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Scientific Report

Application of the NBS profiling technique in *Juglans* spp.

Introduction

J. nigra (Eastern black walnut) and *J. regia* (common or Persian walnut) are both major economically important species in Europe, Asia and North America. The Persian walnut, an indigenous species in Eurasia from the Balkans to southwest China, is cultivated throughout the temperate regions of the world for its high quality wood and edible nuts. Black walnut, native to the Eastern part of North America, is a fast growing species with less valuable wood in comparison to *J. regia*. Since the 17th century, *J. nigra* has been imported from the Eastern and Central hardwood forests of the United States to the European continent for ornamental purposes and subsequently for its rapid growth.

Both common and black walnut are sensitive to soil conditions, developing best on deep, well-drained, moist and fertile soils (Williams, 1990), but they differ in response to pathogenic infection (Weber et al.1980). *J. regia* is susceptible to the damage caused by pests, abiotic factors (flooding, drought) and biotic stresses (e.g. anthracnose, bacteriosis)(McGranahan and Leslie, 1990). Particularly the anthracnose caused by *Gnomonia leptostyla* (Fr.) Ces. is one of the most important diseases of walnut for timber production; symptoms develop on leaves, stem and fruit as irregular necrotic areas that are often surrounded by small chlorotic halos. In severe cases, these lesions may cause premature defoliation , fruit drop or poorly filled nuts (Funk et al. 1980; Woeste et al. 2001).

Although phylogenetic analysis based on nuclear RFLP, *matK* and ITS sequence has demonstrated that black walnut and Persian walnut belong to different sections of genus *Juglans*, *Rhysocaryon* and *Dioscaryon* respectively (Fjellstrom and Parfitt, 1955; Stanford et al. 2000) hybridization between them is possible. *Juglans x intermedia* (Carr) is a natural hybrid between *J.nigra* and *J.regia*. Compared to the parental species, most *J. x intermedia* hybrids show increased vegetative vigour, distinct disease resistance, good wood quality, and greater winter-hardiness than *J. regia* (Mapelli et al. 1997; Fady et al 2003). For these reasons there is a great demand for *J. x intermedia* for forestry, especially in Northern Europe. Investigation on the resistance to anthracnose infections of *J. regia*, *J. nigra* and inter-specific hybrids (*J. nigra x J. regia*) plants proved that *J. nigra* is resistant, while the hybrids showed an intermediate behaviour toward *Gnomonia leptostyla* infection (Anselmi et al 2005).

Most resistance to pathogens in plants is based on gene-for-gene model: an R-gene product recognises a pathogen gene product in the host, conferring full or partial resistance. Generally two different classes of molecular markers are applied for the characterisation of genetic diversity: neutral markers and functional markers. Neutral markers, such as AFLP and microsatellite markers, are generally not under selective pressure. They are useful for a lot of applications, including the evaluation of gene flow, population differentiation and inbreeding but correlation to functional traits (ex : resistance disease) occurs by chance. Instead functional markers are targeted to specific, and display the diversity or are correlated with diversity in genes that may affect the trait of interest. Functional markers are generated by methods that target a gene or group of genes; these include the NBS-profiling approach (Van der Linden et al 2004).

During the past decade an increasing number of plant disease (R) genes from different species have been identified, cloned and sequenced. These genes fall into five mainly subclasses (Baker et al. 1997), but the vast majority of them are members of the cytoplasmic nucleotide binding site - leucine rich repeat (NBS-LRR)-containing R-gene family. The NBS region of the characterized R genes contain several highly conserved motifs. These include P-loop (ATP and GTP binding

proteins domain), kinase -2 motif (this domain coordinates the metal ion binding necessary for phosphor-transfer reactions) and GLPL motif (also known as hydrophobic domain, a putative membrane-spanning domain) (Saraste et al. 1990; Meyers et al. 1999). The NBS-LRR proteins seem to be involved in recognizing pathogens and activating signal transduction pathways to induce defences responses in plant.

