Identification of AFLP Markers Associated with Embryonic Root Development in *Populus tomentosa* Carr.

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Abstract

Embryonic root (radicle) development in the mature embryo following germination is essential for the formation of the root organ in plants. In this study a phenotype described by a lack of proper radicle development was identified in an intraspecific hybrid of *Populus tomentosa* Carr.. Association of this trait with Amplified Fragment Length Polymorphisms (AFLPs) markers was investigated in a segregating F_1 population generated by intraspecific-controlled crossing between a highly fertile female *P. tomentosa* clone "5082" and a male *P. tomentosa* clone "JY". A total of 3193 seeds were obtained, and the rate of germination found to be 48.74% at 15 to 20 days. 376 (24%) of seedlings were shown to lack a root organ following visual assessment of the developing radicle. Genetic regulation of this trait appeared to be via a single dominant gene or a set of tightly linked genes, based on the 3:1 ratio of the rooting versus non-rooting seed embryos. A Bulked Segregant Analysis approach using 5600 AFLP markers was applied to this population and revealed 2 AFLP markers, *Eco*RI + $_{GAG}$/*Mse* I + $_{AAT}$-492 and *Eco*RI + $_{GAG}$/*Mse* I + $_{CCA}$-502, that were associated with the radicle development-controlling locus in *P. tomentosa*. The AFLP markers identified have potential for application in hybrid breeding via marker assisted selection, and provide a starting point for map based cloning of the radical development-controlling gene.

Key words: Bulked Segregation Analysis, *Populus tomentosa* Carr., Qualitative Trait, Dominant Gene.

Introduction

Populus tomentosa Carr. is a native species in the section Leuce under *Populus* in China. It is an important species for cultivation in Northern China and has a very long planting history (ZHU and ZHANG, 1997). Many wild and cultivated *P. tomentosa* varieties have emerged during the evolution of the species. This accumulated genetic variation can be utilized to study genetics of valuable traits in *P. tomentosa* has many favorable characteristics such as rapid growth, resistance to disease, and high wood quality (ZHANG et al., 2004). This species has played a key role in timber production and ecological environmental protection along the Yellow River (ZHU and ZHANG, 1997). However, unlike *P. deltoides*, *P. trichocarpa* or *P. nigra*, it is normally difficult to generate a large intraspecific population for selection and cultivation of excellent hybrid seedlings in *P. tomentosa* because its overall ability to sexually reproduce is poor, and particular individuals are often sterile. This has limited research on trait segregation in *P. tomentosa* progeny, and restricted its potential for commercial application in China. To deal with this problem, ZHANG et al. (1992) explored the flowering and bearing habits of clones in the arboretum of *P. tomentosa* of 1054 individuals collected from all over China. Several female and male clones with a higher ability to successfully reproduce sexually were identified. These highly fertile *P. tomentosa* clones provided excellent parent materials for further performing large scale intraspecific hybridization in *P. tomentosa*.

Radicle development has become the subject of considerable attention in recent years because it plays a crucial role in the life cycle of flowering plants. In most angiosperm plant species the primary root and its shoot meristems are established during embryogenesis (NATESH and RAU, 1984; SCHERES et al., 1994; LAUX and JURGENS, 1997; VERNOUX et al., 2000). Upon germination the primary root and shoot meristems initiate post-embryonic development by producing the root organ. In order to understand the developmental mechanisms that regulate root formation, previous researchers made great efforts to study root organogenesis in *Arabidopsis thaliana*. Mutation analysis has identified major regulatory genes controlling meristem and root organ development (MAYER et al., 1991; CHENG et al., 1995; KUBO et al., 1999). A few genes are also known to be required for lateral root development (CELENZA et al., 1995). Several genes are shown to be essential for root meristem formation in the *Arabidopsis* embryo (MAYER et al., 1991; CHENG et al., 1995; KUBO et al., 1999).

