CHAPTER 17

LENSOMICS: ADVANCES IN GENOMICS AND MOLECULAR TECHNIQUES FOR LENTIL BREEDING AND MANAGEMENT

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Abstract: Lentil is a self-pollinating diploid (2n = 14 chromosomes) annual cool season grain legume produced as a high protein food source throughout the world. Several lentil genome maps are available and recent progress towards a consensus map has been made by employing robust locus markers that are derived from the model legume *Medicago truncatula* and other legume genomes. Such markers are co-dominant and will likely be useful across a broad lentil genetic background for marker-assisted trait selection. Candidate trait-associated genes are under investigation, particularly for disease resistance, and these are soon likely to become available for validation against pathogen populations and in differing environments using transgenic approaches. For this, reliable transformation systems have been developed. However, further effort is required to develop a robust and high-throughput full regeneration system for transformant lentil plants. The near future of Lensomics will include further candidate gene characterisation through transcriptome and reverse genetic techniques. These studies will be conducted to uncover genes responsive to biotic and abiotic stimuli as well as those governing desirable seed quality traits, such as size, shape and colour. Furthermore, proteomic and metabolomic approaches will be employed to derive information on the functional mechanisms involved

S.S. Yadav et al. (eds.), Lentil: An Ancient Crop for Modern Times, 275–289.

1. INTRODUCTION

Since lentil is historically grown in areas of the world where funding for genetic research is scarce there is paucity in the development and implementation of molecular techniques into lentil breeding in comparison to cereal and other crop species. Indeed, many of the sought after traits are simply inherited and maybe selected through visual phenotyping more cost effectively than through molecular analysis due to the initial complexities and cost of implementing molecular markers in a breeding program.

Molecular tools have rather recently been employed by several research teams for assistance with breeding by understanding the genetic basis of many traits and for selection against major production constraints such as susceptibility to important foliar fungal disease. Consequently, much of this chapter will focus on the development of molecular markers and the identification of gene sequences associated with resistance to fungal foliar pathogens, as well as the development of advanced technologies such as genetic transformation and transcript profiling. These are techniques that are still somewhat in their infancy in lentil, when compared to the less genetically orphaned crop species. However, marker technologies, phenotyping capabilities and the development of mapping populations have progressed to a stage where rapid and extensive uptake of molecular genotyping should occur in lentil breeding within the next 5 to 10 years.

The implementation of markers and genetically transformed materials into lentil breeding programs must be cost efficient and only employed for the accurate and fast selection/introduction of otherwise difficult to select/absent traits. Markers associated with, or transgenic plants carrying functional genes that code for the genetic mechanisms governing abiotic stresses such as drought, frost, cold, boron, salinity, herbicide tolerance as well as biotic constraints such as ascochya blight, botrytis grey mould, anthracnose, rust, fusarium wilt, stemphylium blight, helicoverpa and bruchids would greatly benefit the global lentil economy. Subsequent to the implementation of the first 'high value' markers, it will become economically attractive to use a larger number of markers that cover a wide range of traits.

2. GENOTYPING AND MAPPING

2.1. Map Progress

In order to identify regions of the genome associated with traits of interest and to subsequently select for those regions and potentially identify the candidate genes responsible, a detailed genome linkage map is sought. The initial *Lens* genetic linkage maps were constructed using morphological and isozyme markers (Zamir and Ladizinsky 1984; Tadmor et al. 1987). The first map comprising DNA-based markers was produced by Havey and Meuhlbauer (1989). Subsequent maps created with either intersubspecific or interspecific crosses were those of Weeden et al. (1992), Tahir et al. (1993), Tahir and Muehlbauer (1994) and Vaillancourt

and Slinkard (1993). With the advent of PCR based markers, the number of mapped markers across the *Lens* genome increased dramatically. The first extensive map comprised 177 random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and morphological markers was constructed using a RIL population from a cross between a cultivated *L. culinaris* ssp. *culinaris* cultivar and a *L. culinaris* ssp. *orientalis* accession (Eujayl et al., 1998). The major reason for using distantly related parents was due to the limited polymorphism detected within the cultivated gene pool (Ford et al. 1997).

