independent, non-identical 60-mer probes per gene model plus control probes and labeling controls for a total of 65,965 probe sets corresponding to 55,970 gene models predicted on the *P. trichocarpa* genome sequence version 1.0 and 9,995 aspen cDNA sequences (*Populus tremula*, *Populus tremuloides*, and *P. tremula*×*P. tremuloides*), was used for expression analysis. The *Populus* version 2.0 oligoarray is fully described in the platform Gene Expression Omnibus (GEO) at NCBI (http://www.ncbi.nlm.nih.gov/geo; GPL2699). Single dye labeling of samples and hybridization procedures were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland) following their standard protocol. Expression data were processed to assure a high specificity in the following manner: (1) all independent 60-mer oligos for the 55,970 genes were blasted against the *Populus* genome v1.1 (Tuskan et al. 2006) available on http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html and only probes with less than 10% homology to gene models other than the gene model target were retained for the further analysis. Due to this stringent filtering, about 30% of the genes (16,667) were excluded since all three independent probes failed the given specificity and (2) fluorescence data were normalized using quantile normalization and Robust Multi-chip Average (Irizarry et al. 2003) implemented in the ARRAYSTAR software (DNASTAR, Madison, WI) and 3) A Student's t test, with FDR (Benjamini-Hochberg) multiple testing corrections, was applied to the duplicate sets on the array and the three biological repetitions using ARRAYSTAR software. Transcripts with a significant p value (<0.05) and ≥5.0-fold change in transcript level were considered as differentially expressed between *P. trichocarpa* ECM and *P. deltoides* ECM. Average expression levels from the three biological repetitions were calculated for each gene and were used for the calculation of the expression ratio. The complete expression dataset is available as series (accession number GSE26416) at the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/).

Results

Mycorrhizal colonization

Fourteen weeks after inoculation, root systems of the 300 progeny and two parental genotypes were exclusively colonized by *L. bicolor*. No greenhouse inhabiting contaminant ectomycorrhizal fungi, such as *Thelephora terrestris*, were observed on the roots. Progeny and parental clones could be ranked based on their degree of mycorrhizal colonization (Fig. 1). The two parents greatly differed in their mycorrhizal colonization rate; *P. trichocarpa* exhibited a colonization rate of 45%±2, whereas *P. deltoides* showed a rate of 15%±3. The percentages of ECM colonization among the progeny varied from 8%

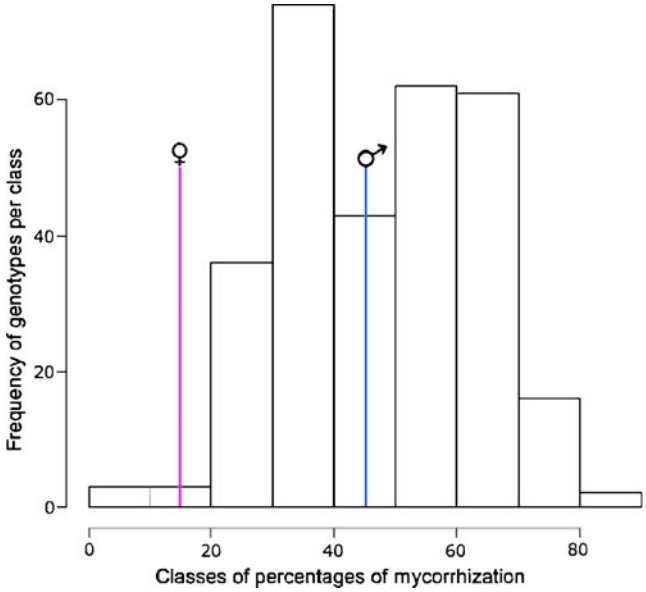

Fig. 1 Distribution of *Populus deltoides* (female) and *Populus trichocarpa* (male) and their F_1 progeny following their root colonization by *Laccaria bicolor*. The *bars* represent the number of genotypes distributed in each class according to their percentages of mycorrhizal colonization. The percentage of *P. deltoides* is identified by the *pink line* while that of *P. trichocarpa* is identified by the *blue line*

(hybrid clone No. 661300230) to 88% (hybrid clone N° 661300592), with an average of 47%. The genetic variation in this interspecific hybrid family was assessed by a linear mixed model (Table 1). ANOVA results showed a significant genotype and observer effects. No block effect was detected. The broad-sense heritability of the percentage of mycorrhizal colonization was 0.40 (CV_G= 20.82%) before correction of the observer effect while after correction it was 0.38 (CV_G=22.94%).

