Eucalyptus grandis resistance genes: an *in silico* search for potential targets in response to Myrtle Rust.

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Abstract

Plants have evolved multiple layers of defence responses to pathogens. A first line of defence is the recognition and response to common pathogen-associated molecular patterns. Plants also exhibit pathogen-specific responses, which are mediated by expression of resistance genes (R-genes). R-gene products effectively block disease progression. A significant plant pathogen, which causes Myrtle Rust, has spread rapidly along the east coast of Australia since its first identification on the NSW Central Coast in 2010. This incursion is of concern due to the dominance of the Myrtaceae family in Australian vegetation. The recently published genome of *Eucalyptus grandis* has provided a valuable resource for the identification of potential resistance genes in this important Australian genus. In this study a search was conducted against the *E. grandis* genome for one important class of R-genes that incorporate nucleotide binding sites and leucine-rich repeat protein domains (NB-LRR). Predicted NB-LRR coding sequences were identified and analysis of the coding sequences revealed 11 gene models within the *E. grandis* genome. Scrutiny of these gene models, through *E. grandis* peptide homologues, identified a further 412 genes which contained both NB and LRR domains. The identification of these R-genes may provide researchers with a targeted approach to defence response studies to Myrtle Rust and other pathogens in *E. grandis*.

Key words

NB-LRR, R-genes, defence response, Myrtle Rust.

Introduction

Pathogen response genes in plants have been identified within several species such as *Arabidopsis thaliana* and *Oryza sativa* (rice) (Bergelson et al., 2001; Meyers et al., 2003). This has provided researchers with a useful framework to investigate gene homology and variance across species with regard to defence genes. Key aspects of plant defence are responses to conserved pathogen motifs and the more specific response to pathogen avirulence factors (Jones & Dangl, 2006). The conserved pathogen associated molecular patterns (PAMPs) initiate PAMP triggered immunity (PTI) with responses ranging from the increased expression of pathogenesis-related proteins (Edreva, 2005) and phytoalexins (Hammerschmidt, 1999) to callose deposition and reactive oxygen species (Karuppanapandian et al., 2011). Pathogen avirulence molecules, also termed effectors due to their attenuation of PTI responses in the host, initiate effector triggered immunity (ETI). ETI responses are highly specific to the pathogen effectors and often lead to localised necrosis in the host tissue known as hypersensitive response as well as stimulating systemic resistance (Mur, Kenton et al., 2008) .

ETI defence response is mediated by resistance proteins, known as R-proteins, which specifically recognise and bind to effectors, thereby disrupting their pathogenicity. This class of protein is predominantly identified by a leucine rich repeat region (LRR) which allows for ligand specificity in the β-sheet between the repeated leucine rich domains (Gassmann, 2008). Another recurring feature of these R-proteins is a nucleotide binding site (NBS or NB) domain. Meyers et al. (2003) identified over 149 genes encoding these NBS-LRR proteins in Arabidopsis using an *in-silico* analysis. As the LRR motif is present in multiple genes that are not related to defence, complete NB-LRR sequences are a better predictor for resistance

genes (R-genes)(Meyers et al., 2003). One of the major targets for research into crop plants is the identification of resistance genes to breed into crops for resilience against pathogens (Rouse, Nava et al., 2012).

In Australia, a newly arrived fungal pathogen (Hirsch, 2011), *Puccinia psidii* sensu lato, infects plants of the Myrtaceae family, causing Myrtle Rust. This has raised concerns about potentially devastating impacts on the natural vegetation, which is dominated by Myrtaceae species (Carnegie & Cooper, 2011). In April 2010 the National Management Group declared Myrtle Rust to be ineradicable and recommended a plan to study, monitor and manage pathogen spread. This plan was endorsed and implemented by the Australian Federal Government (2011). While early studies into susceptibility of various species have indicated some levels of resistance, the outcome for natural populations is still unclear (Zauza et al., 2010).