Van der Linden (2004) proposed a new strategy to amplify a large collection of R-gene and R-gene Analogues (RAG) fragments and samples polymorphism in these genes. NBS-profiling approach is based on PCR amplification using simultaneously, an adapter primer matching a restriction enzyme site and a degenerate primer targeting the conserved domains present in the NBS region. In this way it is possible to obtain DNA profiles with markers mainly present in R-genes and RGAs. The NBS profiling method is a technique that has proven to be useful in a large number of crop as apple (Calenge et al. 2005), lettuce, potato, barley, tomato (Van der Linden et al. 2004; Malosetti et al 2007) and wheat (Mantovani et al. 2004). It is a technique that requires optimal DNA quality and optimal components for each step in the procedure. NBS profiling was developed at Plant Research International, Wageningen, Netherlands, and is used routinely in the Business Unit Biodiversity and Breeding.

Objective

The aim of this work was to set up all necessary steps for a preliminary NBS-profiling application in *J. regia*, *J.nigra* and hybrids. Once the optimal primer-enzyme combinations have been identified, subsequently NBS profiling would be applied on several walnut genotypes in order to provide molecular markers tightly linked to R-gene and RGAs involved in anthracnose resistance.

Research activity

Material

In spring 2007 young leaves were sampled directly from the crown of 14 plants conserved in the experimental field of the CRA-Unità di Ricerca Forestale, Poplar Research Institute, Rome (Italy) and stored at -20°C until molecular analysis. According to morphological observations, 7 samples were classified as *J. nigra* (352JN, 355JN, 378JN, 371JN, 369JN, 361JN, 356JN) and 2 samples as interspecific hybrid (HT18; HU18). The others leaves were picked from 5 *J. regia* trees (RA3, RA21, RA5, RA1.3, RA22.5). In RI.SELV.ITALIA, project, “Area 1-Risorse Genetiche Forestali”, sottoprogetto 1.1. , “Biodiversità e produzione di materiale vegetale da propagazione”, Prof. Anselmi proved that these seven *J. nigra* genotypes are resistant to antracnose infection. On the contrary the sampled *J. regia* and interspecific hybrid plants resulted susceptible (except for RA1.3 that is tolerant) and tolerant toward *Gnomonia leptostyla* infection respectively.

DNA extraction

Genomic DNA was extracted from leaf tissue using the DNeasy Plant Mini Kit (QIAGEN), suspended in 100 µL of Buffer AE (Qiagen) and stored at -20°C. DNA presence was monitored by subjecting sample to 1% agarose gel electrophoresis in 0.5 x TBE buffer. The amount of DNA was spectrophotometrically determined and was brought to a working concentration of 20 ng / µL.

NBS –profiling application (Van der Linden et al. 2004; PRI protocol)

1. Restriction digestion and adaptor ligation

Restriction digestion and adaptor ligation were performed in a single reaction. Two hundred nanograms of DNA was digested with either MseI or RsaI restriction enzyme in the appropriate buffer for 3 h at 37°C. An adapter was ligated to the restriction fragments using high concentration of ligase (1U for MseI-sticky enzyme and 5U for RsaI- blunt enzyme). The reaction was stopped by heat inactivation (15 minutes at 65°C). The adapter consisted of a long arm which was identical in

1-21 bp sequence region with the adapter primer and a short arm that was blocked by an amino group at the 3' end.

Adapter sequence:

