OPTIMIZING PCR-BASED TECHNIQUES TO STUDY WHITE LUPIN GENETICS

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Summary

DAF was used to evaluate Lupinus germplasm. Template DNA quality and concentration, the primer to template ratio, Mg^{++} concentration, and annealing temperature were critical factors affecting DAF patterns. Patterns fell into two categories, one phylogenetically conserved and the other genotype specific, thus enabling characterization of genetic relationships of Lupinus germplasm.

Key words : *Lupinus albus L., genetic markers, polymerase chain reaction*

Introduction

Random amplified polymorphic DNA (RAPD) and DNA amplification fingerprinting (DAF) are two PCR-based techniques that have been effectively used for germplasm evaluation (1,3). Since the amplification process is dependent upon many components and their interactions, it is important to specify a set of reactions in order to obtain reproducible results for a given species. The objectives of this research were to examine the influence of different PCR parameters on DAF patterns and to explore the feasibility of using DAF procedure to obtain genetic markers in order to evaluate genetic diversity in white lupin.

Materials and methods

Crude genomic DNA was extracted from 250 ng leaves of 6-week old seedlings of 4 *Lupinus* species and 13 L. *albus* L. (white lupin) accessions with the G Nome Isolation Kit (Bio 101, Inc., La Jolla, CA, USA). The crude DNA was then purified by 3 successive phenol:chloroform (1:1) extractions followed by ethanol precipitations. One hundred and ten 8-mer primers of arbitrary sequence were synthesized and quantified. Each amplification reaction was performed in a 25 µl volume consisting of template DNA (100 ng), one primer (15 µM), $MgCl_2$ (4 mM), 5 units of Stoffel Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA), 5.0 µl mixed dNTP (200 µM each), and 2.5 µl of 10x Stoffel reaction buffer (Perkin Elmer Cetus). The solution was overlaid with 15 µl of light mineral oil to prevent evaporation and amplified in a Hybaid thermal reactor (National Labnet Co.,Woodbridge, NJ, USA) under the following temperature conditions: 96 °C for 10 min, followed by 35 cycles of 96 °C for 5 s, 45 °C for 20 s, 72 °C for 30 s, and 1 min at 72 °C. Amplification products were assayed following the procedure described in (1). The reproducibility of DAF results was tested by independently isolating and purifying template DNA of each accession at two different times and performing the PCR reaction at three separate times.

Results and methods

Crude DNA samples resulted in poor or lack of amplification while purified DNA samples gave reproducible amplifications. Of 110 primers tested, 56 yielded amplified DNA fragments but differed in the number of bands amplified. The ratio of primer to template concentration was found to be critical with an optimum range of a 5 to 10-fold excess of primer. A template concentration of 100 ng and primer concentration of 15 µM gave the best amplification. Of six $MgCl_2$ concentrations tested (1-6 mM), the maximum number of bands was observed at 4 mM. An increase or decrease in the concentration of $MgCl_2$ resulted in the progressive loss of bands. Amplification was sensitive to changes in annealing temperature (30-65 °C in 5°C increments) and time (20 to 60 s in 10 s increments). For most of the primers tested, the best combination was annealing at 45 C for 20 sec. Changes in other parameters including enzyme concentration (3 to 10 units), denaturing temperature (94 or 96 °C), time of strand extension (30 to 60 s), and cycle number (30 to 45) had little influence on DAF banding patterns, in accord with the results reported by others (2).

Thirteen of 56 primers produced well-resolved and highly reproducible polymorphisms in four *Lupinus* species. Intriguingly, the DAF pattern of *L. albus* is was similar to that of *L. graecus*, the closest wild relative and one of several putative ancestors of white lupin. DAF patterns fell into two categories, one phylogenetically conserved and the other genotype specific.

The characteristic amplification patterns indicate the usefulness of DAF in genotype fingerprinting, providing a means to distinguish between species and between related lines within a species.

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