ADVENTITIOUS ROOT AND SHOOT REGENERATION *IN VITRO* IS UNDER MAJOR GENE CONTROL IN AN F_2 FAMILY OF HYBRID POPLAR (*POPULUS TRICHOCARPA* X *P. DELTOIDES*)

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ABSTRACT

In vitro adventitious shoot and root regeneration was studied in a three generation pedigree of *Populus trichocarpa X P. deltoides* hybrids. When cultured on Woody Plant Medium supplemented with μM trans–zeatin (shoot regeneration) or 10 μM indole butyric acid (root regeneration), the parents showed extreme difference in both shoot (100% vs. 0%) and root (67% vs 0%) regeneration. In the F_2 and backcross generations, continuous segregating variation from 0% – 100% was found for both traits. Quantitative genetic analysis suggests that in vitro adventitious shoot and root regeneration in Populus are controlled by major genes. These tissue culture responses were highly heritable (broad sense heritability H_2 = 0.72 and 0.68; for root and shoot regeneration, respectively). One major quantitative trait locus (QTL) that controls shoot regeneration frequency was identified on linkage group C of the map shown in BRADSHAW *et al.* (1994). This single QTL accounted for 34.5% of the genetic variance. A major QTL affecting root regeneration frequency, which accounts for 51.1% of the genetic variance, was found on linkage group N. Correlation analysis and QTL mapping experiments suggest that root and shoot regeneration are controlled by separate genetic mechanisms.

Key words: *Populus*, QTL, RFLP, regeneration, tissue culture, genetics.

Many plant cells retain a latent capacity for complete plant regeneration via adventitious organogenesis – the production of shoot or root meristems from single cells. The regeneration of intact plants from plant cells in tissue culture is controlled by their genetic makeup, epigenetic factors, and culture environment. Manipulation of environmental factors such as the physiological condition of donor plants, components of the culture medium, and incubation conditions has expanded the range of plant species (and genotypes within those species) that can be regenerated. However, the internal physiological and biochemical processes through which heredity and culture environment interact to determine plant regeneration are not fully understood. Elucidation of a genetic basis for plant regeneration *in vitro* might provide clues about the molecular mechanisms involved, as well as guide breeding efforts to improve their suitability as platforms for genetic engineering.

The genetic basis of shoot regeneration in plant tissue culture has been studied for many years. BINGHAM *et al.* (1975) found that the alleles responsible for shoot regeneration in diploid alfalfa (*Medicago sativa* L.) were fixed after two cycles of selection, resulting in considerably increased regenerability over the base population. Other studies in plant species such as cucumber, tomato, and wheat have also shown that regenerability is under strong genetic control (H^2 = 0.43 to 0.87; KOORNNEEF *et al.* 1987; KOMATSUDA *et al.* 1989; NADOLSKA–ORCZYK 1989). Consistent with the ease of breeding and high heritability, segregation analyses from F_1, F_2, and/or backcross populations have indicated that two dominant genes are responsible for shoot regeneration in *M. sativa* L. (REISCH & BINGHAM 1980; WAN *et al.* 1988), *Lycopersicon peruvianum x L. esculentum* Mill. (KOORNNEEF *et al.* 1987), *Cucumis sativus* L. (NADOLSKA–ORCZYK & MALEPSZY 1989), and *Petunia* spp. (DULIEU 1991).

The genetic basis of adventitious root formation, unlike shoot regeneration, has not been studied extensively. Adventitious rooting is a key step in

micropropagation of elite genotypes in many crop and forest tree species. While little literature is available about the genetic basis of adventitious root formation *in vitro,* a study of field–planted eastern cottonwood (*P. deltoides*) cuttings indicated that adventitious root development was under fairly strong genetic control (H^2 = 0.33 – 0.44) and therefore responsive to clonal selection (WILCOX & FARMER 1965).