5'-ACTCGATTCTCAACCCGAAAGTATAGATCCCA-3'....long arm



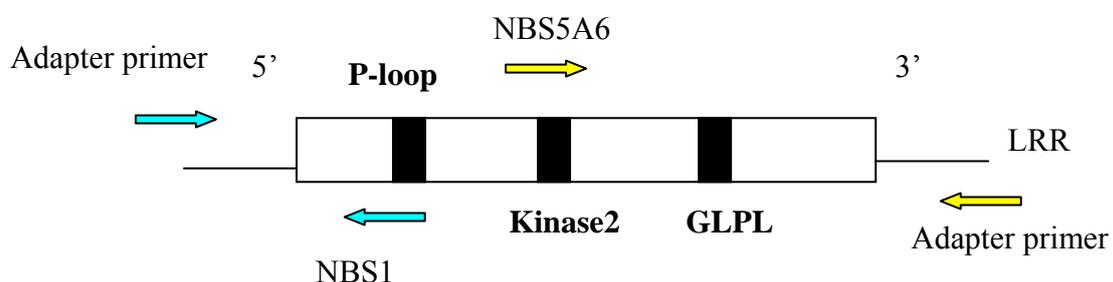
Adapter primer sequence:

5'-ACTCGATTCTCAACCCGAAAG-3'

In the short arm the amino-group hindered the elongation of the 3' end by Taq polymerase and P-group facilitated the ligation to blunt-end fragments. In this way at the start of PCR amplification, the adapter primer could not anneal to the DNA template but only when the specific NBS primer annealed and was elongated. The annealing of adapter primer relied on the synthesis of a complementary strand. This prevented the amplification of adapter-adapter fragments.

2. First Amplification

Two different NBS-specific primers, NBS1 and NBS5A6 (NBS 5 combined with NBS6) were used for a total of 4 primer-enzyme combinations. Amplification of NBS-specific fragments was performed in a single polymerase chain reaction with NBS-primer and adapter primer rather than two-step PCR procedure present in the original protocol (Van der Linden et al. 2004). These primers were designed from a part of the conserved P-loop motif for NBS1 and of kinase-2 for NBS5A6; NBS1 primer amplified DNA towards the 5' end of the targeted genes, outside the NBS region, meanwhile NBS5A6 towards the 3' end, inside the NBS region.



Polymerase chain reaction (PCR) was done in 25 μ L of reaction volume adding 5 μ L of restriction-ligation template (diluted two times), 2.5 μ L of HotStartTaq PCR buffer 10 X, 200 μ M dNTPs, 20 pmol for each primer and 0.4U of HotStartTaq polymerase (Qiagen, Germany). Reactions were performed in a GENEamp 9700 Thermocycler according to the following procedure: 15 min at 95°C (to activate HotStartTaq polymerase) followed by 30 cycles of 30 sec at 95°C, 1.40 min at 55°C, and 2 min at 72°C; then a final extension step at 72°C for 20 min. The amount and estimated size of the amplified fragment was checked testing 15 μ L aliquot of the amplified reaction by electrophoresis on 1% agarose gel in 0.5x TBE buffer, and stained with ethidium bromide.

All 4 combinations (MseI-NBS1; MseI-NBS5A6; RsaI-NBS1; RsaI-NBS5A6) amplified in *J. regia*, *J. nigra* and hybrid genotypes. A smear with several distinct bands in the size range of 100-1000 bp was observed in the agarose gel (1%) for each combination.

3. Labelling PCR

The PCR products were re-amplified using labelled adapter primer (fluorescence, IRD-700). Polymerase chain reaction (PCR) was done in 10 μ L of reaction volume adding 5 μ L of 10 X diluted PCR mixture, 1 μ L of PCR buffer 10X, 200 μ M dNTPs, 3 pmol of NBS primer, 0.6 pmol of IRD-labelled adapter primer and 0.2 U of SuperTaq DNA polymerase (Qiagen, Germany). Reactions were performed in a GENEamp 9700 Thermocycler according to the following procedure: 3 min at 95°C followed by 35 cycles of 30 sec at 95°C, 1.40 min at 55°C, and 2 min at 72°C; then a final extension step at 72°C for 20 min. The labelled PCR products are mixed with an equal volume (10 μ L) of formamide-loading buffer (98% formamide, 10mM EDTA pH 8.0 and 0.1% Bromo Phenol Blue) and an aliquot (0.3 μ L) was analyzed in the LI-COR 4300 DNA Analysis System (LI-COR Biosciences). The 4300 System is an instrument based on LI-COR's highly sensitive infrared fluorescence detection technology. The labelled PCR products were separated on a 6% polyacrylamide gel, and the individual fragments visualized by fluorescence detection.

