Program 3: Identification and characterisation of novel sources of blackleg resistance

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Aims

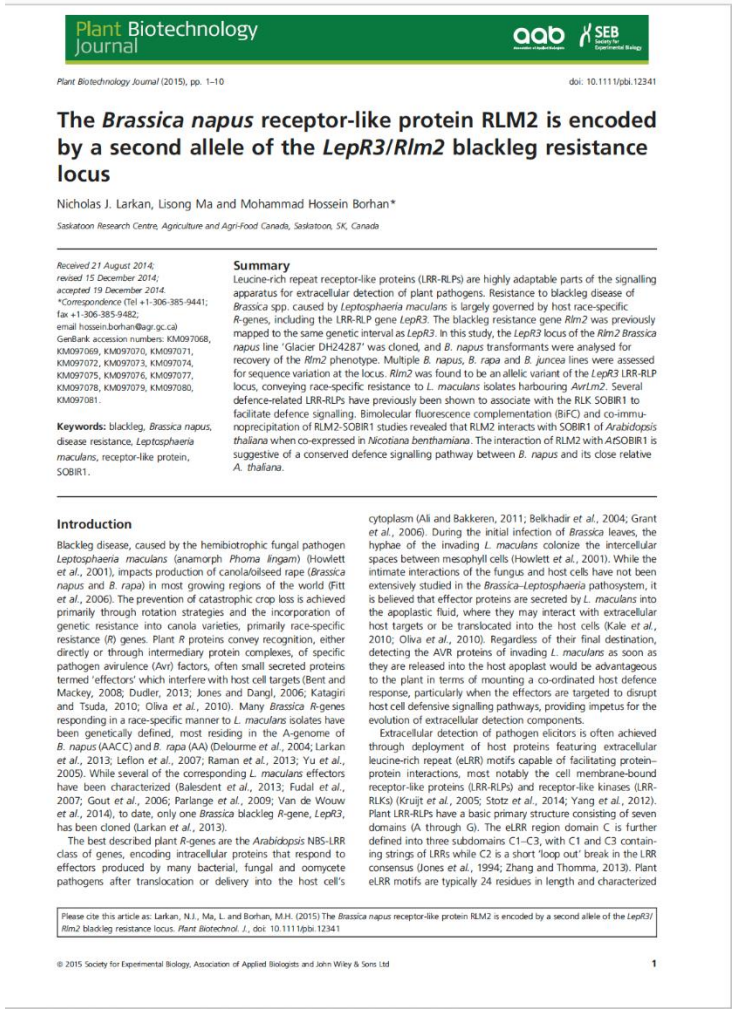
- Develop molecular markers for routine screening of blackleg resistance genes
- Identify candidate genes for genetically mapped resistance genes
- Deliver novel sources of qualitative blackleg resistance

Molecular marker development

- Screened 112 current and past Australian varieties
- Undertook genome sequencing
- Correlated information with phenotypic screening

Rlm1, Rlm4, LepR1	2018	72	72A1	310	29.60		200	PCR#01	72A	(-)	(+)	(-)	(-)	Spring	Hybrid	Rlm1, Rlm4, LepR1	Correct
			72A2		33.80	1.89	200	NovaSeq									
Rlm4	2016-4	73	73A1	430	157.00		35	PCR#01	73A	(-)	(+)	(-)	(-)	Spring	OP	Rlm4	Correct
			73A2		238.00	1.88	35	NovaSeq									
Rlm4, Rlm6	2016-4	74	74A1	330	128.00	1.84	35	NovaSeq	74A	(-)	(+)	(-)	(-)	Spring	Hybrid	Rlm4, Rlm6	Correct
			74A2		46.00		35	PCR#01									

Molecular marker development



Plant Biotechnology Journal

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The *Brassica napus* receptor-like protein RLM2 is encoded by a second allele of the *LepR3/Rlm2* blackleg resistance locus

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GenBank accession numbers: KM097068, KM097069, KM097070, KM097071, KM097072, KM097073, KM097074, KM097075, KM097076, KM097077, KM097078, KM097079, KM097080, KM097081.

Keywords: blackleg, *Brassica napus*, disease resistance, *Leptosphaeria maculans*, receptor-like protein, SOBIR1.