The analytical techniques used in radicle development research in *Arabidopsis* are not well suited for identification of the loci controlling radicle development in forest trees, due to trees have inherent characteristics, such as dioecious, long generation intervals, lack of available mutants and near-isogenic lines. However, identification of randomly generated molecular markers based on sequence polymorphisms linked to the trait of interest is well suited to forest trees. Associations between molecular markers and economically important traits were first reported by SAX (1923). The development of the Bulked Segregant Analysis (BSA) method (MICHELMORE et al., 1991) allowed tree breeders to combine molecular markers with the BSA strategy to reliably detect markers linked to economically important traits in forest trees (CERVERA et al., 1996; VILLAR et al., 1996; WILCOX et al., 1996; KONDO et al., 2000; STIRLING et al., 2001).

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In this paper, we describe the Mendelian segregation of a seed radicle development trait in intraspecific hybrid progeny of *P. tomentosa* and use BSA method to identify the AFLP markers linked to the gene(s) controlling radicle development. To our knowledge, this is the first report on genetic analysis of seed radicle development and its association with Amplified Fragment Length Polymorphisms (AFLPs) markers based on a large intraspecific population in *P. tomentosa.*

Materials and Methods

Plant materials and measurements

In 2001, a controlled intraspecific hybridization was made between a highly fertile female clone "5082" of *P. tomentosa* (collected in the arboretum of *P. tomentosa* at Guanxian County, Shandong province, China) and a male clone "JY" of *P. tomentosa* (collected at Xi'an, Shannxin province, China), which gave rise to 3193 seeds. Furthermore, other parental materials TB01 (female) and TB11(male) ,which are the hybrids between *P. tomentosa* and *P. bolleana*, were used in this study.

The seeds were sterilized in 0.1% hydrargyrum chloride for 30 s and then with 75% ethanol for 2 min. After five washes in sterile distilled water, seeds were germinated on flask containing 0.5 x Murashige and Skoog (MS) salt mixture, pH 5.8, in 0.8% agar. Flasks were incubated in a near vertical position at 22°C, 70% humidity and a cycle of 16 hr light/8 hr dark.

Measurements of phenotype were made at 15 to 20 days after germination, and the rate of germination was 48.74%. We obtained a total of 1555 seedlings with 376 showing the novel phenotype (lacked a visible root). Chi-square tests (d. f. = 1, $P < 0.05$) were conducted to check the Mendelian ratio of normal rooting seedlings versus aberrant (lacking a visible root) rooting seedlings. The normal rooting seedlings were then sown in soil while the seedlings lacking a visible root were fixed in a solution of ethanol and acetic acid (75% ethanol and 25% acetic acid).

Marker assessments

Total genomic DNA was isolated from young leaves of 98 progeny individuals, of which 60 progenies were randomly sampled from rooted seedlings and 38 from non-rooted ones, using the CTAB method (HOISINGTON et al., 1994). The template DNA concentration was estimated by comparing the fluorescence intensities of ethidium bromide-stained samples to those of λ-DNA standards on 0.8% agarose gel.

The AFLP protocol developed by VOS et al. (1995) for amplifying the selective restriction-ligation fragments was followed with minor modifications. Total genomic DNA (0.40 µg) from each individual was digested and ligated with 3.0 U *Eco*RI, 3.0 U *Mse*I and 1.5 U T_4 DNA ligase in 20 µl reaction mixtures containing T_4 ligase buffer (New England Biolab), 5 pmol *Eco*RI adapter and 50 pmol *Mse*I adapter. The restriction-ligation reaction was incubated at 37°C overnight.

The pre-amplification reaction was performed with 4 µl of template DNA (1:10 solution diluted from the restriction-ligation mixture), using a pair of primers (E00/M00) with nucleotide sequence complementary to those on the *Eco*RI and *Mse*I adapters. The reaction mixtures (20 µl) consisted of 0.6 U *Taq* polymerase (Promega), 30 ng E00 primer, 30 ng M00 primer, 1x PCR buffer (Promega) and 0.2 m*M* each of all four dNTPs (Promega). PCR amplification consisted of 30 cycles of a 30-s denaturation at 94°C, 30-s annealing at 56°C and a 60-s extension at 72°C with 5-min final extension at 72°C. Four microliters of a 1:20 dilution of the final pre-amplification DNA mixture was then selectively amplified using 40 ng each of different *Eco*RI/*Mse*I primer combinations with three selective nucleotides at the 3' end in 20 µl reaction mixtures containing 0.8 U *Taq* polymerase (Biostar), 1 x PCR buffer (Promega) and 0.2 m*M* each of all four m*M* dNTPs (Promega) using the PCR-cycle profile described by VOS et al. (1995) with 5-min final extension at 72°C. All PCR reactions were performed using a 9600 Perkin Elmer thermo-cycler.

