Biotechnology and gene mapping in lentil

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Abstract: Genomic tools and genetic mapping are assisting the understanding of the lentil genome and have made possible the use of marker assisted selection for breeding purposes. Although some important traits are conferred by single genes most are determined by quantitative trait loci (QTL) and influenced by environmental factors. Genes for several traits have been genetically mapped and shown to be linked to molecular markers. These include resistance to fusarium wilt, ascochyta blight, anthracnose, and stemphylium blight. Winter hardiness and tolerance to frost have also been mapped. It is now feasible to use the linked markers in a marker assisted selection breeding program. Proteomics and metabolomics are emerging technologies that can be used to better characterize the functional mechanisms behind breeding targets.

Key words: abiotic stress resistance, disease resistance, functional genes, genetic mapping, metabolomics, molecular markers, proteomics, quantitative trait loci, recombinant inbred lines

Introduction

Significant advances in the availability of genomics tools towards understanding the function and selection of specific components of the lentil genome have recently been made. Several advanced breeding programs worldwide have implemented and are currently using molecular assisted breeding technology. However, this has to date been limited to the selection of rather few traits, mostly likely due to lack of resources for broad validation and implementation. Nevertheless, high throughput marker generation and genotyping that is functionally associated, together with novel tools such as next generation sequencing and available genome maps, are illuminating the complex and intertwined nature of responses to biotic and abiotic stimuli in the lentil genome.

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Genomics and functional gene identification

Global gene expression profiling at the mRNA level has been used to identify functionally-associated genes. Characterization of the RNA population under a particular environmental and/or developmental condition enables understanding of the dynamic functioning of genes as well as their mutual role in specific regulatory networks. This approach may be used to dissect regulatory mechanisms and transcriptional networks involved in defence responses to pathogen and physiological responses to abiotic stress such as drought, cold and salinity.

Differential gene expression methods include cDNA-amplified fragment length polymorphism (cDNA-AFLP) (1), suppression subtractive hybridization (SSH) (6), serial analysis of gene expression (SAGE) (30), differential display (18. 31), massively parallel signature sequencing (MPSS™) and microarray technology (25). Of these, microarrays have become the method of choice for large scale systemic analysis of differential gene expression profiling. This method is semi-quantitative, sensitive to low abundance transcripts that are represented on a given array and has been successfully used to study plant responses to various biotic and abiotic factors in *Arabidopsis thaliana* (3, 22, 26), *Medicago truncatula* (11, 17), soybean (*Glycine max*) (19, 28) and chickpea (*Cicer arietinum*) (5, 20).

Most recently, this method was used to elucidate the functional response to attack from Ascochyta blight, caused by *Ascochyta lentis* Vassilievsky, an important fungal disease worldwide (8).

Differentially expressed genes were identified among resistant (Ill7537) and susceptible (ILL6002) genotypes, which may serve as accurate selection tools in the future development of varieties with increased and sustainable resistance. For this, a cDNA microarray was used to observe substantial difference in functional category and timing of gene expression among the two genotypes, often referred to as the Pathogen/Microbe-Associated Molecular Pattern (P/MAMP). In particular, large differences were observed in early up-regulation of Resistance Gene Analogues (RGA; Figure 1), as well as several classes of mycotoxic producing genes such as PR4 and PR10. In ILL7537 (resistant), RGAs were switched on very early and quickly down regulated before being up-regulated again. Conversely, the same genes were up-regulated 24 hours later in ILL6002 (susceptible) and at much higher levels. Thus the question arises as to whether these genes act as 'surveillence molecules' or recognition/receptors to quickly initiate subsequent defence signalling cascades in the resistant genotype and it's a case of a little too much, too late in the susceptible genotype? Perhaps the failure to quickly recognise the invading pathogen prior to colonisation leads to the high susceptibility response.

Similarly, in the early stage of invasion, several other classes of defence responses are seen to be initiated much faster in the resistant ILL7537 genotype. In fact, the classic symptoms associated with an hypersensitive response (HR), such as browning of tissue and necrosis around the point of invasion, is not seen at all in ILL6002, and less frequently in ILL5588 (cv. Northfield; moderately resistant), when compared to ILL7537. Early evidence of this differential response is seen by tracking expression of superoxide dismutase, a enzyme used in the "mopping up" process of reactive oxygen species (ROS) following an oxidative burst, whereby the gene is expressed much sooner and at higher levels in ILL7537 than in ILL6002 (Figure 2).