Molecular markers, such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPDs, WILLIAMS *et al.* 1990), could facilitate the study of molecular mechanisms underlying plant regeneration. Recently, RFLP analyses and linkage maps derived from them have been used to identify chromosomal regions important for shoot regeneration in maize (*Zea mays* L., ARMSTRONG *et al.* 1992) and to map a gene controlling shoot regeneration in tomato (*Lycopersicon peruvianum*, KOORNNEEF *et al.* 1993). RAPD markers associated with somatic embryogenesis in alfalfa (*Medicago sativa* L.) have been identified (YU & PAULS 1993).

Biotechnology can serve as a tool to increase the rate of genetic gain in tree improvement programs. To realize the maximum benefit from biotechnology, the ability to use mature tree genotypes as a starting material for *in vitro* culture is vital because most economically important traits are expressed only in mature trees, and juvenile–mature phenotypic correlations are often low. When introduced into *in vitro* culture systems, the variation in both genetically and epigenetically determined levels of endogenous growth substances and sensitivity to exogenous growth regulators make the development of optimum culture regimes difficult. COLEMAN and ERNST (1990) showed that the adventitious shoot regeneration competence states of *Populus deltoides* stem explants were genotype–dependent. Some of the genotypes that were not competent initially to regenerate could be induced by the manipulation of experimental conditions, while others could not. HAN *et al.* (1990) found that the genotype specific responses of mature black locust (*Robinia pseudoacacia* L.) persisted through a cycle of dedifferentiation (in callus culture) and regeneration (into intact trees). These studies indicate a strong genetic or epigenetic control of tissue culture traits in some woody plants. No multiple generation genetic studies, however, have been reported for the tissue culture response of tree species, mainly because of the problems inherent to forest tree genetics such as long generation interval. In the present study of adventitious shoot and root regeneration in poplar, we took advantage of a three generation (parental, F_1, and F_2 / BC_1) pedigree of *Populus trichocarpa x P. deltoides* hybrids and an ongoing poplar genetic linkage mapping project (BRADSHAW *et al.* 1994). The female *P. trichocarpa* parent responded well to in vitro shoot (100%) and root (67%) regeneration while the male P. deltoides parent showed no response at all under the same conditions. We studied the quantitative genetic aspects of the traits in F_1 hybrids and segregating populations (F_2 and BC_1) and identified the map locations of two quantitative trait loci (QTLs) that are likely involved in control of organogenesis *in vitro*.

MATERIALS AND METHODS

Plant materials

The three generation pedigree was founded in 1981 by interspecific hybridization between *Populus trichocarpa* (T) clone 93–968 and *P. deltoides* (D) clone ILL–129. Two full–sibs from the resulting F_1 family, 53–246 and 53–242, were mated in 1988 to produce the F_2 family 331. The F_1 53–246 was mated to the male parent ILL–129 in 1988 to produce the backcross family (BC_1) 342. Dormant cuttings (20–30 cm long, 1 – 2 cm in diameter) were collected in January of 1991 from Washington State University's Farm 5 in Puyallup, Wash. Greenwood stems that sprouted from the cuttings in the greenhouse were used for the tissue culture experiments.

Tissue culture

Greenwood stems (about 5 mm in diameter) were sterilized in 30% Clorox solution for 15 min followed by 5 washes with sterile distilled water. Sixty stem discs (5 mm thick) from each clone (15 replicates repeated at two different culture shelves) were prepared from internodal segments of the stem and were placed (rightside up) in Petri dishes containing 25 ml of Woody Plant Medium (WPM; LLOYD & MCCOWN 1981) supplemented with 20 $g.l^{-1}$ sucrose, 6 $g.l^{-1}$ agar, and either 5 µM trans–zeatin (shoot regeneration; n = 30) or 10 µM indole butyric acid (IBA, root regeneration; n = 30). Regeneration frequency (% explants producing shoots/roots), number of shoots/roots per explant, and root length were scored after 4 weeks of culture at 26 °C, with 18 hr light/6 hr dark lighting regimes using fluorescent lamps (50 $\mu Em^{-2}s^{-1}$). To test the reproducibility of the tissue culture system, twenty–eight clones were randomly chosen from three representative groups (poor < 33%; 34% < intermediate < 66%; high > 67%) and the experiments were

repeated in the following year under the same conditions.

