IDENTIFICATION OF RAPD MARKERS ASSOCIATED WITH CROWN FORM IN *CUPRESSUS SEMPERVIRENS* BY BULKED SEGREGANT ANALYSIS

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ABSTRACT

Common cypress (*Cupressus sempervirens* L.) appears in two varieties: (a) *C. sempervirens* var. *pyramidalis* and (b) *C. sempervirens* var. *horizontalis* which present different crown forms. The phenotype of the former variety is characterized by columnar or narrowly conical habit, whereas the phenotype of the latter is characterized by broadly spreading branches. Bulked segregant analysis was used to identify random amplified polymorphic DNA (RAPD) markers associated with crown form. The two pools of individuals from an F_1 population segregating for crown form, and both parental clones were screened with 198 10-mer primers. Out of seven markers that exhibited joint segregation with the trait of interest, one RAPD marker (OPE-12_{570}) was found to be tightly linked to a genomic area associated with crown form. The marker was present in the "*horizontalis* form" bulk and parent and absent in the "*pyramidalis* form" bulk and parent. Further analysis of individual progeny clones, in this particular genetic background, showed that the mapping distance between OPE-12_{570} and the above genomic area was 5.9cM. The tight linkage of this marker to a presumably major gene influencing crown form is an important first step towards the ultimate identification and cloning of such a gene. It may further permit early selection of forms at nursery or *in vitro* culture stage particularly in this family. It might also facilitate breeding programs and studies on the introgression of the two forms in natural populations.

Keywords: *Cupressus sempervirens*, crown form, bulked segregant analysis, RAPD markers, breeding programs.

INTRODUCTION

Crown form variability in cypress (*Cupressus sempervirens* L; *Cupressaceae*) has been recognized since the time of Theophrastus (4th century BC) and yields two typical varieties: (a) variety *horizontalis* with broad spreading branches, broadly conical crown, and wide angles (approaching vertical) between branches and stem, and (b) variety *pyramidalis* with columnar or narrowly conical crown habit forming acute angles between branches and stem. Cypress natural populations are comprised by the *horizontalis* variety. The variety *pyramidalis*, a popular ornamental variety, is the most widely planted of all cypresses (JOHNSON 1974). Often *C. sempervirens* var. *pyramidalis* forms naturalized stands derived from a number of introduced individuals.

The two varieties are interfertile and can give progenies which, apart from the parental forms, present different intermediate types of crown structure (PANETSOS 1967). The two varieties are characterized by some particular morphological and geometrical features: relative branch length, branch rectitude or curvature, initial branch insertion angle, occurrence and importance of reiteration. As crown form and especially branch angle are significant determinants of technical wood quality and quantity, the establishment of molecular markers linked to form is of paramount practical importance. Such markers would be useful in early selection of nursery or *in vitro* cultured individuals designated for reforestation and landscape architecture applications. The existence of the two crown varieties provides an excellent model framework into which genetic and molecular hypotheses concerning crown

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form control could be assessed. Conclusions could be subsequently evaluated or extended to other forest or horticultural species in which plant form is of economic importance.

Forest trees have long generation times and are still in the earliest stages of domestication. They are typically highly heterogeneous and few extended pedigrees are available. Because of these limitations, there is a relatively greater potential for DNA markers to improve genetic analysis and to accelerate breeding in forest trees, compared to highly domesticated crops. However, DNA markers have not been extensively applied to practical tree improvement due to technical and theoretical limitations, such as high levels of heterozygosity and marker linkage equilibrium in populations (STRAUSS *et al.* 1992). Recent progress has shown that PCR based DNA markers can be widely used to map individual forest trees. Some quantitative trait loci (QTLs) have already been identified in forest trees for commercially important traits. Randomly amplified polymorphic DNA genetic markers (RAPDs) were used to rapidly identify markers linked to genes or genomic regions of interest by bulked segregant analysis (MICHELMORE *et al.* 1991). Using this method WILCOX *et al.* (1996) identified a dominant gene that confers resistance to fusiform rust disease in loblolly pine by genomic mapping. In eucalypts, RAPD markers were used for genetic mapping of QTLs controlling vegetative propagation (GRATTAPAGLIA *et al.* 1995), while in oaks the molecular differentiation between *Q. petraea* and *Q. robur* was evaluated with RAPD markers (MOREAU *et al.* 1994). In the present study we used bulked segregant analysis (BSA) of RAPD products to identify markers linked to tree form and branch habit gene(s) in cypress.