The first intraspecific lentil map was reported by Rubeena et al. (2003) and comprised 114 RAPD, inter simple sequence repeat (ISSR) and resistance gene analogue (RGA) markers. A subsequent intraspecific linkage map was constructed using a F_2 population from a cross between ILL7537 (ascochyta blight-resistant) and ILL6002 (ascochyta blight-susceptible). The map comprised 72 markers (38 RAPD, 30 AFLP, 3 ISSR and one morphological), and spanned a total length of 412.5 cM at a LOD score of 4.0 and a maximum recombination fraction (θ) of 0.25 (Rubeena et al. 2006). Two quantitative trait loci governing resistance to the fungal pathogen *Ascochyta lentis* were identified on linkage groups I and II, respectively, at which dominant and partial dominant gene action was observed. These QTL may represent the effects of the two major dominant genes previously reported to be responsible for *A. lentis* resistance in ILL7537 (Nguyen et al. 2001), however, the underlying candidate resistance genes are yet to be isolated from these QTL regions.

PCR based markers that are inherited in a co-dominant fashion, as well as markers that originate from known gene sequence, have enabled the very recent development of transferable and function-associated lentil genome maps. Such maps are not only applicable across multiple genetic backgrounds (genotypes) but also enable the direct association of specific genome regions with predicted gene function. Short sequence repeat (SSR; microsatellite) markers are particularly useful because they are unilocus and multi-allelic, being produced from amplification of the repeat region between flanking primers. SSR markers may be sourced from within known gene sequences, making them useful for future function association.

SSR markers have previously been used for genotyping in soybean (Rongwen et al. 1995), field pea (Ford et al. 2002) and chickpea (Winter et al. 1999). Several suites of SSR markers have been developed from the genomic sequence of the ubiquitous accession ILL5588 (Australian cultivar Northfield; Zavodna et al. 2000; Hamwieh et al 2005; P. Inder, unpublished). For the construction of the SSR marker sequence libraries, genomic DNA was bonded onto a nylon membrane and hybridized with radiolabeled oligonucleotide repeats; namely $(GT)^{10}$, $(GA)^{10}$, $(GC)^{10}$, $(GAA)^{8}$, $(TA)^{10}$ and $(TAA)^{5}$. Recently, several of these markers were successfully transferred across the genetic backgrounds of elite Australian cultivars (Table 1). This database will play an important role in seed typing for quality assurance in the domestic breeding program, cultivar integrity in commercial production and commercial export certification.

Table 1. A rudimentary genotype database constructed for 10 elite Australian lentil accessions using SSR amplification profiles and fluorescent capillary electrophoresis

	SSR 107		SSR 204		SSR 48		SSR 80	
	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2	Allele 1	Allele 2
Northfield	169	169	194	194	174	174	163	163
Indianhead	131	131	202	202	193	193	151	151
Digger	131	131	202	202	191	191	163	163
Boomer	133	133	194	194	191	191	147	147
ILL7537	131	131	196	197	191	199	165	165
ILL6788	127	127	194	194	174	174	143	143
Palouse	188	188	200	200	199	199	143	143
ILL2024	133	133	196	196	203	203	143	143
Nipper	169	169	194	194	174	174	163	163
Nugget	131	131	196	196	191	191	163	163

The first *Lens* sp. map to include a SSR marker was that of Durán et al. (2004). Recently, Hamwieh et al (2005) added 39 SSR and 50 AFLP markers to the map constructed by Eujayl et al. (1998) to produce a comprehensive *Lens* map comprising 283 genetic markers and covering 715 cM. Subsequently, the first lentil map, that contained 18 SSR markers as well as 79 intron-targeted amplified polymorphic (ITAP) gene-based markers, was constructed using a F_5 RIL population developed from a cross between ILL5722 (Australian cultivar Digger) and ILL5588. The map comprised seven linkage groups that varied from 80.2 cM to 274.6 cM in length and spanned a total of 928.4 cM (Phan et al. 2006a).