QTL detection

A single QTL (Myc_d1 on linkage group LG II d), explaining 10.9% of the variation of the mycorrhizal colonization, was detected (LOD score=1.4, significance= 0.018; Table 2) on the genetic map of *P. deltoides* (Jorge et al. 2005). On the genetic map of *P. trichocarpa* (Jorge et al. 2005), three QTLs—Myc_t1, Myc_t2m, and Myc_t3—respectively explaining 28.3%, 3.0%, and 1.8% of the mycorrhizal colonization trait, were detected on the respective linkage groups LG I t (LOD score=4.0, significance=0.002), LG III t (LOD score=1.1, significance=0.048), and LG XI t (LOD score=1.1, significance= 0.024; Table 2). None of these QTLs co-localized with QTLs for partial resistance to *Melampsora*× *columbiana* or *M. larici-populina* (Tagu et al. 2005; Yin et al. 2004). The nearest flanking SSR and/or SNP markers allowed the positioning of these QTL regions onto the *Populus* genome v2.0 (Tuskan et al. 2006). These QTL intervals encompassed 15 Mb on the *Populus* chromosome II (Myc_d1), 4.2 Mb on the chromosome I (Myc_t1), 2.4 Mb on the chromosome III (Myc_t2), and 1.7 Mb on the chromosome XI (Myc_t3).

Analysis of microarrays transcript profiling

Among all genes, 2,034 myccorhiza-regulated transcripts corresponded to the QTL intervals, i.e., Myc_d1 (1528), Myc_t1 (264), and Myc_t2 (242). No significant transcripts were found for QTL Myc_t3. By considering a change in gene expression at a ≥5-fold difference between *P. trichocarpa* ECM and *P. deltoides* ECM, we found 210 transcripts associated with Myc_d1 (154), Myc_t1 (27), and Myc_t3 (29) out of a total of 8,546 differentially expressed across the entire genome. In a second step involving the four QTLs, we identified 41 transcripts which displayed a significant (p value≤0.05) ≥5-fold gene expression among the 1,543 (p value≤0.05; ≥5.0-fold) differentially expressed genes between the mycorrhizas of the two parents (Table 3). Five of the 41 transcripts were among the ten most differentially represented transcripts between *P. trichocarpa* ECM and *P. deltoides* ECM (Table 4). Of these 41 transcripts, 27 corresponded to genes of known function and 14 to genes of unknown function.

Discussion

The current study confirmed the results of Rosado et al. (1994) and Tagu et al. (2001; 2005), who showed that the ability to form ectomycorrhizas in *Pinus elliotii* and *P. trichocarpa*×*P. deltoides*, respectively, was partially under the genetic control of the host. Using 146 progenies from the 54B family, Tagu et al. (2001, 2005) found that the heritability of the mycorrhizal development ranged between 0.49 (Tagu et al. 2001) and 0.09 (Tagu et al. 2005). With a larger progeny (300 clones), our statistical analyses have shown a significant genotype effect on ectomycorrhizal formation and a broad-sense heritability of the percentage of mycorrhizal colonization of 0.38 (CV_G=22.94%), indicating moderate genetic control of the trait. Our results, as in previous studies (Tagu et al. 2001; 2005), indicate a large difference in the response of the two parents to *L. bicolor* colonization (45% for *P. trichocarpa* and 15% for *P. deltoides*). The mean of the progeny in our study was 47% (vs. 35% in Tagu et al. 2005) was not significantly different from that of the *P. trichocarpa* parent. We thus hypothesize that the genes involved in ECM colonization are dominant and inherited from the *P. trichocarpa* parent, just as the bimodality in the distribution might be explained by the