Eucalyptus grandis W. Hill ex Maiden is an Australian Myrtaceous forest species that is grown for timber in many parts of the world. As an important forestry species it was selected as a model Eucalyptus specimen to have its genome sequenced (Neale & Kremer, 2011). The draft genome for *E. grandis*, from a 17 year old inbred tree clone, BRASUZ1 (genome size of 620Mbp, 11 haploid chromosomes), was released in 2011 and is expected to promote further research into this genus (Myburg et al., 2011). The availability of genomic information for *E. grandis* makes it a model Myrtaceae plant for genetic studies into potential defence responses to Myrtle Rust infection. Previously an *in-silico* study of the transcriptome of Eucalypts identified 210 homologous R-gene clusters and predicted that some of these may be structurally unique (Barbosa-da-Silva et al., 2005). Recent evidence also suggests that pairs of NB-LRR genes are required for resistance to effectors

and that encoded polypeptides arising from these gene clusters arrange to form duplexes (Sinapidou et al., 2004).

Scrutiny of R-genes in pathogen challenged plants may allow targeted studies to be developed. The current study investigated the presence of R-gene homologues in the *E. grandis* genome based on NB-LRR type disease resistance genes from other eudicotyledons. The aim of the study was to identify potential R-gene targets for further investigation in response to pathogen challenge.

Methods

Genomic search

An initial nucleotide search for NB-LRR and NBS-LRR gene homologues was conducted using NCBI (National Centre for Biotechnology Information) nucleotide nr database. The nucleotide sequences of 22 genes from this search, from a variety of eudicotyledons, were downloaded and used as queries to search for homologous genes within the *Eucalyptus grandis* genome. This search was performed using the nucleotide BLAST (Basic Local Alignment Search Tool) function (Goodstein et al., 2012) of the Phytozome browser (http://www.phytozome.net/).

The *E. grandis* BLAST searched for candidate genomic regions with expected match results of less than e-10 and base pair (bp) alignments greater than 500bp. The predicted protein domains and nucleotide sequences of the transcripts matches were then further analysed. Genes that matched the criteria for the major class of resistance genes were sought. These criteria were the presence of a leucine-rich repeat (LRR) domain encoding

sequences, as well as a nucleotide binding domain (NB) encoding sequences. Transcripts matching these criteria were analysed for the presence of LRR and NB domain sequences. Much of the current genomic data for this species remains unverified at the transcriptional and the predicted polypeptide stages. The transcripts for many of these homologous regions are therefore not currently available from the Phytozome genome browser.

Peptide homologue search

Translated *E. grandis* NB-LRR genes, identified from the BLAST search, were then used to search for homologous peptides within the genome using the Phytozome database.

Reverse database search

A reverse nucleotide BLAST of the gene Eucgr.B01032, with homology to a NB-LRR disease resistance gene of *Medicago truncatula*, was conducted against the NCBI databases for verification of potential disease resistance gene homologues.

Probe design

Nucleotide sequences of transcripts for *E. grandis* genes from NB-LRR search (Eucgr.F02383, Eucgr.H03559 and Eucgr.B01032) were used to design gene probes. Transcript or predicted coding sequences (CDS) were selected for probe design using Primer3 version 0.4.0 (http://frodo.wi.mit.edu/).

Probe BLAST

Probes for predicted NB-LRR genes were tested against the genome, using BLAST, to identify whether they were complementary to multiple genomic locations and to look for previously unidentified potential R-genes as well as NB-LRR homologues from other species.

Results

Genomic search results

Genomic searches for NB-LRR genes on NCBI nucleotide database within eudicotyledons produced 22 potential genes, which were used as query sequences to BLAST against the *Eucalyptus grandis* genome on Phytozome. The nucleotide BLAST results of eight of these genes against *E. grandis* produced 13 significant gene homologues as shown in Table 1. Criteria for candidate gene sequences contained NB and LRR domains, as well as e-values less than -10 and a length of greater than 500 base pairs. Suitable candidate genes were Eucgr.B01032, Eucgr.F02383, Eucgr.F04249, Eucgr.H01478 and Eucgr.H03559 (Table 1). Although Eucgr.K01668 and Eucgr.J00667 indicated good homology with NB-LRR genes from *Medicago truncatula,* their translated sequences did not incorporate either NB or LRR protein domains (Table 1 and Figure 1).