Summary

Leucine-rich repeat receptor-like proteins (LRR-RLPs) are highly adaptable parts of the signalling apparatus for extracellular detection of plant pathogens. Resistance to blackleg disease of *Brassica* spp. caused by *Leptosphaeria maculans* is largely governed by host race-specific *R*-genes, including the LRR-RLP gene *LepR3*. The blackleg resistance gene *Rlm2* was previously mapped to the same genetic interval as *LepR3*. In this study, the *LepR3* locus of the *Rlm2 Brassica napus* line 'Glacier DH24287' was cloned, and *B. napus* transformants were analysed for recovery of the *Rlm2* phenotype. Multiple *B. napus*, *B. rapa* and *B. juncea* lines were assessed for sequence variation at the locus. *Rlm2* was found to be an allelic variant of the *LepR3* LRR-RLP locus, conveying race-specific resistance to *L. maculans* isolates harbouring *AvrLm2*. Several defence-related LRR-RLPs have previously been shown to associate with the RLK SOBIR1 to facilitate defence signalling. Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation of RLM2-SOBIR1 studies revealed that RLM2 interacts with SOBIR1 of *Arabidopsis thaliana* when co-expressed in *Nicotiana benthamiana*. The interaction of RLM2 with *At*SOBIR1 is suggestive of a conserved defence signalling pathway between *B. napus* and its close relative *A. thaliana*.

Introduction

Blackleg disease, caused by the hemibiotrophic fungal pathogen *Leptosphaeria maculans* (anamorph *Phoma lingam*) (Howlett *et al.*, 2001), impacts production of canola/oilseed rape (*Brassica napus* and *B. rapa*) in most growing regions of the world (Fitt *et al.*, 2006). The prevention of catastrophic crop loss is achieved primarily through rotation strategies and the incorporation of genetic resistance into canola varieties, primarily race-specific resistance (*R*) genes. Plant *R* proteins convey recognition, either directly or through intermediary protein complexes, of specific pathogen avirulence (Avr) factors, often small secreted proteins termed 'effectors' which interfere with host cell targets (Bent and Mackey, 2008; Dudler, 2013; Jones and Dangl, 2006; Katagiri and Tsuda, 2010; Oliva *et al.*, 2010). Many *Brassica R*-genes responding in a race-specific manner to *L. maculans* isolates have been genetically defined, most residing in the A-genome of *B. napus* (AACC) and *B. rapa* (AA) (Delourme *et al.*, 2004; Larkan *et al.*, 2013; Leflon *et al.*, 2007; Raman *et al.*, 2013; Yu *et al.*, 2005). While several of the corresponding *L. maculans* effectors have been characterized (Balesdent *et al.*, 2013; Fudal *et al.*, 2007; Gout *et al.*, 2006; Parlange *et al.*, 2009; Van de Wouw *et al.*, 2014), to date, only one *Brassica* blackleg *R*-gene, *LepR3*, has been cloned (Larkan *et al.*, 2013).

The best described plant *R*-genes are the *Arabidopsis* NBS-LRR class of genes, encoding intracellular proteins that respond to effectors produced by many bacterial, fungal and oomycete pathogens after translocation or delivery into the host cell's cytoplasm (Ali and Bakkeren, 2011; Belkhadir *et al.*, 2004; Grant *et al.*, 2006). During the initial infection of *Brassica* leaves, the hyphae of the invading *L. maculans* colonize the intercellular spaces between mesophyll cells (Howlett *et al.*, 2001). While the intimate interactions of the fungus and host cells have not been extensively studied in the *Brassica–Leptosphaeria* pathosystem, it is believed that effector proteins are secreted by *L. maculans* into the apoplastic fluid, where they may interact with extracellular host targets or be translocated into the host cells (Kale *et al.*, 2010; Oliva *et al.*, 2010). Regardless of their final destination, detecting the AVR proteins of invading *L. maculans* as soon as they are released into the host apoplast would be advantageous to the plant in terms of mounting a co-ordinated host defence response, particularly when the effectors are targeted to disrupt host cell defensive signalling pathways, providing impetus for the evolution of extracellular detection components.

Extracellular detection of pathogen elicitors is often achieved through deployment of host proteins featuring extracellular leucine-rich repeat (eLRR) motifs capable of facilitating protein–protein interactions, most notably the cell membrane-bound receptor-like proteins (LRR-RLPs) and receptor-like kinases (LRR-RLKs) (Kruijt *et al.*, 2005; Stotz *et al.*, 2014; Yang *et al.*, 2012). Plant LRR-RLPs have a basic primary structure consisting of seven domains (A through G). The eLRR region domain C is further defined into three subdomains C1–C3, with C1 and C3 containing strings of LRRs while C2 is a short 'loop out' break in the LRR consensus (Jones *et al.*, 1994; Zhang and Thomma, 2013). Plant eLRR motifs are typically 24 residues in length and characterized