Table 2 QTLs detected by multiple interval mapping in the full-sib *Populus deltoides*×*Populus trichocarpa* F_1 progeny for mycorrhizal colonization evaluated in green house conditions

Trait	Linkage group[a]	Marker	*N*	Significance[a] (on 1,000 runs)	Position (cM)	LOD	Mean[b]	PEV	Effect	
									A	*D*
Myc_d1	LG II d		158	0.018	54.9	1.4	43.31	0.109	5.97	10.7
		PMGC667			35.3	0.4				
		PMGC2088.1			194	0.4				
		PMGC2418			219	0.3				
Myc_t1	LG I t		234	0.002	50.8	4.0	45.95	0.283	4.74	16.9
		E1M7.9			43.3	1.6				
		CCR2			62.5	0.8				
		P1312			86.4	2.4				
Myc_t2	LG IIIb t		120	0.048	24.7	1.1	48.14	0.030	−7.35	5.44
		PMGC486			18.3	0.7				
		PMGC2274			45.1	0.4				
Myc_t3	LG XI t		281	0.024	0	1.1	45.91	0.018	−4.19	4.72
		PMGC2011			0	0.5				
		SNTP1			14.5	0.1				

d Populus deltoides linkage groups, *t Populus trichocarpa* linkage groups, *Marker* nearest marker anchored to the *Populus* genome from the maximum LOD value, *N* number of genotypes used for QTL detection for the marker closest to the QTL LOD peak, *LOD* maximum LOD (logarithm of odd ratio), *PEV* proportion of explained variability by the QTL, *A* the additive effect, *D* the dominant effect

[a] $p \leq 0.05$

[b] In percentage of mycorrhizal colonization

dominant hypothesis (Fig. 1). Interestingly, 32% of the progeny, such as the clone 661300592, showed transgressive segregation and a higher percentage of mycorrhizal colonization than that of their parents. This result may be due to the phylogenetic distance that exists between the two parents, which functionally could lead to the formation of heterozygotes in the hybrid progeny and heterosis for this trait. If validated, this suggests that the ability to form ectomycorrhizas in *Populus* could be manipulated via breeding.

In the current study, a QTL analysis identified four QTLs, one on the maternal *P. deltoides* map and three on the paternal *P. trichocarpa* map (Jorge et al. 2005). Until a saturated, higher resolution genetic map of *P. deltoides* and *P. trichocarpa* can be created, there will remain a difficulty in narrowing the QTL intervals associated with ECM colonization while increase in the observed population would elicit also more recombinant in the genomic regions of interest. The genomic regions corresponding to the detected QTLs encompass several megabases. In order to identify candidate genes which may be playing a role in the mycorrhizal development, we selected genes differentially expressed between *P. trichocarpa* and *P. deltoides* ECM and located in the QTL genomic regions. Among the 8,546 and 1,543 (p value$\leq$0.05; $\geq$5.0-fold) differentially expressed genes in the entire genome between the mycorrhizas of the two parents, 41 (p value$\leq$0.05; $\geq$5.0-fold) transcripts were located in the QTL genomic regions: 20 in Myc_d1, 14 in Myc_t1, and seven in Myc_t2.

Among these 41 transcripts, 25 were overrepresented in *P. deltoides* and 16 were overrepresented in *P. trichocarpa*. Based on our inoculation results, we hypothesize that the genes whose transcripts are overrepresented in *P. trichocarpa* play a positive role in ectomycorrhizal development, while the genes whose transcripts are overrepresented in *P. deltoides* play a negative role. That is, the most overrepresented gene in *P. trichocarpa* mycorrhizas compared to *P. deltoides* mycorrhizas, found in the QTL Myc_t2 on the chromosome III, encodes for a protein, EREBP-4, an ethylene-responsive binding protein transcription factor (Leubner-Metzger et al. 1998; Ohta et al. 2000; Solano and Ecker 1998), known to play an important role in the regulation of pathogenesis related genes (Hao et al. 1998; Ohme-Takagi and Shinshi 1995). Moreover, Hirota et al. (2002) has shown that the auxin-regulated AP2/EREBP *PUCHI* gene, encoding a putative APETALA2/ethylene-responsive element binding protein transcription factor, is required for the coordinated pattern of cell divisions during lateral root formation in *Arabidopsis thaliana*. Finally, it is well-known that phytohormones are involved in ectomycorrhizal development (Barker et al. 1998; Slankis 1950; Gay et al. 1994; Karabaghli-Degron et al. 1998; Tagu et al. 2003). Rupp and Mudge (1985) showed that ethylene and