The selective amplification reaction products were mixed with 0.5 x volume of loading buffer (98% formamide, 10 m*M* EDTA, 0.05% bromphenol blue, and xylene cyanol), denatured at 95°C for 10 min, loaded on a 6% denaturing polyacrylamide gel (7.5 *M* urea) and electrophoresed at constant power (95 W) in 1x TBE buffer (50 m*M* Tris, 50 m*M* boric acid, 1 m*M* EDTA, pH 8.0). Products of the selective amplification reaction were detected using the silver staining method as described by TIXIER et al. (1997).

Bulked Segregant Analysis

Bulk Segregant Analysis (BSA) method for detecting AFLP markers associated with loci controlling seed radicle development was performed as described by MICHELMORE et al. (1991). The two bulks were made by mixing equal amounts of pre-amplified DNA products from 15 normal rooting (bulk 1) and 15 non-rooting (bulk 2) seedlings, respectively. The primer combinations generating polymorphic loci between the two bulks were subsequently used to detect each individual DNA in the F_1 progeny.

Figure 1. – Seedling appearance after seed germination in *P. tomentosa* Carr. (Top: seedling with non-visible root organ; Bottom: seedling with normal root organ).

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Table 1. – Trait segregation in F_1 population derived from different crossing combinations.

Cross combinations	No. of seeds	Germination rate (%)	No. of rooting seedlings	No. of non-rooting seedlings
Clone "5082"×clone "JY"[a]	354	42.10	109	39
Clone "5082"×clone "JY"[b]	3193	48.74	1179	376
clone "5082"×clone "TB11"[a]	386	84.50	326	0
clone "TB01" ×clone "JY"[a]	3801	85.20	3238	0

[a] in 2000
[b] in 2001

Linkage analysis was performed using the formulas r = nCol/(nCol + nLer) described by MATHER (1938) where r is the recombination rate, n is the numbers of the progenies, Col is the recombination frequency, Ler is the non-recombination frequency, and the genetic distances were calculated using the Kosambi mapping function with centiMorgans (cM) (KOSAMBI, 1944).

Results

Phenotypic and genetic analysis of radicle development

Different crossing combinations were designed and performed in order to establish a mapping population for construction of genetic maps in *P. tomentosa* Carr. (ZHANG et al., 2003). Seedlings with non-visible root organ were identified in the progeny derived following a preliminary intraspecific cross between the female clone "5082" and male clone "JY" (*Fig. 1*). As shown in *Table 1*, 354 seeds were obtained from this cross. After germination, we observed 39 seedlings with the non-rooting phenotype, which accounted for 26.35% of the total seedlings. However, seedlings without a visible root organ were not obtained in progeny derived from two other interspecific crossing combinations (clone "5082" clone "TB11" and clone "TB01" x clone "JY"). To test the reproducibility of the observed ratio of the rooting to non-rooting phenotype in progeny of clones "5082" and "JY", a controlled cross was repeated using the same materials as in the preliminary trial. Phenotypic segregation for a radicle development trait was observed in the new progeny. A total of 3193 seeds were obtained and the rate of germination was 48.74% (1555 seedlings) (*Table 1*). 1179 seedlings showed normal radicle development and 376 seedlings lacked a visible root organ. The segregation ratio of normal rooting to non-rooting seedlings was 3 : 1 in *P. tomentosa* and chi^2 = 0.557 ($P_{0.05}$ = 3.84). This segregation ratio suggested that seed radicle development character is a qualitative trait to be controlled by a single locus with two alternative alleles of which one allele is completely dominant to the other. Segregation analysis of the F_1 progeny of

Table 2. – *Eco*RI $_{+3}$/*Mse* I $_{+3}$ primer combinations assayed in *P. tomentosa* Carr..