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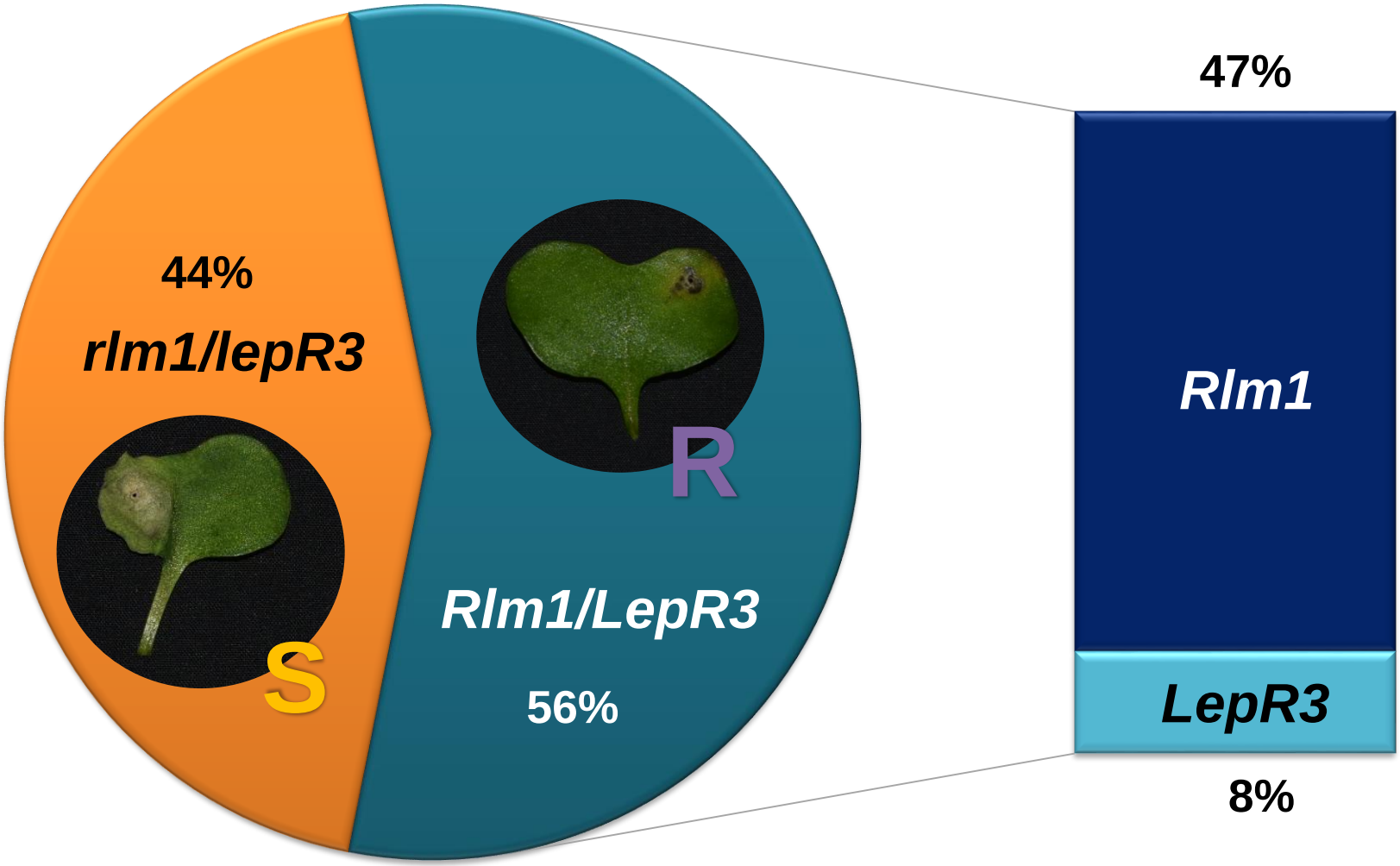
LepR3 screening