Table 3 Microarrays transcript profiling corresponding to the regions of the detected QTLs

Protein ID	Expression ratio delt/trich	Position on the poplar genome v1.1	*p* value	Putative function
Differentially expressed transcripts corresponding to the QTL Myc_d1 (on LG II d)				
412601	59.0	LG_II:15301824-15302404	0.00424	Epoxide hydrolases
830358	58.2	LG_II:11022536-11024605	0.0000936	Flavonol reductase
755638	54.8	LG_II:15987878-15991132	0.000929	Glycosyl hydrolase family 3
552266	16.7	LG_II:13072796-13076702	0.000535	Ion transport protein (K^+)
830240	13.1	LG_II:7335242-7338550	0.00000295	Multi-antimicrobial extrusion protein (MatE)
754447	11.7	LG_II:4552579-4554054	0.00696	Unknown
409930	11.1	LG_II:8891580-8893724	0.00000811	NADH dehydrogenase
754777	9.33	LG_II:7462387-7463916	0.00008	PPR repeat
644137	8.67	LG_II:9585263-9591052	0.0000591	Cinamoyl-Coa reductase
411764	7.69	LG_II:6648928-6651247	0.000121	Calcium-binding EF-hand
816402	7.28	LG_II:7844838-7851663	0.00144	Phosphoenolpyruvate carboxykinase
754607	7.08	LG_II:6033373-6034896	0.00553	GRAS transcription factor
644752	6.06	LG_II:15004945-15009133	0.00811	Unknown
755810	0.03	LG_II:18234821-18235260	0.0000103	Unknown
551488	0.08	LG_II:6082658-6084346	0.0000149	Flavonol synthase (FLS)
412508	0.08	LG_II:7051326-7052889	0.000042	UDP-glucose glucosyltransferase
552582	0.09	LG_II:16695749-16698440	0.0000349	Serine/threonine phosphatase 2 C (PP2C)
816547	0.13	LG_II:10624397-10629736	0.00000997	t-complex polypeptide 1
411729	0.14	LG_II:12077325-12079059	0.0000719	Riboflavin biosynthesis protein ribG
643641	0.15	LG_II:5986249-5987114	0.0000675	Transcription factor with AP2 domain
Differentially expressed transcripts corresponding to the QTL Myc_t1 (on LG I t)				
548125	23.9	LG_I:5041805-5042362	0.00028	AtKT1I protein
750943	17.0	LG_I:4447457-4448212	0.000333	Gag-pol polyprotein
750935	8.31	LG_I:4403704-4404186	0.000259	Unknown
640058	8.08	LG_I:4273316-4279167	0.000219	WRKY-DNA binding protein
750915	6.80	LG_I:4168534-4168743	0.0471	Unknown
548126	6.60	LG_I:5046517-5051754	0.0278	AtKT1I protein
179061	6.30	LG_I:3921853-3924767	0.00257	Cytochrome p450
179380	5.24	LG_I:4920840-4923277	0.000351	FKBP-type isomerase
639958	0.02	LG_I:3547285-3547784	3.98E-07	Unknown
797239	0.06	LG_I:5366392-5369808	0.00121	Lipolytic enzyme
179173	0.08	LG_I:4198566-4198742	0.0246	MADS-box protein AGL3
814871	0.15	LG_I:3683042-3684777	0.00785	Chalcone synthase
708629	0.17	LG_I:3590282-3593590	0.00761	Putative phloem-specific lectin
640148	0.18	LG_I:5110195-5111127	0.00146	Unknown
Differentially expressed transcripts corresponding to the QTL Myc_t2 (on LG III t)				
413017	21.6	LG_III:17355138-17361585	0.000655	Peptide transporter
830999	8.17	LG_III:17698625-17704159	0.00013	Unknown
758242	7.22	LG_III:18074607-18076370	0.000139	Methionine lyase
647351	5.59	LG_III:17638210-17639620	0.000158	PPR repeat
800187	0.01	LG_III:16731851-16733071	0.00000658	Protein EREBP-4
758186	0.07	LG_III:17642081-17643094	0.0131	Steroid sulfotransferase
555048	0.12	LG_III:17431514-17433457	0.00152	Serine threonine-specific kinase