Frequency data were arcsin transformed (STEEL & TORIE 1980) before analysis to improve normality. All data were analyzed using analysis of variance (ANOVA) from the GLM procedure in SAS (SAS Institute, 1990). The analysis model was $y_{ij} = \mu + \alpha_i + \beta_j + e_{ij}$ (i = 1, 2, ..., t; j = 1, 2, ..., r), where y_{ij} represents the mean of clone i in replication j; μ is the grand mean; α_i is the effect due to clone i; β_j is the effect due to replication j; e_{ij} is the residual. Broad–sense heritabilities for regeneration frequency, number of shoots/roots, and root growth were calculated from variance components derived from the ANOVA model (WU *et al.* 1991). Correlations between pairs of the traits were tested using Pearson's correlation coefficient from the CORR procedure in SAS (SAS Institute, 1990).

Linkage map construction

The *Populus* genetic map consists of 343 RFLP, STS, and RAPD markers, and has been described elsewhere (BRADSHAW *et al.* 1994). The map was made in the same pedigree used for phenotypic measurements. To increase the number of informative markers available for determination of QTL genotypes, additional marker loci found on the linkage groups described previously (BRADSHAW *et al.* 1994) were added to the maps using the "place" command in MAPMAKER 3.0, and the most likely marker placement was accepted. These markers had originally been excluded from the published map because they could not be ordered with confidence exceeding a LOD score of 1.3 vs. alternative orders. The map used for QTL identification is shown in Fig. 2.

The 19 largest linkage groups, representing approximately half the estimated length of the *Populus* genome (BRADSHAW *et al.* 1994), were scanned with MAPMAKER/QTL 1.1 (PATERSON *et al.* 1988; LINCOLN *et al.* 1992). Based on the scanned length and the average spacing between mapped markers, a LOD threshold of 2.9 was chosen from Fig. 4 of LANDER and BOTSTEIN (1989) to reduce to approximately 5% the probability of falsely assigning a QTL. All genotypic data were encoded as though the pedigree were a cross between inbred lines; i.e. only two alleles, derived either from *P. trichocarpa* (T) or *P. deltoides* (D), were recognized at any locus. The mode of gene action was estimated by comparing LOD scores of the various models (dominant, recessive, additive) in MAPMAKER/QTL.

RESULTS

Shoot regeneration

Under the culture conditions used in this study, 100% of stem segments from the female parent *P. trichocarpa* 93–968 produced shoots in vitro, while those from the male parent *P. deltoides* ILL–129 produced none. Their F_1 hybrids, 53–242 (male) and 53–246 (female), showed intermediate responses (87 and 60%, respectively), but higher than the average response (51%) for the F_2 population (Figure 1). Shoot regeneration frequency was significantly different among the F_2 clones ($F = 5.11$, $p < 0.001$). Segregation analysis using the F_2 population showed neither a normal distribution nor discrete phenotypes. This suggests that the adventitious shoot regeneration is controlled neither by many genes nor by a single gene. Both shoot regeneration frequency and shoot number were highly heritable (H^2 = 0.68 and 0.46, respectively; Table 1). Among clones capable of regenerating shoots, shoot number per explant varied significantly ($F = 8.7$, $p < 0.001$). Clones with higher shoot regeneration frequency tended to produce more shoots per explant (r = 0.649; Table 2). Shoot regeneration frequency, however, showed no significant correlation with any rooting traits (Table 2).