MATERIALS AND METHODS

Plant material

A full-sib family, resulting from a cross of a *C. sempervirens* var. *horizontalis* and a *C. sempervirens* var. *pyramidalis* individual, segregating in a 1:1 ratio ($\chi^2 = 0.891$, $p = 0.345$) of the parental forms was used. In particular the progeny of this cross consisted of 24 *horizontalis* and 31 *pyramidalis* individuals. The material was derived from an experimental plantation of the Institute of Mediterranean Forest Ecosystems and Forest Products Technology, near Patras, Greece. Fifty-five full-sib progeny as well as parental individuals were sampled for genetic analysis. This full-sib family was regarded as representing a pseudo-testcross population for BSA purposes. The age of the progeny (6-year old) was considered as adequate for full expression of the tree form, which was scored according to the Bouroulet test (BOUROULET *et al.* 1993).

DNA extraction and quantification

Genomic DNA from the two parent trees and the 55 progeny individuals was isolated according to DOULIS *et al.* (2000). The protocol for isolation of DNA from cypress needles with the QIAGEN DNeasy Plant Mini Kit (Cat. 69104) was modified and optimized for 100 mg of starting material. Needles were ground under liquid nitrogen and a volume of 400 µl of buffer AP1 (lysis buffer) and 4 µl of RNase A stock solution (100 mg· ml^{-1}) were added to the ground plant tissue, vortexed vigorously, incubated for 10min at 65 °C and mixed for 2–3 times by tube inversions. A volume of 130 µl of buffer AP2 (precipitation buffer) was added to the lysate, mixed, and incubated for 5 min on ice. The solution was centrifuged for 5 min at maximum speed using a Sorvall MC microcentrifuge. A volume of 570 µl of the lysate was applied to the QIAshredder™ spin column and centrifuged for 2 min at maximum speed. The flow-through fraction of a volume of 430 µl was transferred to a new Eppendorf tube, then 215 µl of buffer AP3 (binding buffer) plus 430 µl ethanol were added, and mixed. Of the above mixture 650 µl were applied onto DNeasy mini spin column and centrifuged for 1min at 8000 rpm. The procedure was repeated for the remaining 425 µl. The DNeasy column was placed in a new collection tube, and a volume of 500 µl of buffer AW (washing buffer) was added onto the column and centrifuged for 1min at 8000 rpm. This step was carried out twice. The column was transferred to a new collection tube and spinned for 5min under a vacuum, to ensure complete dryness of the column membrane. Lastly, two elutions with 100µl each of buffer AE (elution buffer), were made by using two different Eppendorf tubes, resulting in 200µl DNA stock solution.

Bulked segregant analysis

Two bulked DNA samples from eight *pyramidalis* and eight *horizontalis* progeny were constructed (MICHELMORE *et al.* 1991) from the segregating population, each bulk therefore representing the alternate phenotypic states of the loci controlling the selected trait against a random genetic background of several loci. Equal amounts of DNA from 8 individuals (200 ng DNA per individual) with identical form (*pyramidalis* or *horizontalis*) were mixed to create the respective bulks. One hundred ninety-eight decamer primers

(Operon Technologies, Almeda, California) were used to screen the parents and the two bulked DNA samples based on the method of WILLIAMS *et al.* (1990) with minor modifications. Initially, four PCR amplifications were performed with each primer using DNA from (1) the *horizontalis* bulk, (2) the *pyramidalis* bulk, (3) the *horizontalis* parent, and (4) the *pyramidalis* parent. The presence of a RAPD fragment in one bulk and the corresponding parent and its absence in the others provided evidence for a marker putatively linked to crown form.