Table 1 – Phytozome BLAST results against Eucalyptus grandis *genome using eudicotyledonous plant NB-LRR genes identified from NCBI database.*

NCBI database plant resistance genes and species		**Phytozome BLAST results for Eucalyptus grandis**			
NB-LRR type genes from NCBI nucleotide search	**Species name**	***Eucalyptus grandis* locus**	***E. grandis* Scaffold/ feature**	**BLAST E-value**	**Current functional annotation from Phytozome and base pair homology**
>gi|357509266| NB-LRR type (MTR_7g089080) mRNA, complete cds	*Medicago truncatula*	Eucgr.K01668	11	2.3 E-35	Cyclin N and C terminal domains >1000bp (Cell cycle regulation)
>gi|357509266| NB-LRR type (MTR_7g089080) mRNA, complete cds	*Medicago truncatula*	Eucgr.J00667	10	1.2 E-32	Cyclin N and C terminal domains >500bp (Cell cycle regulation)
>gi|357495078| NB-LRR type Rps1-k-2 (MTR_5g095910) mRNA, complete cds	*Medicago truncatula*	Eucgr.B01032	2/4	9.2 E-15	LRR and NB-ARC domains >500bp (Recognition and binding)
>gi|323370546| ADR1 mRNA, complete cds	*Solanum tuberosum*	Eucgr.F02383	6/1	4.4E-61	LRR and NB-ARC domains >1000bp (Recognition and binding)

>gi|156229391| partial mRNA for Ran GTPase activating protein (ranGAP1 gene)	*Nicotiana benthamiana*	Eucgr.H01478 Eucgr.H03559	8/1 and 2	0	LRR, NACHT and Ran GTPase-activating protein domain >1000bp (Recognition and binding)
>gi|156229389| mRNA for Ran GTPase-activating protein 1 (ranGAP1 gene), cultivar Desiree	*Solanum tuberosum* subsp. *tuberosum*	Eucgr.H01478 Eucgr.H03559	8/1 and 2	0	LRR, NACHT and Ran GTPase-activating protein domain >1000bp (Recognition and binding)
>gi|147882992| RAN GTPase-activating protein 2 (RanGAP2) mRNA, complete cds	*Nicotiana benthamiana*	Eucgr.H03559	8/1	0	LRR, NACHT and Ran GTPase-activating protein domain >1000bp (Recognition and binding)
>gi|147882992| RAN GTPase-activating protein 2 (RanGAP2) mRNA, complete cds	*Nicotiana benthamiana*	Eucgr.H01478	8/2	0	LRR, NACHT and Ran GTPase-activating protein domain >1000bp (Recognition and binding)
>gi|147882994| RAN GTPase-activating protein 1 (RanGAP1) mRNA, complete cds	*Nicotiana benthamiana*	Eucgr.H01478	8/1	0	LRR, NACHT and Ran GTPase-activating protein domain >1000bp (Recognition and binding)
>gi|147882994| RAN GTPase-activating protein 1 (RanGAP1) mRNA, complete cds	*Nicotiana benthamiana*	Eucgr.H03559	8/2	0	LRR, NACHT and Ran GTPase-activating protein domain >1000bp (Recognition and binding)
>gi|3309618| NBS/LRR disease resistance protein (RFL1) and resistance to Pseudomonas syringae protein 5 (RPS5) genes, complete cds	*Arabidopsis thaliana*	Eucgr.F04249	6/6	3.7E-10	LRR and NB-ARC domains <500bp (Recognition and binding)

Eucalyptus grandis peptide homologues

Peptide homologues for NB-LRR candidate genes indicated 412 further *E.grandis* genes that matched the predicted protein domain criteria (Table 2). Many of these genes contained both NB and LRR domains when predicted transcripts were reviewed.

Table 2 – Eucalyptus grandis *gene loci for NB-LRR genes, the gene length and number of peptide homologues present within the genome. Most of the peptide homologues contained both NB and LRR domains.*

***E. grandis* loci containing NB-LRR genes**	**Gene length (bp)**	**Number of *E. grandis* peptide homologues**
Eucgr.B0132	3165	94
Eucgr.F02383	3740	160
Eucgr.F04249	3440	149
Eucgr.H01478	3498	4
Eucgr.H03559	1647	5