We tested the reproducibility of the tissue culture system for shoot regeneration with sub–samples in the

Table 1 Means, ranges, environmental and genetic varioances and broad-sense heritabilities for tissue culture triats estimated from F_2 family 331

Characters	Means	Ranges of means	σ^2_g	σ^2_e	Heritability $\sigma^2_g/(\sigma^2_g+\sigma^2_e)$
Shoot Regeneration (%)	51.1	0 – 100	507	244	0.68
Shoot number	6.8	0 – 25	37.6	44.7	0.46
Root Regeneration (%)	39.3	0 – 90	440	171	.072
Root Number	3.3	0 – 7.3	1.95	4.06	0.32
Root Length (%)	30.7	0 – 58.2	162	394	0.29

Table 2 Pearson's correlation coefficients and significance level for relationship between characters

Characters[1)]	% Shoot	% Root	Shoot Number	Root Number	Root Length
% Shoot	1.000**[2)]				
% Root	ns	1.000**			
Shoot Number	0.649**	ns	1.000**		
Root Number	ns	0.477**	ns	1.000**	
Root Length	ns	0.328*	ns	0.372**	1.000**

[1)] Characters: % shoot / root (% of explants producing shoot / root); # shoot / root (mean number of shoot / root per explant); Root Length (average root length growth)

[2)] * significant at p = 0.005; ** significant at p = 0.01; ns – not significant