RAPD assays

RAPD reactions were completed in a Gene Amp® PCR System 2400 (PERKIN-ELMER®) in PCR certified cycle plates TM–24 (Robbins Scientific). The thermal cycles used were: 1 cycle of 4min at 97.5 °C, followed by 45 cycles of 1min at 94 °C, 1min at 40 °C and 1min at 72 °C, and finally 1 cycle of 4min at 72 °C for the final extension. For a reaction volume of 25 µl, the following were used: 20 ng DNA template, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.2 µM Operon primer, 2.5µl 10× Stoffel buffer (10 mM Tris-HCl, pH 8.3, 10 mM KCl), 4 mM $MgCl_2$, 0.2 units of Stoffel fragment.

Scoring and analyzing RAPD data

Amplified PCR products (i.e., RAPD bands) were resolved using 1.8 % (w/v) agarose 1X TBE gel containing 0.1µg·ml^{-1} ethidium bromide and electrophoresed under constant voltage (90 V) for 3.5 h in a horizontal gel electrophoresis system (20 × 25 cm gel mould) which can accommodate 60 samples simultaneously. A 100 bp DNA standard (GIBCO–BRL) was used to assign base pair size to individual RAPD bands. Photographs of each gel were taken in UV transilluminator using polaroid film (type 667). Gels were screened visually for the presence of polymorphic bands in the *horizontalis* bulk and *horizontalis* parent and their absence in the *pyramidalis* bulk and *pyramidalis* parent, or vice versa. For each primer yielding possible polymorphisms, duplicate PCR reactions were run to exclude false positives. In case of a confirmed result of the BSA further PCR reactions were performed using the DNA of the 24 F_1 *horizontalis* and the 31 F_1 *pyramidalis* individuals, in order to determine the linkage of the PCR product to the target genomic region.

The RAPD markers were named according to the convention used by PARAN *et al.* (1991). For example, the fourth primer in Operon Kit H yielding a RAPD fragment with a size of 800 bp would be termed "OPH-04_{800}".

Linkage analysis

Linkage was tested based on joint segregation and independent assortment using the c^2 test. When linkage was detected maximum-likelihood estimation was used to calculate the recombination frequency and the associated standard error (ARAVANOPOULOS 1998). In the consideration of linkage we introduced two additional thresholds: (1) the recombination frequency to be $\theta < 0.25$, (2) the associated probability to be $p < 0.01$. Mapping distances were estimated by using the function suggested by KOSAMBI (1944), with a standard error according to OWEN (1950).

RESULTS AND DISCUSSION

Bulked segregant analysis for screening candidate markers

Previous attempts to dissect the genetic control of crown form in cypress (PANETSOS 1967; BASSIOTIS 1985) suggested possible hypotheses (PANETSOS 1967), but were not conclusive. In the present study, BSA was used as a rapid way to identify markers linked to a genomic region associated with crown form in the common cypress. Of the 198 random decamer primers used in the bulked segregant analysis, 196 (99%) revealed amplified DNA products in the tested family, while the other 2 failed to amplify. The primers amplified a total of 1632 fragments of scorable quality, producing an average of 8.1 scorable fragments per primer (Fig. 1). During this survey we identified 403 polymorphic fragments that distinguished the *horizontalis* from the *pyramidalis* parent. Nevertheless, only 3 were specific for the *horizontalis* parent and the *horizontalis* bulk, and 5 for the *pyramidalis* parent and the *pyramidalis* bulk, yielding a total of eight putative markers.

Confirmation of linkage for putative markers

The presence of a fragment in one bulk and corresponding parent and absence in the other bulk and parent is an indication for a putatively linked locus, as shown for fragment OPE-12_{570} (Fig. 2). The primers that produced phenotype-specific RAPD fragments are presented in Table 1. The distribution of the eight polymorphic fragments within each bulk was determined following amplifications of individual genotypes (Table 2).

their valuable comments and suggestions.

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