Rlm1/LepR3 screening

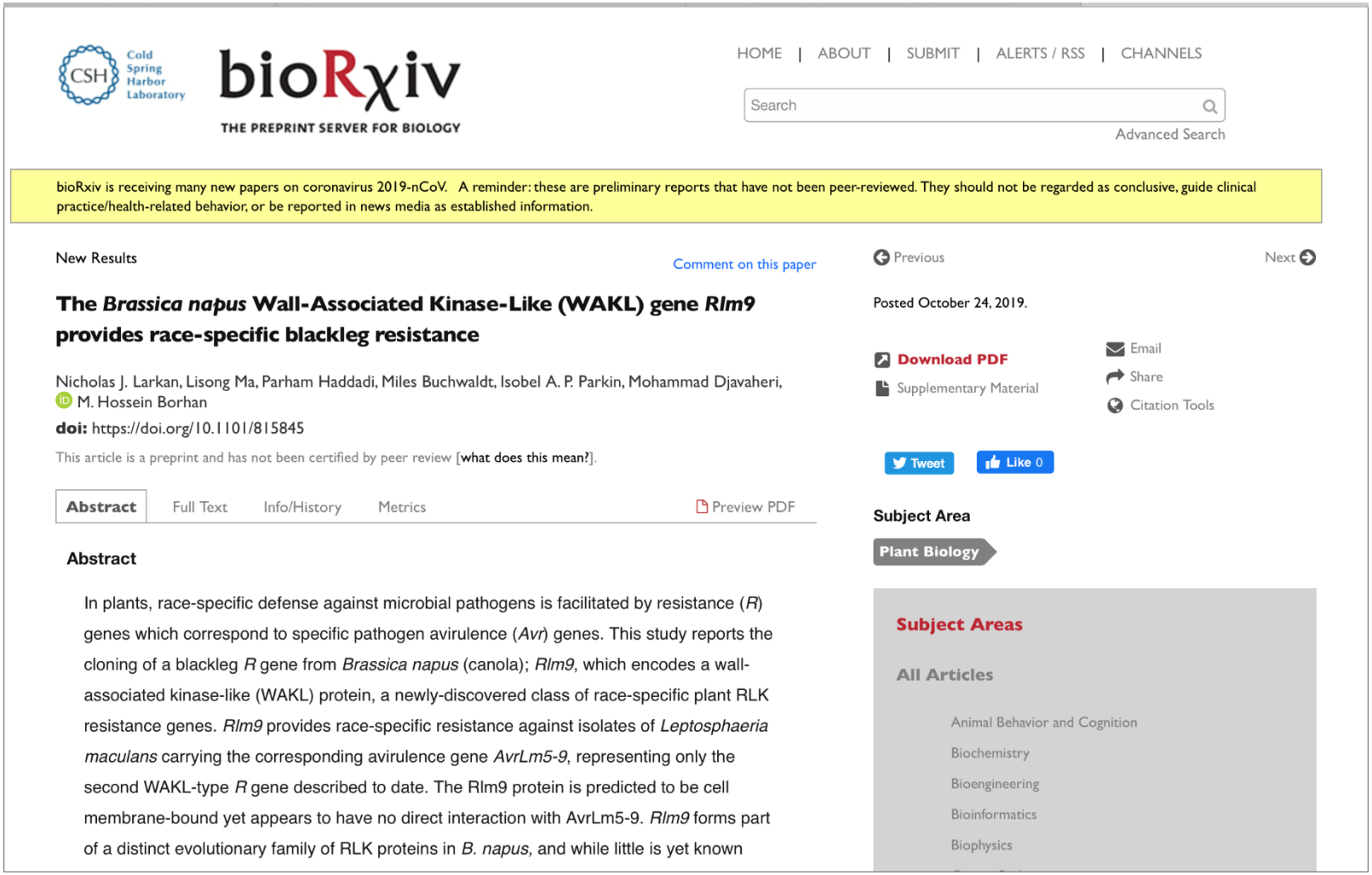
- **5** cultivars were previously thought to have *Rlm1*
- **1** cultivar was thought to have *LepR3*

Proposed Genotype	***LepR3*** **Amplification**
LepR3, RlmS	Yes
LepR3, RlmS	Yes
Rlm1, Rlm4	Yes
Rlm1, Rlm4, Rlm9	Yes
Rlm1, Rlm4, LepR1	Yes
LepR3, RlmS, Rlm4	No
Rlm1	Yes
LepR3, RlmS	Yes
Rlm1, Rlm4, LepR1	Yes
Rlm1, Rlm4, Rlm6, LepR1	Yes

Rlm2 screening

- Screening prior to 2018 could not detect *Rlm2*
- Presence of *Rlm2* detected in four cultivars

Rlm9 screening



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The *Brassica napus* Wall-Associated Kinase-Like (WAKL) gene *Rlm9* provides race-specific blackleg resistance

Nicholas J. Larkan, Lisong Ma, Parham Haddadi, Miles Buchwaldt, Isobel A. P. Parkin, Mohammad Djavaheri, M. Hossein Borhan

