

PHYSICAL AND GENETIC ANALYSIS OF GENOME REGION CONFERRING RESISTANCE TO FUNGAL PATHOGENS IN THE NARROW-LEAFED LUPIN

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ABSTRACT

Screening of the narrow-leafed lupin nuclear genome BAC library has been carried out to find clones carrying genes involved in resistance to pathogenic fungi. Three lupin diseases were considered in this study: Anthracnose (*Colletotrichum lupini*), Phomopsis stem blight (*Diaporthe toxica*) and rust (*Uromyces lupinicolus*). Radioactively labelled probes, designed on the basis of sequence defined markers linked to the respective traits, were applied for several hybridisation series with high-density macroarrays containing the whole nuclear genome lupin BAC library. The clones showing positive hybridisation signals were verified by PCR with DNA isolated from the BAC as template and primer pair specific to the marker sequence. Subsequently, the ends of all confirmed clones were sequenced. In order to find candidate genes the sequencing data were functionally annotated using several bioinformatic tools integrated into the COBALT system to find homology links, conserved coding domains and motives characteristic to known genes involved in resistance to pathogenic fungi. Furthermore, the fingerprint analysis was carried out using the *Eco130I* restriction enzyme and the FingerPrinted Contigs software (FPC V8.5.3) to construct contigs of the analysed genome region.

KEYWORDS

Lupinus angustifolius, BAC library, Anthracnose, phomopsis, rust, candidate genes

INTRODUCTION

Narrow-leafed lupin (*L. angustifolius*) is considered to be a crop rich in seed protein for animal feed and green manure and with very promising potential in the crop rotation system of sustainable agriculture. One of the significant obstacles in the intensification of agricultural production of lupins is their susceptibility to diseases caused by pathogenic fungi. Among them, *Colletotrichum lupini* causing Anthracnose presents the highest hazard to lupin species. In recent years international efforts have been made to search for natural sources of Anthracnose resistance (Sweetingham

et al. 2005), which resulted in the creation of new tolerant cultivars and localisation of markers linked to resistance gene in the LG2 on the first genetic map of the lupin genome (Boersma *et al.* 2005). Studies on the genetic basis of resistance to other major disease, Phomopsis stem blight caused by *Diaporthe toxica* (Shankar *et al.* 2002) made it possible to develop molecular markers linked to one of the resistance genes (Yang *et al.* 2002). From among many other minor fungi affecting lupin plants, the pathogen *Uromyces lupinicolus* causing lupin rust is potentially threatening (Etheridge and Bateman, 1999). A molecular marker linked to rust resistance has been established recently (Hua'an Yang – personal communication). The information on sequence defined molecular markers linked to resistance to rust, Anthracnose and Phomopsis stem blight was the starting point of this study. The final objective was to determine the structure and organisation of *L. angustifolius* genome region containing resistance genes to these diseases.

MATERIALS AND METHODS

HYBRIDISATION PROBES

Hybridisation probes were prepared on the basis of marker sequences linked to the following resistance loci: Anthracnose *Lanr-1* – probes AntjM1 and AntjM2 (You *et al.* 2005), Phomopsis *Phr-1* - probe Ph258M2 (Yang *et al.* 2002) and rust - the RustM1 probe (Hua'an Yang – personal communication). Probes were PCR amplified with *L. angustifolius* genomic DNA as a template, purified using a QIAquick Kit (Qiagen) and radiolabelled by HexaLabel (Fermentas) incorporating 50 µCi of [α -³²P]-dCTP.

LIBRARY SCREENING AND BAC-END SEQUENCING

Labelled probes were hybridised with macroarrays containing the nuclear genome BAC library of *L. angustifolius* cv. Sonet comprised 55 296 clones (Kasprzak *et al.* 2006). With an average insert size of 100 kb, the library represented six haploid genome equivalents. Hybridisation was carried out for 16h at a temperature of 65°C. High stringency washes in 0.1xSSC and 0.1% SDS at 58°C were conducted. Macroarrays were scanned using a Typhoon 8600 phosphoimager (Pharmacia). Verification of clones

giving positive hybridisation signals was performed by PCR with insert DNA as template and sequence specific primers. The ends of all positively verified BAC clones were sequenced on an ABI PRISM 3130 XL Genetic Analyser (Applied Biosystems Hitachi) using appropriate pIndigoBAC5 Sequencing Primers.