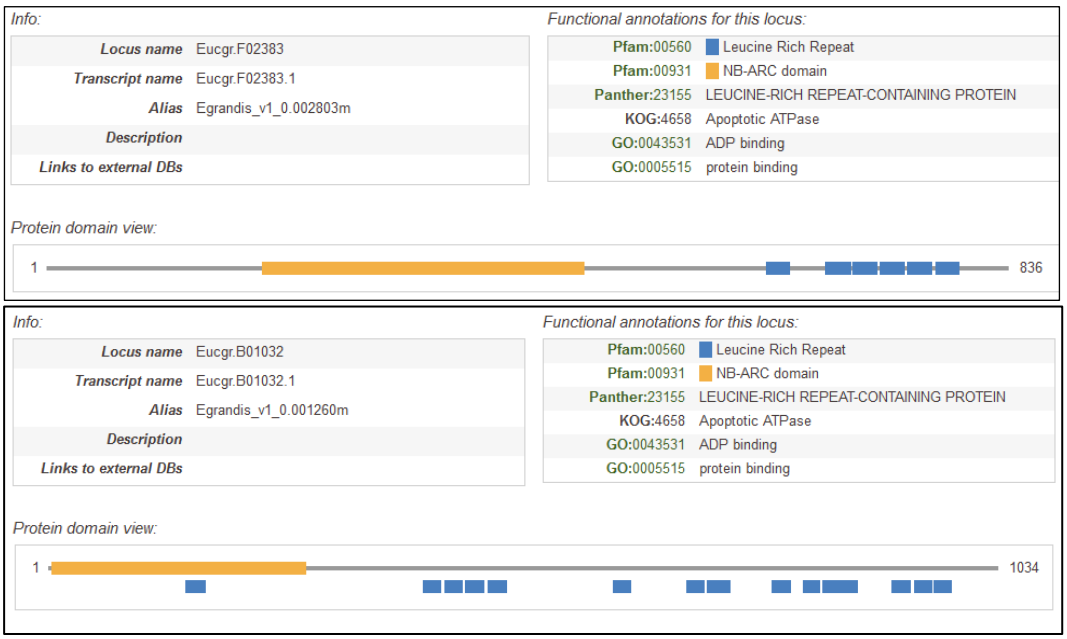
Reverse database search

A reverse nucleotide BLAST search was conducted using *E. grandis* NB-LRR sequence (Eucgr.B01032) as the query sequence. Although Eucgr.B01032 was originally identified as a homologue of a *Medicago truncatula* NB-LRR sequence (NB-LRR type Rps1-k-2), the BLAST search revealed two homologues sequences in the nucleotide nr database. Both of these sequences were from *Vitis vinifera*, and were identified as putative resistance RPP-13-like proteins (Table 3). Interestingly the original gene homologue from *Medicago truncatula*, (NB-LRR type Rps1-k-2), did not appear in the BLAST results.

Table 3 – Results of BLAST using Eucgr.B01032 gene sequence from Phytozome against NCBI. Only two homologous regions were identified, both from Vitis vinifera *(80-200bp alignment score). The second accession for* Vitis vinifera *whole genome sequence is likely to include the first accession for the disease resistance gene.*

Accession	Description	Max score	Total score	Query coverage	E-value	Max identity
XM 002271517.1	PREDICTED:Vitis vinifera putative disease resistance Rpp13-like protein	91.6	91.6	2%	3e-14	84%
AM436252.1	Vitis vinifera, whole genome shotgun sequence, contig VV78X101622.7	91.6	91.6	2%	3e-14	84%

Figure 1 – Predicted protein domains of putative R-genes in Eucalyptus grandis*, showing leucine rich repeats (LRR mostly in blue), nucleotide binding (NB, NB-ARC and NACHT mostly in yellow) and Cyclin domains. All images from the Phytozome website* http://phytozome.net/.



Probe BLAST against E. grandis genome

Eucgr.B0302, Eucgr.F02383 and Eucgr.H03559 sequences were analysed and the conserved regions were used to design PCR primers using the Primer 3 software. The BLAST function on Phytozome was used to determine the specificity of these probes for their targets. The results indicated that the primers were homologous to 141 NB-LRR sequences from various species (for Eucgr.B0302 based primers), 33 disease resistance protein homologues (for Eucgr.F02383 based primers) and five RanGTPase activating protein homologues (for Eucgr.H03559 based primers). This in silico analysis suggested that the primer sequences were specific for their intended targets and are therefore good probes for amplifying NB-LRR targets from *Eucalyptus grandis* genomic DNA.