2.2. Toward a Lentil Consensus Linkage Map

A lentil consensus map will comprise a set of robust and transferable markers from which genetic distance can be measured and compared across different genetic backgrounds. This will enable the tracking of gene recombination events for the building of superior genotypes. In order to construct a consensus map, previously constructed genome maps may be anchored with a common set of genetic markers that span the representative linkage/chromosome groups. Also, orthologous markers that are transferable between related legume species will enable rapid generation and anchoring of maps in species such as lentil where there is little pre-existing genomic information. So far, seven morphological markers have been mapped in lentil of which only four (cotyledon colour *Yc*, anthocyanin pigmentation in stem *Gs*, seed coat pattern *Scp* and pod dehiscence-indehiscence *Pi*) have been placed on multiple maps. Recently, Rubeena et al. (2006) anchored seven linkage groups with those of a previously published map (Rubeena et al. 2003). For this, 22 RAPD and two ISSR markers were transferred among populations. Of more use in map anchoring, due to their stability and reproducibility, will be the newer SSR and ITAPS markers. Phan et al. (2006a) compared ESTs from phylogenetically distant

M. truncatula, *Lupinus albus*, and *Glycine max* species to produce 500 intron-targeted amplified polymorphic (ITAP) markers. They also used 126 *M. truncatula* cross-species markers to generate comparative genetic maps of lentil (*Lens culinaris* Medik.) and white lupin (*Lupinus albus* Linn.) (Phan et al 2006b). Subsequently, Phan et al (2006b) used 18 common SSR markers to join the new map with another pre-existing comprehensive lentil map (Hamwieh et al 2005). Comparative mapping was also conducted that enabled the visualisation of a macrosyntenic relationship between lentil and the model genome *M. truncatula* (Phan et al 2006b; Figure 1). The composite lentil map will serve as a foundation for the future use of genomic and genetic information in lentil genetic analysis and breeding for traits such as pod indehiscence, flower colour, seed coat pattern, seed shape, fusarium wilt, ascochyta blight, botrytis grey mould and virus resistance and flowering responses.

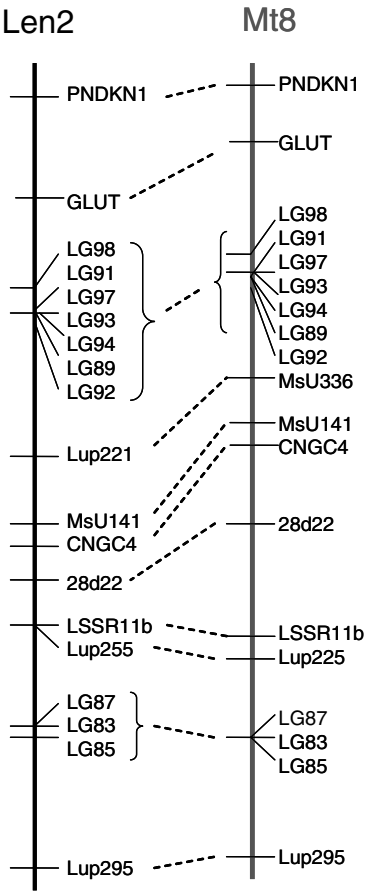

Figure 1. Evidence of simple homologeous and conserved macrosynteny between *Medicago tuncatula* (linkage group 8) and lentil (linkage group 2). Reproduced from Phan et al (2006b)

3. MARKER-ASSISTED BREEDING

Eujay et al. (1998) first identified markers suitable for the selection of a simply inherited disease resistance trait loci for fusarium wilt resistance (*Fw*). Subsequently, Ford et al (1999) identified RAPD markers that were close and flanking the major dominant locus for ascochyta blight resistance in the ILL5588 accession (*Ral*1/AbR_1). Chowdhury et al (2001) also developed RAPD markers that flanked the recessive ascochyta blight resistance locus in the cultivar Indianhead (*ral*2). More recently, markers have been identified that also flank the codominant ascochyta blight resistance loci in ILL7537 (Rubeena et al. 2006) and Tullu et al (2003) identified markers linked to the anthracnose resistance locus in accession PI320937 (*LCt*-2). The most recent report of markers developed to select for disease resistance were those reported by Hamwieh et al (2005), who identified close and flanking markers for the *Fw* locus in ILL5588.