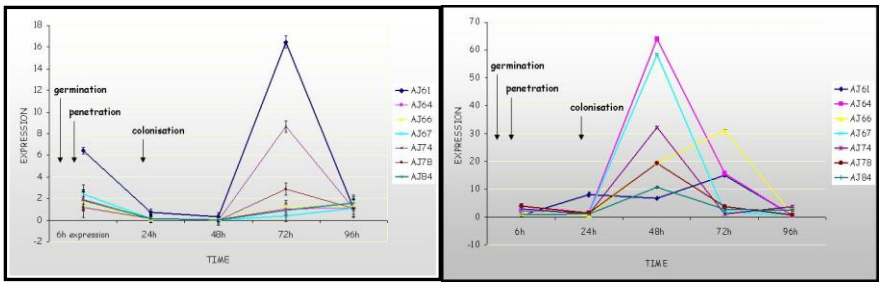

Figure 1. The differential timing of expression of RGA sequences among seedlings inoculated and un-inoculated with *Ascochyta lentis* (left) ILL7537 and (right) ILL6002 genotypes

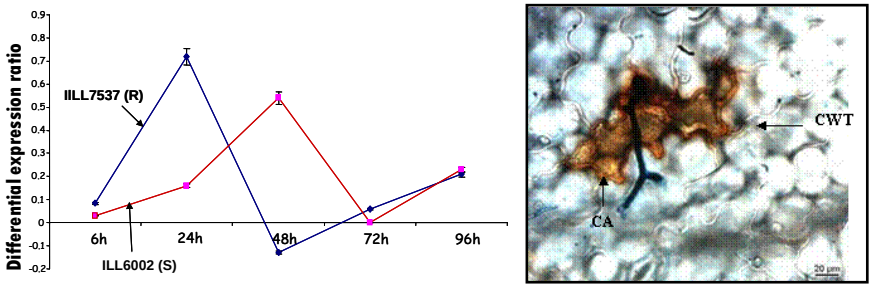

Figure 2. Evidence of (left) a differential early HR between ILL7537 and ILL6002 to Ascochyta lentis inoculation and (right) HR symptoms seen in ILL7537 including browning, necrosis, cell wall thickening (CWT) and cytoplasmic aggregation (CA).

A major current limitation of microarray technology for lentil is the lack of pre-requisite lentil-specific functional genome data (cDNA/EST sequences) to place as probes upon the arrays. However, several research teams (AgriFood, Canada and VicDPI, Australia) are preparing large lentil EST data sets, as well as developing single nucleotide polymorphism (SNP) markers that may be used for genotype-phenotype association and validation study. Once these tools are available, high-throughput functional genomic assessment using arrays will be next leap in lentil biotechnology towards faster, smarter and more sustainable trait selection. However, prior to the accurate use in selection programs of molecular markers, that have been functionally validated, their genomic positioning is required.

Mapping the lentil genome

Although some agronomically important traits are governed by single genes, most are governed by quantitative trait loci (QTL), influenced by both genetic and environmental factors. Since the expression of a QTL is likely to vary among populations and environments, their genomic location and effect must be determined for a specific genetic background and environment (2). The previous "orphan" status of the lentil genome has meant that most existing framework genome maps contain many non-functional RAPD, AFLP, ISSR and SSR-type markers, which are effective for saturating the entire genome but are not directly related to desirable traits or QTL. However, the newly developed gene/locus specific EST and SNP markers are reproducible and represent definite genomic regions. Their placement on existing maps will draw together the functional and physical association for ultimate accurate trait selection.

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The current status of marker-assisted breeding

Using non-functional markers (7), there were first mapped five QTL for height of the first ramification, three for plant height, five for flowering, seven for pod dehiscence, one for shoot number and one for seed diameter. Subsequently, QTL have been identified conditioning winter survival and injury, however, only one of five QTL was expressed in all environments assessed. QTL conditioning resistance to ascochyta blight (23), stemphylium blight (24), rust and white mould have also been mapped. Also, the major QTL underpinning physical seed quality traits such as size, shape and colour have been mapped (Inder et al., Melbourne University, unpublished). However, ideally, the "candidate" gene(s) actually controlling a trait of interest would be used for marker-assisted selection (MAS). Hence, genomic regions where the trait is mapped should be characterized at high resolution (since recombination rates may vary at different genomic regions) and be validated across genetic backgrounds, in order to determine their utility in MAS and to potentially uncover the functional gene(s) themselves. This has been made more of a possibility with next generation sequencing of genomic fragments, such as BACs, associated with the QTL region of interest.

