EVALUATING GENETIC DIVERSITY OF WHITE LUPIN USING POLYMERASE CHAIN REACTION BASED RAPD TECHNOLOGY

Qiu, J.; Van Santen, E. and **Tuzun, S.**
Dep. of Agronomy and Soils and Dep. of Plant Pathology, Alabama Agricultural Experiment Station, Auburn University AL 36849 U.S.A.

ABSTRACT

White lupin is a potential new proteineous grain crop for the United States. However, only a few adapted cultivars are available and breeding programs are needed to develop the full potential of this crop. Information on genetic diversity of crop germplasm is needed to utilise the available diversity most effectively for improvement programs. Random amplified polymorphic DNA (RAPD) markers, a novel strategy based on polymerase chain reaction (PCR) technology, has been used in germplasm evaluation. We have obtained more than 700 accessions of white lupin from all over the world and used PCR-based RAPD technology to study genetic diversity among these accessions. We have developed a standard RAPD protocol for white lupin. Ultra-pure lupin DNA was prepared with successive phenol:chloroform extractions and DNA fragments were amplified utilising synthetic 8-mer primers in a Hybaid thermal reactor (National Labnet Co., Woodbridge, NJ 07095). The reaction mixture in a 25-µL volume consisted of 2.5 µL 10x Stoffel buffer, 4.0 µL $MgCl_2$ (25 mM), 2.5 µL mixed dNTP (200 µM of each), 10 ng of template DNA, 1.0 µg of primer, and 0.5 µL (or 5 units) of Taq polymerase. The reaction mix is overlaid with 45 µL of light mineral oil and amplified 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 ending with 1 min at 72°C. Reliability of the template was evaluated by isolating template DNA from combined leaf samples from at least three individual seedlings per accession. Reproducibility of PCR results was tested by performing the reaction twice under standard conditions. Well- resolved and high levels of reproducible polymorphisms were detected in six commercial white lupin cultivars. The polymorphism patterns of three French cultivars (Adam, Alban, and Lunoble) were very similar but quite different from the other cultivars (Primorski, Tifwhite 78, and Ultra). Furthermore, three advanced selections from plant introductions also differed from each other and from the six cultivars. The characteristic amplification patterns suggest their usefulness in genotype fingerprinting, allowing us to distinguish between related cultivars. The complexity of the banding pattern suggests that primer annealing occurs at multiple sites within this diploid genome, allowing us to examine a number of sites for polymorphisms per reaction. The relative ease of the assay, in comparison to RFLPs, should make this system amenable for detecting genetic variation and facilitating selection.

INTRODUCTION

Plant introduction has long been recognized as the basis of crop improvement and information on genetic diversity is needed in order to use the germplasm most effectively. Currently, there are no molecular markers available that allow simple and accurate determination of genetic relationships in white lupin. PCR (Saiki et al., 1988) based DAF has been developed to determine genetic diversity in several plant species (Caetano-Anollés et al., 1991). The objective of this research is to study the feasibility of using DAF as genetic markers and examine the influence of different parameters on DAF in white lupin.

MATERIALS AND METHODS

DNA extractions and purifications

Thirteen white lupin entries were studied. Among them, Adam, Alban, and Lunoble are from France; Primorski from Russia; Tifwhite-78 from USA; Ultra from Australia; La84 from

Greece; PI 457942 and PI 457939 from Spain; PI 481558 from Syria; PI 255471 from Yugoslavia; BGRC3939 from Germany; and PI 289160 from Hungary. Genomic DNA from each of the entries were extracted from leaf tissues using a G Nome Isolation Kit (Bio 101, Inc., La Jolla, CA) with successive phenol:chloroform extractions followed by ethanol precipitations for further purification and the purified DNA were then quantified.

Synthesis of primers

Eight-base-long nucleotide PCR primers were synthesized using a DNA Synthesizer (Cyclone Plus DNA Synthesizer, Millipore Co., Marlborough, MA) and were quantified. The sequences (5'-3') of thirteen primers used were: 1) AACGGGTG; 2) GTAACGCC; 3) GTATCGCC; 4) CTTCCCAT; 5) CTAACCCG; 6) CCCACACC; 7) TCTCCTCG; 8) ATCCATCC; 9) CTCTATCC; 10) ATCGGGCT; 11) CAGGCCCT; 12) TCTCCTCG; and 13) CTCCTCCT.

PCR conditions

In a 25-µl reaction volume, there were 2.5 µl 10x Stoffel buffer (Perkin-Elmer Co., Norwalk, CT), 4.0 mM of $MgCl_2$, 2.5 µl mixed dNTP (200 µM each of dATP, dCTP, dGTP and dTTP), 10 ng of template DNA, 1.0 µg of primer, and 0.5 µl (or 5 units) of Stoffel fragment (Perkin-Elmer Co.). The reaction mix was overlaid with 35 µl of light mineral oil and amplified in a Hybaid thermal reactor (National Labnet Co., Woodbridge, NJ) 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 ending with 1 min at 72°C.