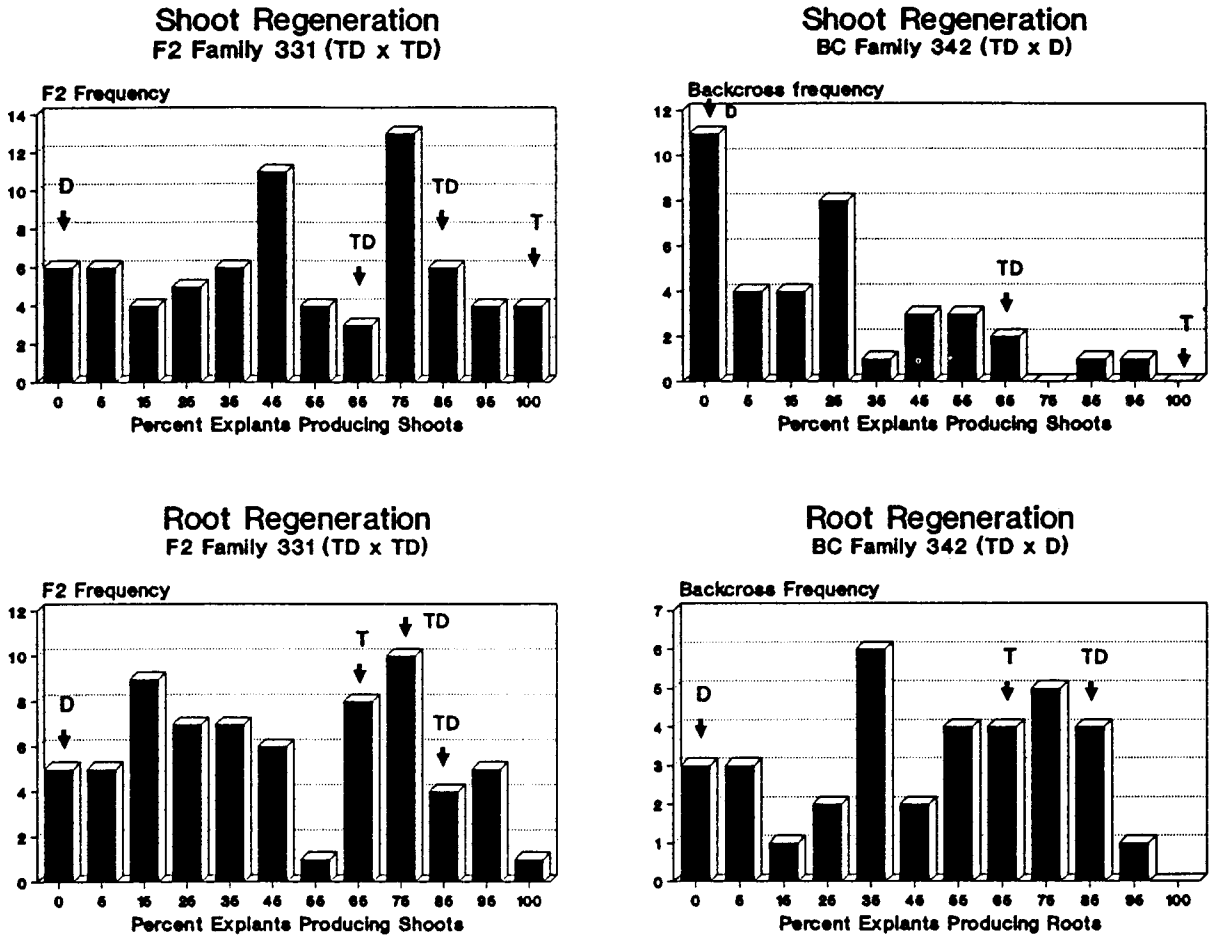
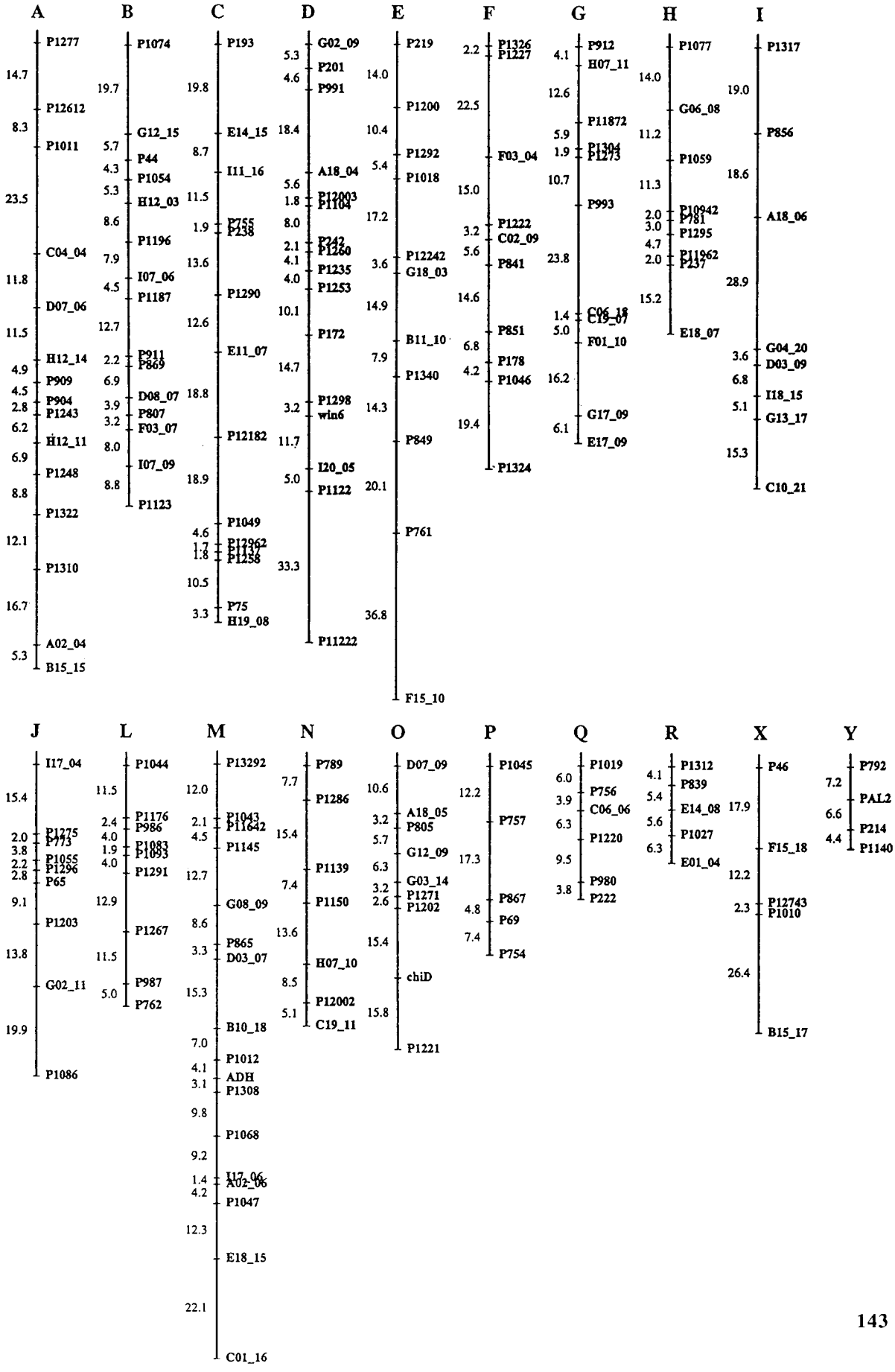