	Eco$_{+AAG}$	*Eco*$_{+ACC}$	*Eco*$_{+CAT}$	*Eco*$_{+CTG}$	*Eco*$_{+GAG}$	*Eco*$_{+GTC}$	*Eco*$_{+TCA}$	*Eco*$_{+TCG}$	*Eco*$_{+TTA}$
Mse$_{+AAC}$	√a	√	√	√	√	√	√	√	√
Mse$_{+AAT}$	√	√	√	√	√	√	√	√	√
Mse$_{+AGC}$	√	√	√	√	√	√	√	√	√
Mse$_{+CAG}$	√	√	√	√	√	√	√	√	√
Mse$_{+CCA}$	√	√	√	√	√	√	√	√	√
Mse$_{+CTG}$	√	√	√	√	√	√	√	√	√
Mse$_{+GTC}$	√	√	√	√	√	√	√	√	√
Mse$_{+TAC}$	√	√	√	√	√	√	√	√	√
Mse$_{+TGT}$	√	√	√	√	√	√			

[a] selected primer combination.

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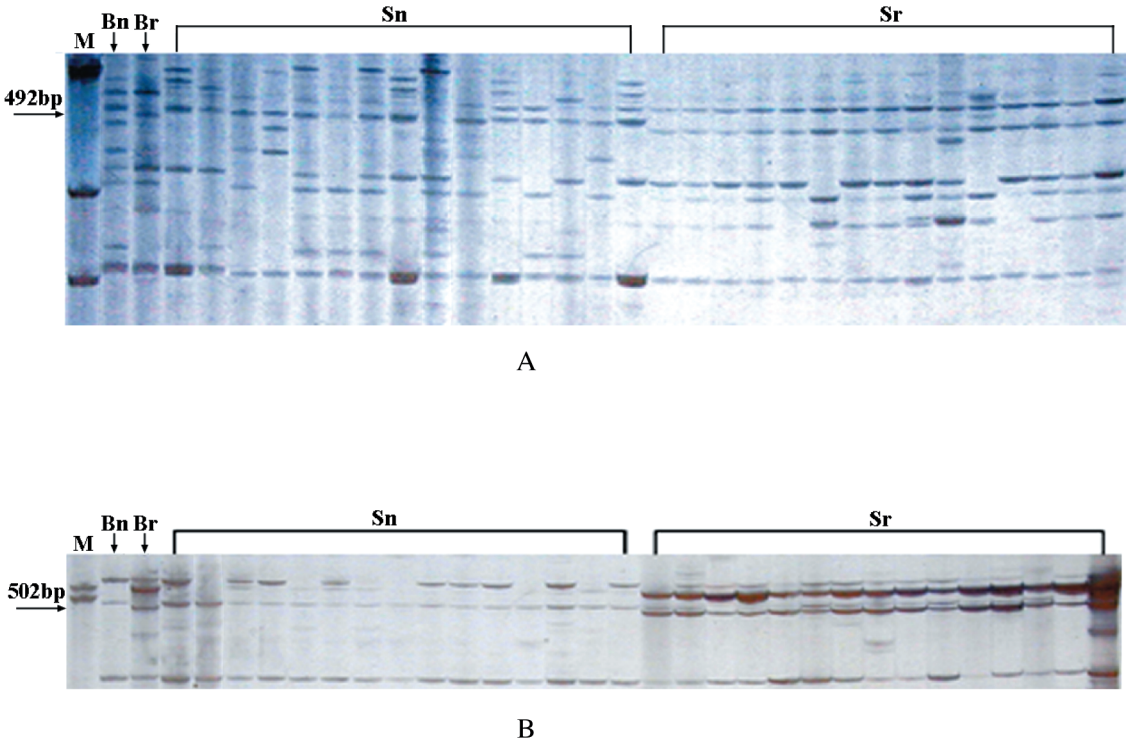