doi: https://doi.org/10.1101/815845

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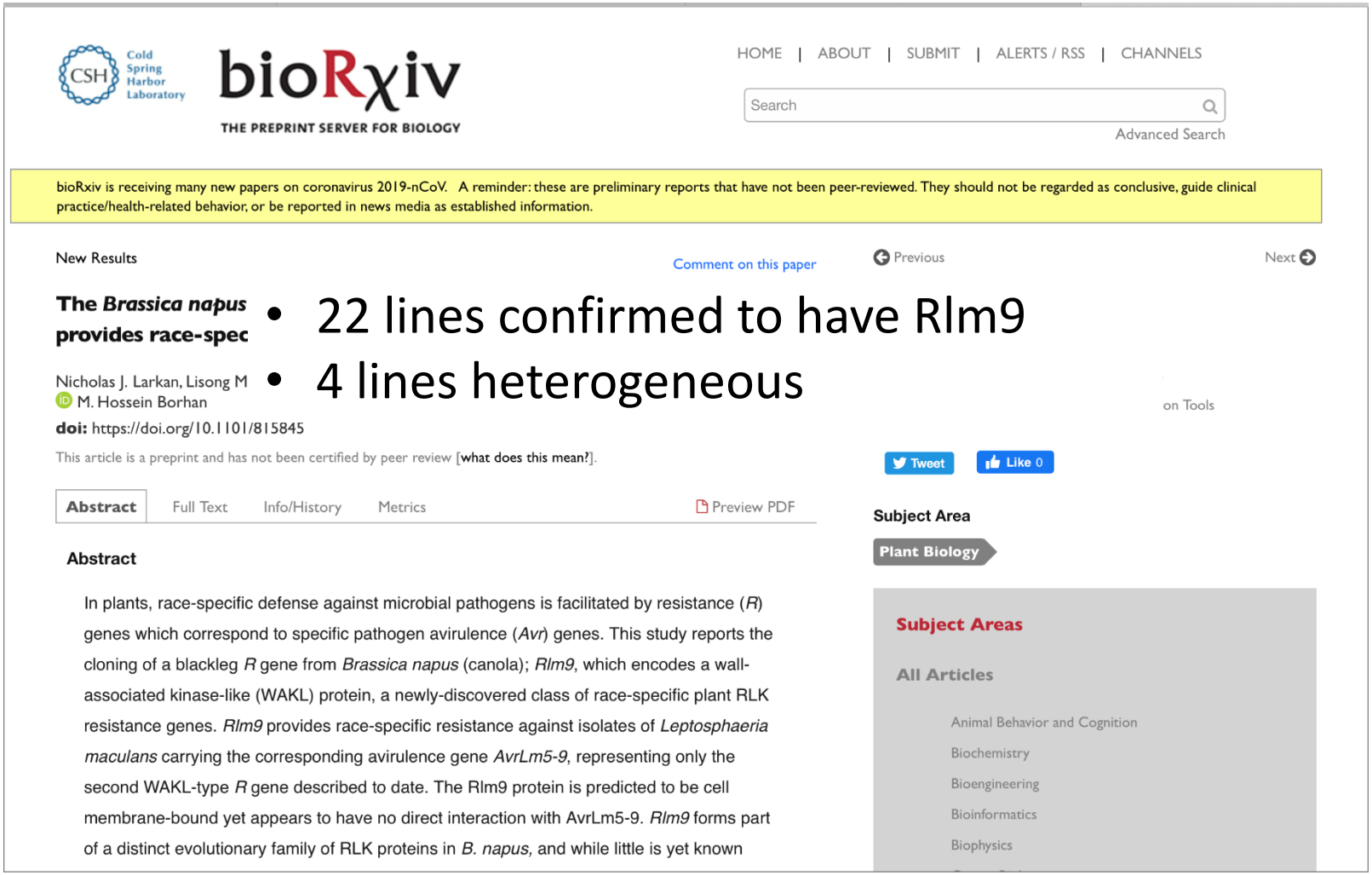
In plants, race-specific defense against microbial pathogens is facilitated by resistance (*R*) genes which correspond to specific pathogen avirulence (*Avr*) genes. This study reports the cloning of a blackleg *R* gene from *Brassica napus* (canola); *Rlm9*, which encodes a wall-associated kinase-like (WAKL) protein, a newly-discovered class of race-specific plant RLK resistance genes. *Rlm9* provides race-specific resistance against isolates of *Leptosphaeria maculans* carrying the corresponding avirulence gene *AvrLm5-9*, representing only the second WAKL-type *R* gene described to date. The Rlm9 protein is predicted to be cell membrane-bound yet appears to have no direct interaction with AvrLm5-9. *Rlm9* forms part of a distinct evolutionary family of RLK proteins in *B. napus,* and while little is yet known

Rlm9 screening



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New Results

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The *Brassica napus* provides race-spec

- 22 lines confirmed to have Rlm9
- 4 lines heterogeneous

Nicholas J. Larkan, Lisong M
M. Hossein Borhan

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doi: https://doi.org/10.1101/815845

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Plant Biology

Abstract

In plants, race-specific defense against microbial pathogens is facilitated by resistance (*R*) genes which correspond to specific pathogen avirulence (*Avr*) genes. This study reports the cloning of a blackleg *R* gene from *Brassica napus* (canola); *Rlm9*, which encodes a wall-associated kinase-like (WAKL) protein, a newly-discovered class of race-specific plant RLK resistance genes. *Rlm9* provides race-specific resistance against isolates of *Leptosphaeria maculans* carrying the corresponding avirulence gene *AvrLm5-9*, representing only the second WAKL-type *R* gene described to date. The Rlm9 protein is predicted to be cell membrane-bound yet appears to have no direct interaction with AvrLm5-9. *Rlm9* forms part of a distinct evolutionary family of RLK proteins in *B. napus,* and while little is yet known