Figure 1 Distribution of phenotypes for adventitious shoot and root regeneration in the F_2 and backcross (BC_1) populations. Values for parental (D and T) and F_1offsprings are shown by arrows

►►

Figure 2 Linkage map of the *Populus* genome used for QTL mapping. Only the largest linkage groups were used. Marker loci beginning with "P" are RFPL (e.g P1277), RAPD loci are named according to their Operon number and approximate fragment size (e.g., Co4–04 is a ~400 bp fragment from C4), and a few loci of known identity is named (e.g. ADH is alcohol dehydrogenase). A more complete description of the map may be found in BRADSHAW *et al.* (1994)


A
P1277
14.7
P12612
8.3
P1011
23.5
C04_04
11.8
D07_06
11.5
H12_14
4.9
P909
4.5
P904
2.8
P1243
6.2
H12_11
6.9
P1248
8.8
P1322
12.1
P1310
16.7
A02_04
5.3
B15_15
B
P1074
19.7
G12_15
5.7
P44
4.3
P1054
5.3
H12_03
8.6
P1196
7.9
I07_06
4.5
P1187
12.7
P911
2.2
P869
6.9
D08_07
3.9
P807
3.2
F03_07
8.0
I07_09
8.8
P1123
C
P193
19.8
E14_15
8.7
I11_16
11.5
P755
1.9
P238
13.6
P1290
12.6
E11_07
18.8
P12182
18.9
P1049
4.6
P12962
1.7
P1137
1.8
P1258
10.5
P75
3.3
H19_08
D
G02_09
5.3
P201
4.6
P991
18.4
A18_04
5.6
P12003
1.8
P1104
8.0
P242
2.1
P1260
4.1
P1235
4.0
P1253
10.1
P172
14.7
P1298
3.2
win6
11.7
I20_05
5.0
P1122
33.3
P11222
E
P219
14.0
P1200
10.4
P1292
5.4
P1018
17.2
P12242
3.6
G18_03
14.9
B11_10
7.9
P1340
14.3
P849
20.1
P761
36.8
F15_10
F
P1326
2.2
P1227
22.5
F03_04
3.2
P1222
5.6
C02_09
P841
14.6
P851
6.8
P178
4.2
P1046
19.4
P1324
G
P912
4.1
H07_11
12.6
P11872
5.9
P1304
1.9
P1273
10.7
P993
23.8
C06_18
1.4
C19_07
5.0
F01_10
16.2
G17_09
6.1
E17_09
H
P1077
14.0
G06_08
11.2
P1059
11.3
P10942
2.0
P781
3.0
P1295
4.7
P11962
2.0
P237
15.2
E18_07
I
P1317
19.0
P856
18.6
A18_06
28.9
G04_20
3.6
D03_09
6.8
I18_15
5.1
G13_17
15.3
C10_21
J
I17_04
15.4
P1275
2.0
P773
3.8
P1055
2.2
P1256
2.8
P65
9.1
P1203
13.8
G02_11
19.9
P1086
L
P1044
11.5
P1176
2.4
P986
4.0
P1083
1.9
P1093
4.0
P1291
12.9
P1267
11.5
P987
5.0
P762
M
P13292
12.0
P1043
2.1
P11642
4.5
P1145
12.7
G08_09
8.6
P865
3.3
D03_07
15.3
B10_18
7.0
P1012
4.1
ADH
3.1
P1308
9.8
P1068
9.2
I17_06
1.4
A02_06
4.2
P1047
12.3
E18_15
22.1
C01_16
N
P789
7.7
P1286
15.4
P1139
7.4
P1150
13.6
H07_10
8.5
P12002
5.1
C19_11
O
D07_09
10.6
A18_05
3.2
P805
5.7
G12_09
6.3
G03_14
3.2
P1271
2.6
P1202
15.4
chiD
15.8
P1221
P
P1045
12.2
P757
17.3
P867
4.8
P69
7.4
P754
Q
P1019
6.0
P756
3.9
C06_06
6.3
P1220
9.5
P980
3.8
P222
R
P1312
4.1
P839
5.4
E14_08
5.6
P1027
6.3
E01_04
X
P46
17.9
F15_18
12.2
P12743
2.3
P1010
26.4
B15_17
Y
P792
7.2
PAL2
6.6
P214
4.4
P1140