Research has also focused on the conversion of arbitrary markers to sequence-specific markers that are reproducible and robustly transferable among genetic backgrounds (Nguyen et al. 2001). Although several SCAR markers have been developed and validated among genotypes, they do not select for the genes specifically governing the traits of interest. Rather, the newer function-associated molecular maps that are being developed (Phan et al. 2006a, b), will enable direct selection of the actual candidate genes. Together with knowledge of the genomic regions that quantitatively account for genetic portions of particular phenotypes (Rubeena et al. 2006), these maps will enable accurate selection of multiple gene traits, for future trait/gene pyramiding (Tar'an et al. 2003) and adaptation to various environments.

4. GENETIC ENGINEERING

Applications of genetic engineering can play important roles in solving fundamental challenges faced in classical breeding. Through the targeting of specific genetic pathways or expression of known functional genes, transgenic approaches may aid in increased yield by improving agronomic traits, such as enhancing pest, stress and herbicide resistance. Improvements could also be made in the quality of the crop, including its food and feed characteristics. Thus, genetic engineering technology provides an important adjunct to classical breeding. In the post genomic era, genetic engineering is a key suite of tools used to answer basic biological question such as gene function and their roles in various physiological and developmental processes. Reliable, efficient and reproducible regeneration and transformation systems are prerequisites for assessing the effect of altering genomes with novel genes and their associated functions and also in exploiting the vast knowledge know available from the model crop genomes.

4.1. Cell and Tissue Culture

The grain legumes have been less amenable to manipulation in tissue culture (McClean and Grafton 1989), and generally are more recalcitrant to *in vitro*

regeneration and transformation (DeKathen and Jacobsen 1990) than may other crop species. However, routine protocols for obtaining stable transformants are now available for the major grain legumes such as the common bean (*Phaseolus vulgaris*), soybean (*Glycine max*), pea (*Pisum sativum*), peanut (*Arachis hypogea*), and alfalfa (*Medicago sativa*) as well as the model legume *Medicago truncatula* (Puonti-Kaerlas et al. 1990; Christou 1992; Russell et al. 1993, Trick et al. 1997). For lentil, limited research has been conducted in developing useful and stable transformation and regeneration protocols.

Organogenesis and somatic embryogenesis are the two common methods used for regeneration of complete plants in tissue culture. Somatic embryogenesis involves the production of a bipolar structure with root and shoot axis and a closed vascular system. This involves the induction of embryogenic callus and development of these cells into embryos by manipulating culture conditions including media and growth regulators. Somatic embryos originate from single cells and thus are an excellent target for transformation systems, and have been successfully used in the genetic transformation of the legume relative, soybean (Trick et al. 1997). In lentil to date there is only one report of successfully achievement of stimulated somatic embryogenesis (Saxana and King 1987). In general, the efforts to achieve somatic embryogenesis have failed due to the embryo not proceeding beyond the characterised globular and heart shapes.