Figure 2. – AFLP markers linked to the loci of radicle development-controlling in *P. tomentosa* Carr.. A represents marker $E_{EAG}/M_{AAT-492}$; B represents marker $E_{EAG}/M_{CCA-502}$; M represents 1Kb DNA ladder (NEB Biolab), Bn represents non-visible root seedlings bulks, Br represents normal rooting seedlings bulks, Sr represents the identified markers by BSA that are present in the normal rooting seedlings, Sn represents the identified markers by BSA that are absent in the non-visible rooting seedlings.

these seedlings indicated that a single dominant genetic locus or a set of closely linked loci was responsible for post-embryonic radicle development in *P. tomentosa*. This result implied that the genotype controlling radicle development loci is heterozygous (Aa) in both parents (clone "5082" and clone "JY") and produced segregation ratios of 1AA : 2Aa : 1aa of genotype in the F1 progeny. This genotype resulted in the segregation ratio of 3 : 1 seedlings of normal root versus without visible root for phenotype in the F_1 progeny of *P. tomentosa.*

AFLP linked to radicle development

To expedite the identification of the AFLP markers linked to genes involved in radicle development in the post-embryo for *P. tomentosa*, screening for AFLP-polymorphisms was carried out by bulked segregant analysis (MICHELMORE et al., 1991). Approximately 5600 selectively amplified DNA fragments ranging in size from 40 to 650 nucleotides were scored with selected 78 primer combinations (*Table 2*). Most primer combinations showed no polymorphic loci between rooting and non-rooting bulks. However, primer pairs *Eco*RI + $_{GAG}$/ *Mse* I $_{+AAT}$ and *Eco*RI $_{+GAG}$ / *Mse* I $_{+CCA}$ produced markers that were polymorphic between the two bulks (*Fig. 2*). These two candidate AFLP markers were approximately 492 bp and 502 bp in size, respectively. DNA samples from 98 individuals were then amplified, scored, and the results used to analyze the linkage between the candidate AFLP markers and the radicle development-controlling allele. We identified one recombinant F_1 individual with primer combination *Eco*RI $_{+GAG}$ / *Mse* I $_{+AAT}$, and two for primer pair *Eco*RI $_{+GAG}$ / *Mse* I $_{+CCA}$. Both candidate AFLP markers thus appear to be tightly linked to the radicle development-controlling loci in *Populus tomentosa*. The linkage distance between $E_{GAG}/M_{AAT-492}$, $E_{GAG}/M_{CCA-502}$ and the radicle development-controlling loci was approximately 1 and 2 cM, respectively.

Discussion

Organogenesis is species specific, indicating that it is under strong genetic control. Thus, a better understanding of the genetic basis of organ formation should eventually allow us to modify economically important traits in plants in a controlled manner. The constant activation of meristems results in the formation of new organs in plants. Previous studies have demonstrated that the primary root and its corresponding meristems are laid down during embryogenesis in angiosperms (NATESH and RAU, 1984; SCHERES et al., 1994; LAUX and JURGENS, 1997; VERNOUX et al., 2000). They are committed to post-embryonic development upon germination by producing visible root organ (lateral root and adventitious root). In our study, root organ formation in *P. tomentosa* was due to initiation of cell differentiation more than cell division. The existence of specific mutants in the root organ phenotype demonstrates that this trait is under tight genetic control. A Medelian segregation ratio (3:1) of radicle development in F_1 segregating progeny was observed in *P. tomentosa*. The use of a combination of Bulked Segregant Analysis (BSA) and AFLP methodolo-

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gy helped us identify two markers tightly linked (linkage distance ~ 1-2 cM) to a radicle development trait. These results represent an important first step in the molecular analysis of radicle development trait in *P. tomentosa.*