following year and found no significant difference between the results of the two experiments ($t = 1.88$, $p > 0.05$).

One major QTL was identified (near P1290; LOD = 3.04) for shoot regeneration frequency (arcsin transformed) on linkage group C of the map shown in BRADSHAW *et al.* (1994; Fig 2). As expected from the phenotypes of the *P. trichocarpa* and *P. deltoides* parents and the F_1 offspring, the *P. deltoides* allele at the shoot regeneration frequency QTL reduced shoot regeneration frequency in a partially recessive fashion. This single QTL accounts for 24% of the phenotypic variance and 35% of the genetic variance in shoot regeneration frequency among the F_2 offspring. No QTLs for shoot number were located.

Root regeneration

The male *P. deltoides* parent produced no roots *in vitro* while the female *P. trichocarpa* parent regenerated roots from 67% of the stem discs. Their F_1 hybrids, 53–242 and 53–246, showed higher responses (71 and 87%, respectively) than those of parents. Root regeneration frequency, root number per explant, and root length were significantly different among F_2 individuals ($F = 6.1$, 6.1, and 7.0, respectively; $P < 0.001$). Observations on the segregation patterns of both F2 and backcross populations strongly suggest that the rooting is under major gene control (Fig. 1). Rooting frequency was highly heritable ($H^2 = 0.72$), while root number and growth had relatively low heritabilities ($H^2 = 0.32$ and 0.29, respectively; Table 1). Root regeneration frequency was moderately correlated with both root number and root length growth ($r = 0.477$ and 0.328, respectively; Table 2).

A major QTL (near P1286; LOD = 3.30) affecting root regeneration frequency (arcsin transformed) was found on linkage group N (BRADSHAW *et al.* 1994; Fig. 2). This QTL accounts for 37% of the phenotypic variance and 52% of the genetic variance in the F_2. The root regeneration frequency QTL shows strong indications of apparent overdominance (heterozygous class of offspring superior to either homozygous class) in its mode of action; additive, dominant, and recessive models are more than 100–fold less likely than overdominance. The ratio of dominance to additivity (d/a) is 9.0 (d/a = 0 if additive; d/a = + 1 if dominant). No QTLs were found for root number or length.