Organogenesis describes the processes by which cells and tissues are de-differentiated, leading to the production of shoot or root primordium whose vascular systems are often connected to the parent tissues. The stages involved in complete plantlet regeneration via *de novo* organogenesis include shoot bud formation, shoot development and rooting of the shoots. Bajaj and Dhanju (1979) first reported direct shoot organogenesis in lentil from apical meristems using media containing kinetin (Kin). Shoot bud formation was achieved relatively easily in lentil and this initial report of shoot organogenesis in lentil was followed by many others in which a variety of explants such as apical meristem (Bajaj and Dhanju 1979), stem nodes (Polanco et al. 1988, Sing and Raghuvanshi 1989, Ahmed et al. 1997), cotyledonary node (Warkentin and McHughen 1993, Sarker et al. 2003b), epicotyls (Williams and McHughen 1986), decapitated embryo, embryo axis and immature seeds (Polanco and Ruiz 2001) were used. However, shoot regeneration from leaf tissue has not yet been reported. Explants derived from mature seeds have subsequently been preferred as the explant of choice mainly because of their year-round availability. So far, Murashige and Skoog (MS) salts medium has been the most commonly reported medium for lentil regeneration. Several cytokinins such as Kin, 6-benzylaminopurine (BAP), N-phenyl-N'-1, 2, 3-thiadiazol-5-yl-urea (TDZ) and auxins such as **2**,4-dichlorophenoxyacetic acid (2,4-D), α-Naphthalene acetic acid (NAA) , indol-3-butyric acid (IBA) and indol-3-acetic acid (IAA) have been used for direct or indirect organogenesis. Also, gibberellic acid (GA3) has been used to alter shoot length *in vitro* (Sarker et al. 2004).

An efficient and reproducible rooting protocol is necessary to obtain viable plants from *in vitro* regenerated shoots. Often species, genotype and/or explant dependent,

the success may also be greatly influenced by the media and phytohormones used. Induction of functional roots on *in vitro* formed plantlets has become the most difficult stage in developing a complete and robust lentil regeneration system (Fratini and Ruiz 2002), and to date no reproducible rooting protocol has been reported. In lentil, as for other pulse crops, increasing concentrations of cytokinins in the culture media, mainly BA or TDZ, has resulted in a higher number of shoots regenerated. However, shoot length and subsequent rooting was greatly reduced (Mohamed et al. 1992; Gulati and Jaiwal, 1994; Prakash et al. 1994; Sanago et al., 1996; Polisetty et al. 1997; Subhadra et al. 1998, Sarker et al. 2003b). Using high concentrations of cytokinin to induce shoot bud formation resulted in stunted elongation of shoots that lacked shoot apical meristems and vascular connections. This led to subsequent difficulty in regenerating roots from such shoots. The inhibitory effect of BA on rooting has been well documented by Polanco and Ruiz (1997) via *in vivo* and *in vitro* studies of lentil seedlings. The strong inhibitory effect of this cytokinin on root growth, was demonstrated by a drastic *in vivo* reduction of the mitotic index of the root meristem. Furthermore, Malik and Saxena (1992), observed a progressive decrease in root development with an increase in TDZ concentration. Only stunted primary roots developed on media with TDZ of 5mM or higher, and prolonged exposure for three to five weeks, resulted in callus production from the primary root. Fratini and Ruiz (2002) subsequently found that TDZ and BA inhibited root formation by inducing root swelling and stunting, and at higher concentrations caused callusing of the main root. A continued culture on TDZ induced shoots without a shoot apical meristem, resulting in fused shoots and no plant regeneration. However, through limiting the culture period on TDZ and transferring the regenerated shoots to a growth-regulator-free medium prior to rooting, whole plants were eventually obtained (Fratini and Ruiz 2002).

Sarker et al (2003b) observed 30% root induction on shoots regenerated in the presence of BAP and Kin when shoots were cultured on MS medium supplemented with 25 mg/l IBA. However, roots did not develop from the base of the regenerated shoots but at a level slightly higher than the cut ends. Furthermore, it was observed that once inside the auxin-rich medium, the tip of the roots callused, blocking further growth of the roots and histological study by freeze microtomy showed that the roots did not form vascular bundle connection with the shoot. Fratini and Ruiz (2002) subsequently reported a 95% rooting efficiency by culturing nodal segments of lentil with an axillary bud in an inverted orientation in media with 5 μM IAA and 1 μM Kin, and concluded that the improvement in rooting success was due to polarity. However they were only able to regenerate about two shoots per explants. Most recently Newell et al. (2006) reported a 100% rooting rate from lentil nodal microcuttings by placing them in an inverted fashion in media composed of sphagnum peat, coarse river sand and perlite at a 0.5:2:2 ratio, and concluded that the improved rooting efficiency was due more to aeration than polarity. Studies are required to test the applicability of this rooting procedure for a transformation compatible regeneration system where individual transformed cells first need to be

induced to form shoot primordia requiring the use of cytokinin followed by lengthy exposure to selection media to kill non transformed cells.