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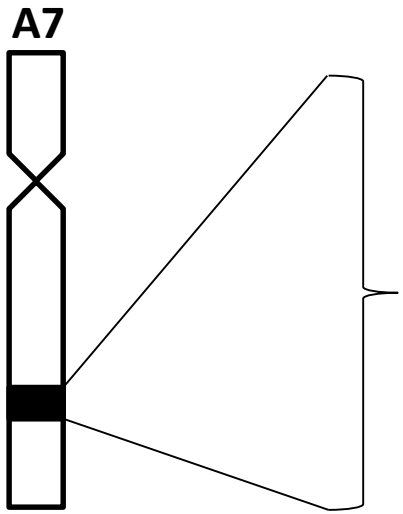
Locating candidate region for Rlm4 and 7 with linked genetic markers



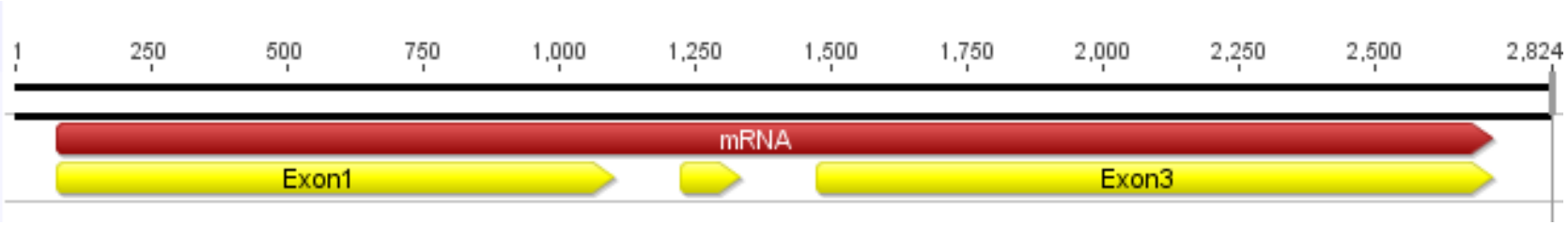
- *Rlm4* and *Rlm7* are closely linked
- *Rlm4* and *Rlm7* interact with the same *AvrLm4-7*
- 8 linked markers to *Rlm4*
- 2 linked markers to *Rlm7*
- The region where the 10 markers overlap covered 100 kbp

Sequencing *Rlm4* candidate region

- The 100kbp candidate region was broken down to 10 10 kbp long-range PCR amplicons
- R gene domains found in one amplicon F8-R9

Minion seq of the amplicon F8-R9

- Identified a candidate gene



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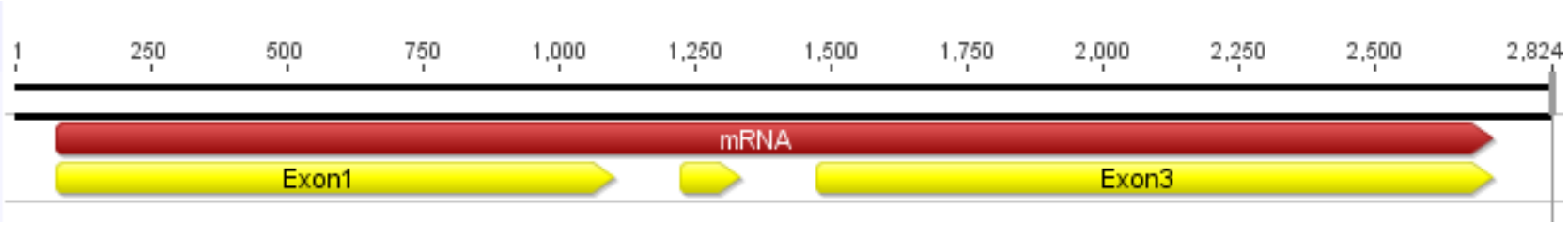
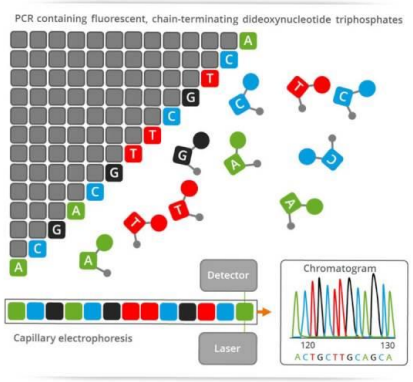
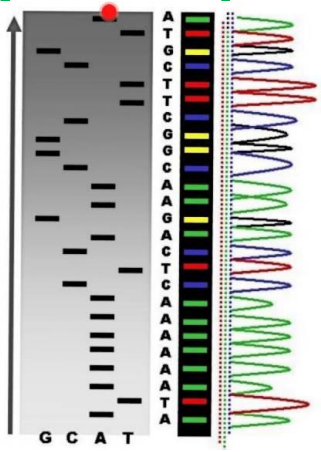