RESTRICTION FINGERPRINTING AND CONTIG ASSEMBLY

Restriction fingerprinting was conducted by applying 2 units of the *Eco130I* enzyme for 1 µg of BAC DNA. The reaction was performed at 37°C for 16 hours. Fingerprinting patterns were developed by electrophoresis on 1% agarose gel (24h, 3V/cm) and visualised by ethidium bromide staining. Contigs were assembled using the Image 3.10b and FPC V8.5.3 software, with a cut-off value set to $1e^{-14}$ and tolerance 3.

FUNCTIONAL ANNOTATION

The precise location and characteristics of various genetic elements encoded in the BES sequence was revealed by annotation. The process included *de novo* detection of specific signals located on the genomic sequence as well as comparative analysis. The procedure was executed with the application of the COBALT system, which represents an analysis pipeline specifically designed for gene discovery and comparative genome research (Karlowski, 2006). The repetitive elements were masked using sequences deposited in RepBase as well as TIGR and MIPS Plant Repeats Collections. The protein coding regions were identified using ESTScan2 using parameters for *Arabidopsis thaliana*. Recognised protein sequences were compared with Swiss-Prot collection on the ExPASy proteomics server (<http://www.expasy.org>).

RESULTS AND DISCUSSION

The *L. angustifolius* genome BAC library was subject to four separate screening procedures, conducted with probes designed on the basis of marker sequences linked to the analysed resistance loci and followed by PCR verification of hybridisation results (Table 1). The PCR products generated for ten randomly chosen BACs were sequenced and aligned with appropriate markers, revealing full identity of sequences. The total number of 60 BAC clones showed PCR confirmed positive hybridisation signals both with the AntjM1 and AntjM2 probes. Taking into consideration the average BAC insert size in the lupin library (100 kb) and published information on flanking the Anthracnose resistance *Lanr-1* gene by markers *AntjM1* (3.5 cM, Yang *et al.* 2004) and *AntjM2* (2.1 cM Boersma *et al.* 2005; You *et al.* 2005) such a large number of clones showing double positive hybridisation signals indicate rather a much closer physical localisation of the examined markers than those suggested by genetic mapping. The subset of BACs selected for Ph258M2 showed positive hybridisation signals with the Anthracnose or rust probes. The high number of clones giving simultaneously positive hybridisation signals with

probes concerning these three resistance genes may suggest that the analysed genome region is of significant importance for resistance to fungal diseases.

Table 1. The number of positive hybridisation signals and the number of clones after verification by PCR reaction with sequence specific primers.

Probe	No. of positive hybridisation signals	No. of clones verified by PCR
AntjM1	243	64
AntjM2	300	117
Ph258M2	120	108
RustM1	120	107

The BAC clones were end-sequenced, producing 241 BAC end sequences (BES) with an average insert length of 619 bp. The initial step of functional annotation revealed a high number of transposon and retrotransposon sequences, predominantly Opie, Machiavelli, hAT, Ty1-copia and Ty3/gypsy. Subsequently, 99 of them were masked as repetitive DNA. In the remaining BES, several coding sequences were recognised, like peroxidase, senescence-associated proteins, cytochrome b5 reductase, and Nudix hydrolase. Details on level of homology between selected BES and protein sequences were provided in Table 2. Some of these coding sequences can act as guidelines for seeking candidate genes involved in resistance traits. The **peroxidase 20** from *A. thaliana* is an enzyme which may be implicated in the systemic response to environmental stresses such as wounding, pathogen attack and oxidative stress. Generally, peroxidases play a significant role in the plant defence response and in conferring resistance to a wide range of pathogens (Bindschedler *et al.* 2006). The one BES putatively encodes **cytochrome b5 reductase**. Studies on *Pinus sylvestris* responses to root and shoot specific pathogens indicate an altered expression of this protein in inoculated roots as compared to non-inoculated controls (Adomas and Asiegbo, 2006). The identified **Nudix hydrolase** is a member of the *A. thaliana* Nudix family of proteins. Enzymes of this family catalyse the hydrolysis of a variety of substrates with a preference for ADP-ribose (Olejnik and Kraszewska, 2005). The mono-ADP-ribosylation may play an important role in regulating protein functions in physiological and pathological conditions (Corda and Di Girolamo, 2003).