4.2. Transformation

Genetic transformation of lentil tissues has been reported using several different gene transfer methods. In particular, several foreign genes have been introduced into lentil protoplast by electroporation, lipofection or PEG treatment (Maccarrone et al 1992a, b, 1993, 1995a, b). Chowria et al. (1995; 1996) reported *in planta* electroporation-mediated transformation of nodal meristems and 20% of the branches that grew from the nodal meristems were chimeric. However, the segregation ratios in the putatively transgenic R2 populations were strongly biased against transgene presence or expression. Also, lentil protoplast were electroporated with an aim to reduce lipoxygenase activity by antisense RNA mediated gene silencing (Maccarrone et al 1995b), however, no plants were regenerated from transformed protoplast-derived callus.

The susceptibility of lentil to *Agrobacterium* was first demonstrated by Warkentin and McHughen (1992) through the production of tumors on lentil stems and shoots apices *in vivo* and *in vitro*. Warkentin and McHughen (1992; 1993) later evaluated a number of explants (shoot apices, epicotyl, root, cotyledons, and cotyledonary nodes) and observed transient GUS expression at all wound sites except the cotyledonary nodes and the axils of the cotyledonary petioles. Sarker et al (2003a) also confirmed that cotyledonary nodes were not suitable for *Agrobacterium*-mediated transformation as multiple shoot regeneration occurred from pre-existing meristems in the explant. *Agrobacterium*-mediated transformation efficiency in lentil varies with genotype and strain used, with the EHA101, EHA105 and GV2260 strains being used most commonly. Mahmoudian et al (2002) reported that vacuum infiltration by *Agrobacterium* improved transformation efficiency while Hoque et al (2003) reported that sonication and vacuum infiltration improved transformation efficiency, as measured by expression of the GUS gene. Sarker et al (2003a) reported obtaining transformed lentil shoots from decapitated embryo explants using *Agrobacterium* strain LBA4404, harboring the pBI121 plasmid. Southern blot analysis was later used to confirm integration of the transgene in the lentil genome (B. Mustafa, unpublished). Selection of transformed tissue was done using 50–200 mg/l kanamycin and the transformation efficiency was between 1.5 and 1.9% (Sarker et al. 2003). Using *Agrobacterium*, Barton et al (1998) produced T1 seed of lentils transformed with a 35S-bar-GUS construct and confirmed the stable transfer of the bar gene in lentil plants grown in glasshouse and screen house experiments.

The first report of gene transfer in lentil using particle bombardment was by Öktem et al (1999) who used cotyledonary node explants. Almost 50% of the bombarded explants showed transient GUS expression at 24 hours after bombardment. Chimeric stable expression was observed in regenerated shoots without selection pressure. Following, Gulati and McHughen (2000) reported

the bombardment of lentil cotyledonary nodes with the pCAMBIA1201 plasmid carrying the GUS and hpt genes. GUS and PCR assays detected putative transformants however no transformed shoots were recovered. Gulati et al (2002) reported fertile transgenic lentil plants after bombarding lentil cotyledonary nodes with a plasmid containing a mutant acetolactate synthase gene (ALS) which confers resistance to sulfonylurea herbicides. Putative transgenic shoots were regenerated on MS media with 4.4 μM BAP, 5:2 μM GA3 and chlorsulfuron. The regenerants were micrografted, successfully transferred to soil and the T0 and selfed progeny plants were screened using metsulfuron herbicide leaflet painting. PCR and Southern hybridisation were used to confirm the survivor T1 transformants.