The *Rlm4* allele is significantly different from the reference (*rlm4*)

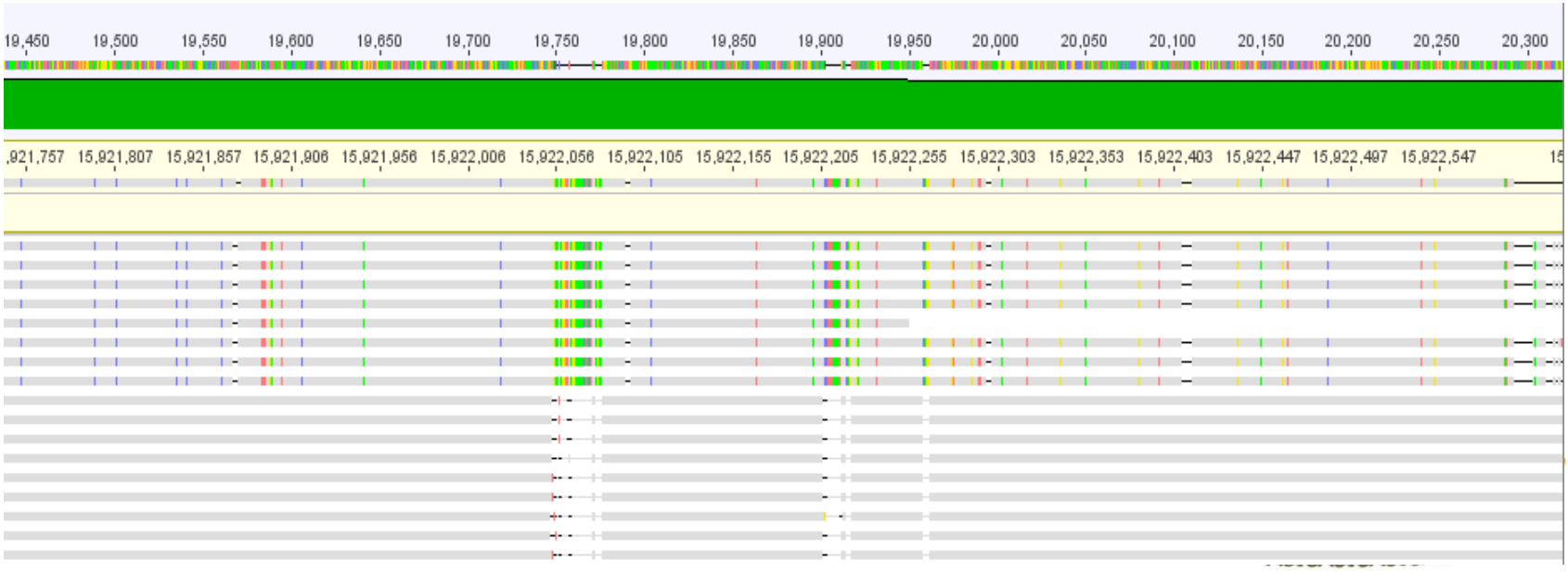
All *Rlm4* lines share the same polymorphisms