Only transcripts with a significant *p* value (≤0.05) and ≥5.0-fold change in transcript level were considered as significantly differentially expressed between *P. trichocarpa* ECMs and *P. deltoides* ECMs. Those corresponding to the detected QTLs appear in *green* and in *pink*. Transcript differentially expressed between *P. trichocarpa* ECMs (trich) and *P. deltoides* ECMs (delt)

Table 4 Microarrays transcript profiling—the top ten transcripts corresponding to the most up- and downregulated genes across the entire *Populus* genome

Top of up-/downregulated gene in whole genome	Protein ID	Expression ratio *P. deltoides*/*P. trichocarpa*	Position on the poplar genome v1.1	*p* value	Putative function
Overexpression in *P. deltoides*					
1	806759	86.6	LG_XIII:952125-953621	0.0011	Epoxide hydrolases
2	412601	59.0	LG_II:15301824-15302404	0.00424	Epoxide hydrolases
3	830358	58.2	LG_II:11022536-11024605	0.0000936	Flavonol reductase
4	755638	54.8	LG_II:15987878-15991132	0.000929	Glycosyl hydrolase family 3
5	753870	54.4	LG_II:348822-349424	0.0000115	Ubiquitin ligase
6	569526	53.3	LG_XII:1635212-1636804	0.00000124	Unknown
7	654260	53.0	LG_VI:18130929-18131375	0.000641	Steroid binding protein
8	679320	50.7	scaffold_594:4284-4879	0.000000548	Isomerase
9	769294	50.6	LG_X:9340218-9341318	0.0102	Protease inhibitor
10	772380	49.2	LG_XII:1447519-1447952	0.00000123	Ubiquitin ligase
Overexpression in *P. trichocarpa*					
1	259627	0.003	LG_XVIII:12445734-12446451	0.0000994	Plastocyanin
2	767919	0.004	LG_IX:6507424-6509210	0.000000399	Alcohol dehydrogenase
3	788513	0.005	scaffold_314:36977-38287	0.00000041	Unknown
4	649437	0.007	LG_IX:3238298-3239099	0.00000239	Unknown
5	669706	0.009	LG_XVIII:11936887-11937445	0.00000132	Unknown
6	675468	0.009	scaffold_243:83053-83671	0.00000306	Unknown
7	800187	0.010	LG_III:16731851-16733071	0.00000658	Protein EREBP-4
8	829444	0.012	LG_I:6809419-6811292	0.0092	Putative flavonol reductase
9	670561	0.012	scaffold_120:512204-517789	0.000000977	Unknown
10	278262	0.012	scaffold_2047:1741-3229	0.00141	Methyltransferase
11	727226	0.012	LG_XI:13839049-13841721	0.000000498	Auxin efflux Carrier

Only transcripts with a significant *p* value (≤0.05) and ≥5.0-fold change in transcript level were considered as significantly differentially expressed between *P. trichocarpa* ECMs and *P. deltoides* ECMs. Those corresponding to the detected QTLs appear in *green* and in *pink*. Transcript differentially expressed between *P. trichocarpa* ECMs (trich) and *P. deltoides* ECMs (delt)

auxin induce mycorrhiza-like roots on *Pinus mugo*. In the *Eucalyptus globulus–Pisolithus microcarpus* ectomycorrhiza, Tagu et al. (2003) showed that many plant genes are overexpressed during the mycorrhizal development and that the expression of the *EgHypar* gene, exhibiting homology with plant auxin-induced glutathione-*S*-transferases, was overexpressed not only in the ectomycorrhiza, but also by exogenous application of auxin. The upregulation of EREBP-4 in our study fits well with the previous knowledge on the involvement of hormonal interactions in ectomycorrhizal establishment.