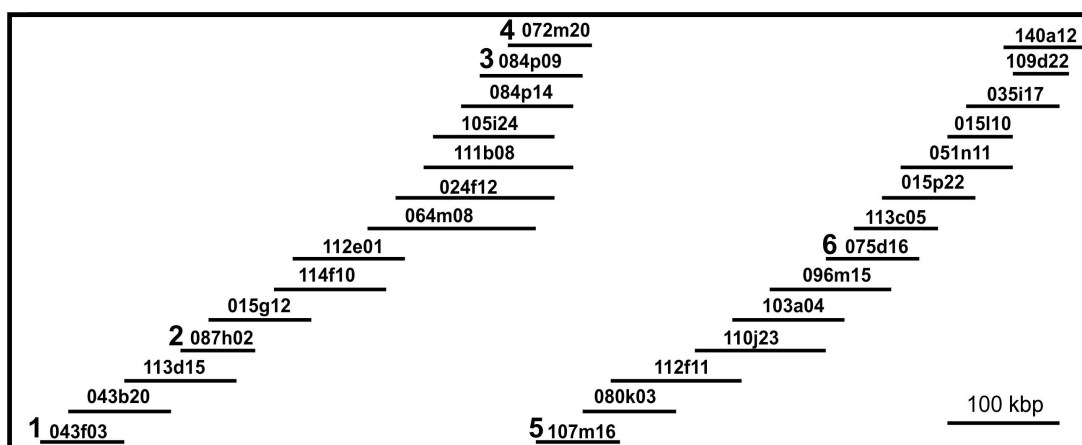
In order to attain the information concerning physical structure of studied genome region the restriction fingerprinting of the 125 BAC clones was carried out. The clones were grouped into two contigs and thirteen singletons. The main contig covering the region of 1.5 Mbp in the genome consisted of 107 clones with an average band size (CB) of about 3.5 kbp. Almost all the functionally annotated genes with were localised here (Fig. 1).

Table 2. The list of genes identified by functional annotation of BAC end sequences.

No.	BAC clones	Genes identified by functional annotation	BAC/protein sequence identity	Blastx expected value
1	043f03	NADH-cytochrome b5 reductase (<i>Arabidopsis thaliana</i>)	60/101 (59%)	1e ⁻¹⁸
2	087h02	Senescence-associated protein DH (<i>Zea mays</i>)	34/59 (57%)	1e ⁻⁴
3	084p09	Similarity to senescence-associated protein (<i>Arabidopsis thaliana</i>)	28/36 (78%)	2e ⁻⁴
4	072m20	Putative senescence associated protein 5 (<i>Oryza sativa</i>)	37/76 (48%)	3e ⁻⁸
5	107m16	Peroxidase 20 (<i>Arabidopsis thaliana</i>)	41/60 (68%)	3e ⁻¹⁶
6	075d16	Nudix hydrolase 19 (<i>Arabidopsis thaliana</i>)	37/69 (53%)	4e ⁻¹⁰
7	072o21	Cytochrome p450 TO54-2 [Fragment] (<i>Taraxacum officinale</i>)	51/53 (96%)	2e ⁻²²

THE NUMBER OF BES ENCODING REPETITIVE ELEMENTS

Opie – 13
Machiavelli – 6 Ty3/gypsy – 11 SHALINE7_MT – 4 hAT – 6 LINE (other) – 3 Ty1-copia – 6 other – 53

**Fig. 1.** The physical organisation of BAC clones forming the contig of analysed lupin genome region. Clones marked by numbers contain coding sequences described in Table 2.

Three BAC clones were assembled into a small additional contig, with one BES carrying a putative gene sequence. The repetitive sequences revealed in numerous BES might result in putatively false overlapping of some clones. It must be emphasised that dQr analysis, forced in the FPC program, splits the large contig into 5-7 smaller ones and transfers some of the identified putative genes beyond the main contig. It may indicate that there is another region in the lupin genome involved in resistance to fungal pathogens, what partially explains the 'false negative' scores of the AntjM1 and AntjM2 markers obtained for an Anthracnose resistant cv. Mandelup (Clements *et al.* 2005). The new MAS applicable markers linked to Anthracnose resistance in Mandelup were designed, but the localisation of this resistance gene on the lupin genome map has not yet been established (Yang *et al.* 2008). The extensive work aiming at *L. angustifolius* genetic mapping of identified clones and genes has already been undertaken to localise regions in the lupin genome encoding putative resistance genes.

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