Sanger Sequencing





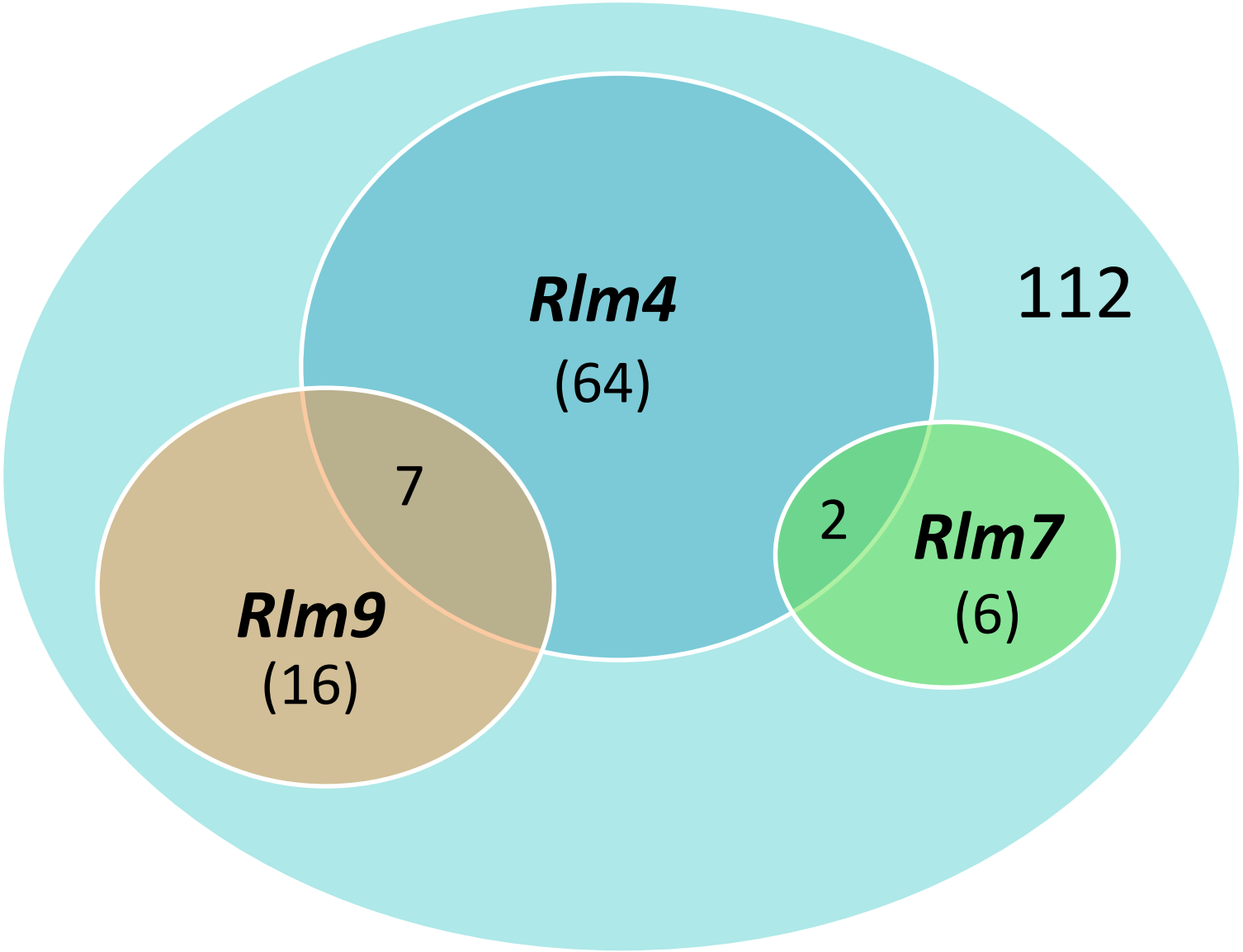
- Sanger sequence and compare the alleles between *Rlm4* and *rlm4* lines



Rlm3 and 7 identification

- Using same method have identified candidate genes for *Rlm3* and *Rlm7*
- Markers developed for *Rlm7*
- *Rlm3* marker development currently underway

Rlm4,7,9 screening



Marker development

- In 90% of the varieties tested, the genotypic and phenotypic data correlated.
- In a further 6% of the varieties, *LepR3* or *Rlm2* were detected using molecular markers but are not detectable phenotypically due to the masking of other resistance genes in the differential isolates used for screening.
- Remaining 4% undergoing ongoing testing to include more replicates to confirm the findings

Marker deployment

- MGP to offer service in 2020 for routine screening for known resistance genes using molecular markers
  - New markers added to set as developed
- Screening all germplasm with four differential isolates that between them are virulent towards the 10 known resistance genes (Rlm1, Rlm2, Rlm3, Rlm4, Rlm6, Rlm7, Rlm9, RlmS, LepR1 and LepR3).
  - If isolates show avirulent reactions on the germplasm then it must harbour either completely new sources of resistance or a resistance source that is known but not currently used in Australian germplasm (e.g. Rlm5, Rlm8, Rlm10, Rlm11).

Screening for novel sources of resistance

- Identified wild lines, synthetic napus and introgression lines for screening
- 200 lines currently selected for screening
  - Initial screening of 20 lines identified a line for further analysis

Summary

- Identification of *Rlm1* and *Rlm6*
- Molecular markers developed for routine screening of *Rlm2, Rlm4, Rlm7, Rlm9* and *LepR3* in *Brassica napus* lines.
- Molecular markers developed for routine screening of *AvrLepR2, AvrLm10* and *AvrLm11* in *L. maculans*
- Pan genomes developed for *Brassica napus* and *L. maculans* for candidate R and Avr gene identification
- Genome sequence obtained for all commercial Australian canola cultivars
- Novel germplasm sourced for future screening for candidate gene identification