Alternatively, microarray analysis in *P. deltoides* mycorrhizas compared to *P. trichocarpa* mycorrhizas detected transcripts for genes encoding proteins such as epoxide hydrolase, multi-antimicrobial extrusion proteins, AtKT1I proteins, WRKY proteins, and cytochrome p450, all reportedly playing roles in plant defenses (Gomi et al. 2003; Kiyosue et al. 1994; Li et al. 2008; Zou et al. 2004). Indeed, the epoxide hydrolase activity has been reported to be involved in the mechanisms of defense in plant–fungus interactions (Gomi et al. 2003). Epoxide hydrolase function in combination with the cytochrome p450 during the reticulation of cutin biopolymers in the cuticle layers of plant cell wall and are thought to create a physical barrier against pathogen attack (Blée and Schuber 1992; Hamberg and Fahlstadius 1992; Pinot et al. 1995). In addition to participation in the cutin biosynthesis, this enzyme produces epoxy fatty acids that may also act as antimicrobial compounds against fungal and bacterial pathogens (Kato et al. 1983, 1985; Masui et al. 1989; Ohta et al. 1990). Similarly, it is known that AtKT1I proteins inhibit the activity of extracellular proteases and can trigger both salicylic acid- and jasmonate/ethylene-dependent defense gene expression (Kariola et al. 2005; Li et al. 2004; Li et al. 2008; Ohme-Takagi and Shinshi 2000). Likewise, WRKY proteins are known to be induced in response to fungal elicitor and play an important role in the regulation of early defense–response genes (Eulgem et al. 1999; Zou et al.

2004). Thus, upregulation of this suite of genes in *P. deltoides* mycorrhizas may explain the lower mycorrhizal rates relative to *P. trichocarpa*. Indeed, the activation of defense mechanisms in *P. deltoides* may inhibit *L. bicolor* colonization, while in *P. trichocarpa* this inhibition may be repressed by the overexpression of EREBP-4 ethylene-sensitive transcription factor. Our results support the conclusions by Felten et al. (2010), Rupp et al. (1989), and Splivallo et al. (2009) that ethylene, released by *Laccaria*, plays a role in primary response of the host plant. Thus, the initial steps in successful colonization may involve *Laccaria* manipulating the host's metabolic machinery and subsequently, decreasing their defenses.

There are limitations to the QTL approach presented in this study. A higher resolution QTL map is needed to narrow down the list of candidate genes. Validation of the detected QTLs different instead of alternate environments and alternate pedigrees is needed. The comparison of the gene expression between the mycorrhizas of the two parents integrates only a part of the interactions which take place during mycorrhizal establishment (i.e., early recognition and signal exchange, physical contact of the mycelium on the root, internal colonization, etc.). Nevertheless, the combination of QTL analysis and whole-genome microarray analysis does provide a list of candidate genes which appear to be involved into mycorrhizal that merit further investigation. In summation, based on our results, the interactions involved in mycorrhizal establishment and colonization are extremely complex and may not be as mutualistic as initially assumed.

Acknowledgments This project was supported by grants from the European Commission project ENERGYPOPLAR and EVOLTREE (to FM). JL was supported by a scholarship from the INRA, Region Lorraine, the ORNL, and the U.S. Department of Energy. Oak Ridge National Laboratory (ORNL) is managed by UT-Battelle, LLC for the US Department of Energy under contract no. DE-AC05-00OR2272. We thank Christine Delaruelle, Béatrice Palin, Judith Richter, Verónica Pereda, Saskia Reinhart, Anne Delaruelle, Simon Duchêne, Aurore Coince, and Jean-Louis Churin for their assistance in the inoculation and in measuring the colonization rates. We also thank Denis Tagu for his help and fruitful discussions.

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