Table 1 Published genetic linkage maps for lentil; mapping populations and types of markers mapped

Population mapped	Marker types mapped	Citation
Interspecific F_2	RFLP, isozymes, morphological	Havey and Muehlbauer, 1989
Inter-subspecific RIL	RFLP, RAPD, AFLP	Eujayl *et al.*, 1998
Intraspecific F_2	RAPD, ISSR,	Rubeena *et al.*, 23
Intraspecific RIL	RAPD, ISSR, AFLP	Kahraman *et al.*, 24
Inter-subspecific F_2	RAPD, ISSR, AFLP, SSR	Durán *et al.*, 24
Inter-subspecific RIL	AFLP, SSR	Hamwieh *et al.*, 25
Intraspecific RIL	SSR, ITAP	Phan *et al.*, 27
Intraspecific RIL	SSR, RAPD, SRAP	Saha *et al.*, 2010

Table 2 Molecular markers closely associated with desirable lentil breeding traits for use in marker-assisted selection

Trait mapped	Associated molecular markers	Citation
Fusarium wilt resistance (*Fw*)	OPK15	Eujayl *et al.*, 1998
Ascochyta blight resistance (*AbR*1)	RV01, RB18, SCARW19	Ford *et al.*, 1999
Ascochyta blight resistance (*ral*2)	UBC227, OPD-10	Chowdury *et al.*, 2001
Ascochyta blight resistance (mapped as a QTL)	C-TTA/M-AC (QTL1 and QTL2), M20 (QTL3)	Rubeena *et al.*, 2003
Anthracnose resistance (*Lct*2)	OPE06, UBC704	Tullu *et al.*, 2003
Frost tolerance (*Frt*)	OPS-16	Eujayl *et al.*, 1999
Winter hardiness	UBC808-12	Kahraman *et al.*, 2004
Fusarium wilt resistance (*Fw*)	SSR59-2B, p17m30710	Hamwieh *et al.*, 2005
Stemphylium resistance	SRAP ME5XR10 and ME4XR16c	Saha *et al.*, 2010

Meanwhile, there are several markers available for different traits that have the potential for use in MAS and gene pyramiding (Table 2). These include SCARW19 and SCARB18 linked to and flanking the *Ab*R1 *A. lentis* resistance loci (27). These enabled successful pyramiding of the *Ab*R1 and *ral*2 *A. lentis* resistance loci together with the *LCt*2 *Colletotrichum truncatum* (anthracnose) resistance loci (23). Most recently the sequence related amplified polymorphism (SRAP) marker, ME4XR16c, has been validated for utility in selecting resistance to stemphylium disease (24).

The future of lentil biotechnology

Without doubt, reports using biotechnology approaches such as proteomics and metabolomics will soon begin to emerge for lentil, in order to discover and better characterize the functional mechanisms behind the breeding targets. This will include a thorough investigation of pathogen effector and host recognition factors involved in disease defence. In particular, the whole genome sequence of the *Ascochyta lentis* genome has recently become available and is currently being annotated (Ford and Lichtensvieg, unpublished). This will be searched for possible effector-related sequences in comparative studies for respective gene expression and protein/metabolite molecules to determine lentil host recognition factors. Also, it is envisaged that next generation sequencing technologies will uncover families of host transcription factors (i.e. *Myb* genes) and downstream genes that are key in the specific biochemical pathways for many stress tolerance and quality traits. With the advancement in functional genomics, expression QTL (eQTL) can be identified for the traits of interest by coupling global genome expression profiling and suitable genetic materials. Since eQTL affect the expression of the genes for the trait of interest, the markers linked to this eQTL will have enormous reliability in MAS compared to the markers identified by traditional QTL analysis. Ultimately, and with sufficient funding, precise formulation of superior and high yielding genotypes will emerge through the combination of lentil 'omics' approaches that will be delivered to a multitude of environments and market preferences. ■

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