4. Preliminary results

All fragments amplified in *J. nigra*, *J. regia* and hybrid plants for each enzyme-NBS primer combination and visualized by LI-COR 4300 DNA Analysis System, were scored. For example the figure 1 shows the NBS profiles of *J. nigra*, *J. regia* and hybrid genotypes with primer NBS5A6 and restriction enzyme MseI, visualized with LI-COR 4300 DNA Analysis System.

The table 1 summarized the number of “common” and/or private NBS fragments detected in each enzyme-NBS primer combination. Out of the total (341 bands), 89 fragments were common to black and Persian walnut and were labelled as “common” In addition, 254 fragments amplifying in *J. nigra* and 128 in *J. regia* only, were classified as species “private NBS-bands”. No private bands were observed in the interspecific hybrids.

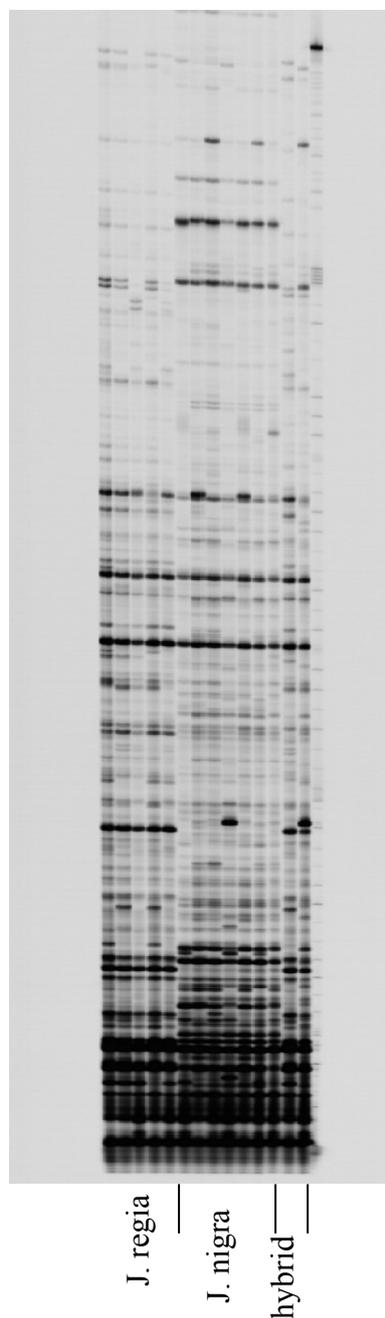


FIGURE 1.

LI-COR 4300 DNA Analysis System Gel for NBS5A6-MseI combination

Combination NBS Primer/Enzyme	Number of total fragments	Number of private NBS-bands		Number of common NBS-bands
		<i>J. nigra</i>	<i>J. regia</i>	
NBS1-RsaI	85	26	32	27
NBS5A6-RsaI	56	19	30	7
NBS1-MseI	84	26	22	36
NBS5A6-MseI	116	55	42	19
Total	341	126	128	89

TABLE 1. Summary of NBS- fragments amplified in *J. nigra* and *J. regia* genotypes in four different enzyme-NBS prime combinations.

This is the starting point to find an association or “linkage disequilibrium” between the NBS markers amplified in walnut germplasm and the resistance for anthracnose disease.

Future Collaboration

These initial results with some test samples of walnut species and hybrids showed a lot of promise. I am planning to visit again the Plant Research Institute (Wageningen) in September/December 2007 using my PhD fellowship in Forest Ecology (Tuscia University, Viterbo, and CNR-IBAF Porano, Italy). I could have the opportunity to extend my NBS profiling experience and to apply the technique on a larger set of walnut genotypes. This may enable to identify molecular markers linked to disease resistance (most notably anthracnose resistance) in walnut species.

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