The allelic mutation affecting radicle development

Recessive mutations in the gene regulating radicle development give rise to seedlings that contain major organs with a distinct seedling appearance that includes the normal cotyledons, hypocotyls and epicotyl, but lacks a visible root organ. The phenotypic ratio of offspring (rooting : non-rooting) was 3 : 1 in *P. tomentosa* and $\chi^2 = 0.557 < \chi_{0.05} = 3.84$. This segregation ratio suggested that radicle development character is a qualitative trait and probably is controlled by a single complete dominant gene or by a set of closely linked genes. This result could be due to allelic mutation (from A to a) for radicle development trait in unique parental materials (clone "5082" and clone "JY") during the long course of natural evolution in the field. For example, A and a are the two alleles which might occupy one locus controlling radicle development in a diploid *P. tomentosa*. A and a are commonly considered to be dominant and recessive genes, respectively. When the individual tree has AA or Aa located in a locus controlling radicle development, the seedlings exhibit normal radicle development and can survive to complete a full life cycle in the field. Conversely, the offspring with aa (lethal recessive mutation) derived from the crossing between both parents with Aa genotype lack a functional root and die because they cannot absorb nutrient materials from soil in the field. The seedlings with a non-rooting phenotype described here are distinct from that of previously described mutants that lacking lateral root or adventitious root (Benfey et al., 1993; Willemsen et al., 1998; Kubo et al., 1999), which have no whole root organ. To our knowledge, this is the first mutant of poplar shown to lack a visible root organ in which the affected seedlings contain a naturally-occurring recessive loss of function mutation. However, such a mutant is an excellent analytical material for studying the expression and function of the relevant gene(s). Therefore, these "without visible functional root" seedlings obtained in these experiments will provide an ideal model system for unraveling the genetic basis of organ development in forest trees.

Identification of AFLP markers linked to loci controlling radicle development

In our study, a combination of Bulked Segregant Analysis and AFLP methodology was successfully employed to identify two AFLP markers, $E_{GAG}/M_{AAT-492}$, $E_{GAG}/M_{CCA-502}$, associated with a radicle development trait. Furthermore, these markers appear to be tightly linked to the loci controlling radicle development in *P. tomentosa*. Bulked Segregant Analysis is a very efficient method for identifying markers associated with monogenic qualitative traits based on a segregating population in poplar. This method allows rapid detection of markers tightly linked to the target trait segregating in the F_1 progeny, not required constructing near-isogenic line, F_2 and backcross population (Michelmore et al., 1991). AFLP markers plus BSA is an excellent "combination" method for conducting linkage analysis in *P. tomentosa*. AFLP is highly reliable and reproducible due to its use of highly stringent PCR, in contrast with RAPD's problem of low reproducibility. AFLP requires no previous sequence or probe information as does RFLP and SSR. Given enough primer pairs spanning the average-frequency restriction sites, an entire genome could be scanned at the DNA level in a reasonable amount of time. In our study, only 78 *Eco*RI $_{+3}$/*Mse* I $_{+3}$ primer combinations were required. The modest number of primers used indicated that AFLP can overcome problems due to low polymorphisms between intraspecific individuals. It is also a fast technique for detecting the specific genomic regions controlling organ development in poplar. Furthermore, the two AFLP markers identified here should be convertible to simple, rapid and low-cost PCR marker types like STS or SCAR. Doing so would enhance and economize the breeding programs, for example, identified markers could assist selection of seedlings homozygous for the normal radicle phenotype, that could be used in further hybridization experiments, and improve the 24% of viable seedlings obtained. However, it will be necessary to isolate and clone the gene(s) controlling radicle development and perform a comparative study of the structures and function of this gene to substantiate these putative results. Therefore, we will construct the high density genetic linkage maps and identify the putative QTLs controlling radicle development trait in *P. tomentosa*. Identification of two molecular markers tightly linked to the target trait is an important first step toward isolation and cloning of the gene(s) controlling radicle development trait in *P. tomentosa.*

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Growth Performance and Variability in Different Clones of *Gmelina arborea* (ROXB.)

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Abstract

A clonal trial of *Gmelina arborea* consisting of seventy clones selected from ten geographical locations in four states of northeast India was evaluated for height, diameter at ground level (DGL) and diameter at breast height (DBH) at the Experimental Station, Rain Forest Research Institute, Jorhat, Assam, India. The performance of all the traits at the age of 24 months showed significant variations between the clones. Broad sense heritability for height, DGL and DBH was 0.31, 0.44

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