Gel electrophoresis

The amplification products were assayed by electrophoresis in a 5% polyacrylamide gel. Electrophoresis were conducted at 105 V for 1.5 h. Gels were fixed with 10% acetic acid and silver stained (Bio-Rad Lab., Richmond, CA). Photographs were taken using Polaroid negatives (type 55 film) and banding patterns were scored on the photographs.

RESULTS AND DISCUSSION

DAF as genetic markers

Well-resolved and highly reproducible banding patterns have been detected among the white lupin entries studied. For instance, when using a primer with sequence of GTAACGCC in DAF analysis of six commercial cultivars, all the cultivars showed several common amplified fragments, particularly, a fragment with approximately 220 basepairs in length (Fig. 1). The amplified DNA patterns of three French cultivars (Lunoble in lane 2, Alban in lane 3, and Adam in lane 4) were very similar but quite different from other cultivars including Tifwhite-78 (USA, lane 1), Ultra (Australia, lane 5), and Primorski (Russia, lane 6).

The characteristic banding patterns indicate the usefulness of DAF in determining genetic diversity in white lupin, allowing us to distinguish between related genotypes.

Influence of different PCR parameters on DAF

DNA samples extracted using the G Nome Isolation Kit as 'crude' DNA resulted in failure of amplification. Template DNA purified using successive phenol:chloroform extractions resulted in successful amplification (Fig. 1). Since only a small amount of

template DNA is needed in PCR, DNA extraction procedures should focus on purity. Template DNA ranging from 1 to 15 ng did not change basic polymorphic patterns. However, increase in template DNA concentration tended to increase the detection of faint bands.

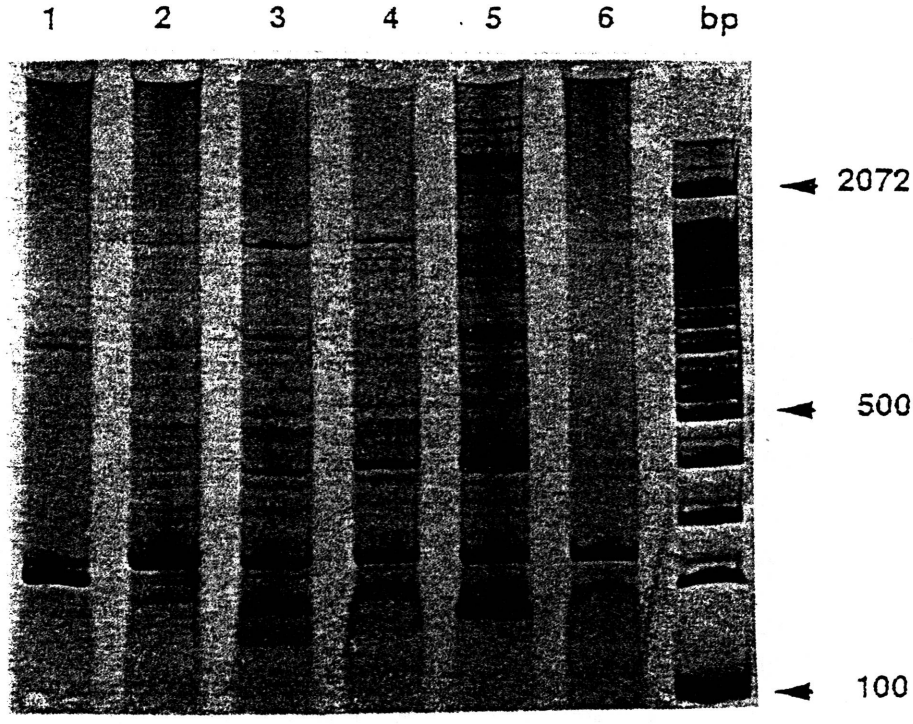

Figure 1. RAPD polymorphism of six white lupin cultivars.

Three of the 13 primers tested showed amplified DNA fragments ranging from 100 to 3000 basepairs in size, but they differed in the number of bands detected. It is presumed that some primers lack suitable priming sites in the white lupin genomic DNA and thus gave no or poorly amplified bands. Therefore, primers should be rigorously tested for priming ability before they are being employed as genetic markers. Primer concentrations ranging from 0.3 to 1.0 ug in a 25 ul reaction volume did not change the basic banding patterns. However, primer concentration lower than 0.1 ug resulted in poor or no amplifications.. Primer annealing temperature and time combinations were found to have a great impact on amplification.

To examine the influence of Mg^{2+} on the efficiency of DAF, $MgCl_2$ was added to the reaction to give final concentrations of 1.0, 2.0, 3.0 and 4.0 mM. Specific and reproducible results were obtained only in the presence of 4.0 mM Mg^{2+}

REFERENCES

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