Table 4 – Phytozome BLAST results against Eucalyptus grandis *genome using probes indicating numbers of plant peptide homologues and* E. grandis *scaffold results.*

Probe for gene sequences (20 nucleotide)	***E. grandis* homology (scaffold)**	**Number of potential targets (50%ID 20%coverage) and (1E-5)**	**Plant peptide homologues of interest**
Eucgr.B0302	2/1	30	*Arabidopsis thaliana* disease resistance proteins (CC-NB-LRR and TIR-NB-LRR)
	2/2	54	*Arabidopsis thaliana* disease resistance proteins (CC-NB-LRR and TIR-NB-LRR) and NBS-LRR disease resistance protein in rice.
	2/3	57	*Arabidopsis thaliana* disease resistance proteins (CC-NB-LRR and TIR-NB-LRR) and stripe rust resistance protein in rice (LOC_Os11g46140.1)
Eucgr.F02383	6	33	Various species including *Arabidopsis thaliana* disease resistance proteins (CC-NB-LRR and TIR-NB-LRR).
Eucgr.H03559	8	5	Arabidopsis thaliana RanGTPase activating protein

Discussion

Plant defence mechanisms are a particularly active area of study due to the promising potential of introducing natural resistance into crop plants to minimise costly intervention or crop losses (An & Mou, 2011; Conrath, 2011). To this end, resistance genes have been incorporated into crops such as wheat and canola with short term positive results (Jin & Singh, 2006; Van de Wouw et al., 2010). R-genes have highly diversifying regions allowing adaptive selection to pathogen effectors (Hammond-Kosack & Kanyuka, 2001). There are also a number of R-genes within plants that appear to have arisen due to horizontal gene transfer and duplication events, thereby allowing for retention of multiple variants of

potential resistance genes (Bergelson et al., 2001). These paralogs allow for the rapid response to pathogen effectors through single base mutations (Fluhr 2001). It is therefore not an unreasonable assumption to seek resistance genes for newly arrived or adapted pathogens, particularly amongst wild populations.

With the recent introduction into Australia of the fungal pathogen causing Myrtle Rust (Carnegie & Cooper, 2011; Carnegie & Lidbetter, 2012), understanding host response in Myrtaceae plants is particularly relevant. Little is currently known regarding defence response variation within natural populations of *E. grandis.* This is of great importance when reviewing potential genetic variation in susceptibility to new pathogens such as *P. psidii*. Studies of differential stress responses are currently being conducted into wild populations of crop plants to identify novel genes that may enhance viability and increase crop potential (Ergen & Budak, 2009). Developing an understanding of molecular defence responses from *E. grandis* may provide novel gene targets for the benefit of the forestry industry. From a conservation perspective it is important to identify these transcriptional responses to enhance an understanding of the likely impacts of this novel pathogen on natural plant communities. Information of response to this pathogen in *E. grandis* will likely facilitate similar studies into other Eucalyptus species that are potentially at risk.

The current study identified 412 potential R-genes corresponding to the NB-LRR motif, the major class of plant resistance genes (Fluhr, 2001), within the draft genome of *Eucalyptus grandis*. Of interest amongst the identified plant peptide homologues was a protein for a putative Stripe Rust resistance protein for *Oryza sativa,* as well as numerous resistance genes from *Arabidopsis thaliana* and other species. A further finding was that although the two gene loci, Eucgr.K01668 and Eucgr.J00667, indicated good homology with

NB-LRR genes from *Medicago truncatula,* the functional annotation did not identify either NB or LRR protein domains. Instead the annotation was for a gene whose protein was predicted to function in cell cycle regulation, a very different predicted function to classical NB-LRRs. This implies that the annotation for either the *M. truncatula* or *E. grandis* gene is currently incorrect and requires revision. Though not presented in this study, the scaffold regions for many identified R-genes were apparently clustered closely. This too requires follow up by greater scrutiny of the loci and the gene sequences. Previous studies (Barbosa-da-Silva et al., 2005) have suggested that the NB-LRR genes may be organised into high density clusters. It may also be that some genes are currently presented more than once due to computational annotation errors. The clustering of R-genes allows for rapid evolutionary response to pathogen effectors (Fluhr, 2001; Sinapidou et al., 2004; Barbosa-da-Silva et al., 2005) and may be involved in the duplex formation of R-proteins (Eitas & Dangl, 2010) that is required for effective response to avirulence proteins.

The findings of this study provide a useful starting point for investigating specificity of host response to pathogen effectors in this newly sequenced species. Evidence for resistance gene homologues within *Eucalyptus grandis* should now be further verified with host-pathogen studies and molecular analysis.

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