DISCUSSION

The ability to regenerate whole plants from unorganized tissues is prerequisite for successful application of biotechnology to genetic improvement programs. The genetic bases of the physiological processes and biochemical pathways involved in plant regeneration *in vitro* are poorly understood. Genetic dissection of the process with linkage maps and segregating progenies may provide a better understanding since the response in tissue culture is dependent on both heritable and non–heritable components. We have studied the genetic control of root and shoot regeneration in segregating populations of a hybrid poplar family. This *Populus* system has several advantages for such studies: 1) the regeneration responses of the parents are extreme in their difference for both shoot (100% vs. 0 %) and root regeneration (67% vs. 0 %); 2) a three generation pedigree segregating for these traits is available; and 3) the genetic linkage map of poplar (BRADSHAW *et al.*, 1994) provides an opportunity to search for molecular markers linked to the traits.

The quantitative genetic analysis using segregating populations to study *in vitro* adventitious root and shoot regeneration of tree species suggests that the determination of regenerability is similar to that of herbaceous crop species. Strong genetic control of tissue culture responses in the *Populus* hybrids is implied. High heritability estimates for *in vitro* adventitious root ($H^2 = 0.72$) and shoot regeneration ($H^2 = 0.68$) from poplar stem segments are consistent with previous estimates for shoot regeneration of various crop species ranging from 0.43 to 0.87 (KOORNNEEF *et al.* 1987; NADOLSKA–ORCZYK & MALEPSZY 1989; KOMATSUDA *et al.* 1989).

Our results demonstrate that *in vitro* shoot regeneration from poplar stem discs is controlled by at least one major QTL with partial dominance of the favorable allele. Dominance of good regeneration capacity has been described before. Genetic analyses of shoot regeneration from callus tissues of alfalfa (REISCH & BINGHAM 1980; WAN *et al.* 1988; YU & PAULS 1993) and tomato (KOORNNEEF *et al.* 1987), leaf explants of cucumber (NADOLSKA–ORCZYK & MALEPSZY 1989), and the upper parts of petunia seedlings (DULIEU 1991) all indicated that this trait is controlled by two dominant genes.

Although rooting is an equally important aspect of adventitious organogenesis, especially in clonally propagated perennial species like *Populus,* genetic studies on *in vitro* adventitious root regeneration have not been reported. However, substantial evidence for genetic control of adventitious rooting from field–planted cuttings exists (HAISSIG 1986). Analysis of clonal variations for rooting traits of western hemlock clones showed that the rooting traits were highly heritable (H^2 = 0.87 – 0.92) and genetic correlations between pairs of traits were high (r = 0.66 – 0.99) (FOSTER *et al.* 1984). We found that *in vitro* rooting from poplar stem discs is highly heritable (H^2 = 0.72) and governed by at least one major QTL with an apparently over dominant mode of action.

Reciprocal grafting studies between normal and mutant phenotypes have shown that the two organs (shoot/root) can affect each other (ZOBEL 1986). Since root characteristics can be affected by shoot genotype, tissue culture using explants may serve as a better system for genetic analysis of adventitious root regeneration.

Correlation data and QTL mapping experiments suggest that root and shoot regeneration are controlled by separate genetic mechanisms. Previous studies have also shown that different tissue culture traits are under different genetic control. Callus culture and shoot regeneration are controlled by different genetic mechanisms in wheat (LAZAR *et al.* 1984), maize (DUNCAN *et al.* 1984), tomato (KOORNNEEF *et al.* 1987), and barley (KOMATSUDA *et al.* 1989).

Traits that appear quantitative in plants (based on surveys of open pollinated families, for example) are not necessarily polygenic. The important aspects are to 1) get an accurate assessment of the phenotype of each individual and 2) look within specific families to limit the number of alleles which are being examined at one time. The simple inheritance of a significant portion of the phenotypic variance for each of the traits suggests that the selection of favorable genotypes be made part of an integrated genetic improvement program. The identified QTLs could be used in dissecting '*in vitro* regeneration' into more understandable entities. With the aid of these molecular markers, it is also possible to screen more readily 'regenerable' genotypes for biotechnological improvement of *Populus*.

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