5. FUTURE PROSPECTS IN LENTIL GENOMICS

The extensive research that has identified genes for use in the transformation of cereals, cotton, soyabeans, canola and other crop species will be of value for lentil production. Examples currently used in agricultural production systems and of benefit for lentil include genes that confer resistance to insects (eg Bt Cotton), herbicide tolerance and virus resistance. Genes currently being evaluated that may have future impact for lentil include those that potentially confer drought or frost tolerance, or non specific disease resistance. Currently, the largest barriers to the use of transgenic lentils are consumers views on transgenics, IP restrictions and the large costs of meeting regulatory requirements for their release.

More tools are becoming available in order to further understand the functional genetic components governing traits of interest and hence aid in selection of the optimal genome fragments in advanced breeding programs. Several of these tools are based on the principle of reverse genetics in which a gene sequence or its expression is altered to study the effect on the phenotype in a particular environment and to compare this to the wild type. Methods such as transposon mutagenesis (Tisser et al. 1999), target induced local lesions in genomes (TILLING) analysis (Henikoff et al. 2003) and post-transcriptional gene silencing (PTGS) through RNA interference (Voinnet 2002) are possible avenues for future lentil functional genomics studies.

Mutagenic lentil populations have been developed for the purposes of studying gene 'knock-out' effects using gamma rays and chemical treatments such as ethyl methane sulfonate (EMS). The TILLING procedure employs an EMS-generated mutant library within which point mutations are sought to provide differentials in enzyme cleavage points. In legumes, TILLING has been applied in the model crop genomes of *Lotus japonicus* (3697 mutant plants; Perry et al. 2003) and *Medicago truncatula* (2000 mutant plants; VandenBosch and Stacey 2003). Furthermore, a population was recently developed in lentil at the Department of Primary Industries, Horsham, Australia (M. Materne, unpublished).

Transcript profiling used to identify genes associated with traits of interest has been applied to the pulse genomes (Muehlbauer et al. 2006). In particular, the cDNA-AFLP technique was used to identify candidate genes for resistance to

Ascochyta rabiei causing ascochyta blight in chickpea (Cho et al. 2005). The flavanone-3-hydroxylase (*F3H*) gene was qualitatively differentially expressed resistant and susceptible plants. Alternatively, the microarray technique has recently been used to identify genes associated with resistance to ascochyta blight in lentil. For this, a cDNA microarray, named the *Pulse Chip* was developed which comprised 565 expressed sequence tags (EST) from a chickpea cDNA library enriched for reaction to *A. rabiei*, 156 ESTs from a *Lathyrus* cDNA library enriched for reaction to *A. pinodes* and 41 lentil ESTs and RGAs from the GenBank database (Coram and Pang 2005). The pulse chip was employed to study expression profiles in the resistant ILL7537 and susceptible ILL6002 lentil genotypes at 6, 24, 48, 72 and 96 hours after inoculation with *Ascochyta lentils*. Key differential genes included; a proline-rich protein (LS0156) for cell wall strengthening, a super oxidase dismutase enzyme (U116) for antioxidant defence, a salicylic acid binding protein (U174), a Snakin-2 antimicrobial protein (U278) and a Bet VI type pathogenesis-related protein (LS481) (Mustafa et al. 2006). Validation of functionality will follow through QRT-PCR and PTGS analyses. This will likely be achieved through the already developed transgenic and *in vitro* shoot regeneration methods.

6. CONCLUSIONS

Lentil is a genetic orphan compared to many larger crop species. However, rapid advances in the development and use of molecular tools in the breeding of lentil is expected in the short to medium term. A consensus map is now available in lentil that can form the basis of a more saturated genetic map for use in mapping genes conferring morphological characteristics, tolerance to abiotic stresses, resistance to pests and diseases and improved quality. Lentil will benefit greatly from genomic research in other species and by its close relationships with the model species, for which much genomic information and tools are available. The lack of haploid technologies in lentil has necessitated the slower and more costly development of mapping populations using single seed descent. However, a large number of populations have been developed in lentil that can be used to map genes for many of the worlds economically important traits. The population used by Phan et al (2006b) alone could be used to develop markers for seed characteristics, resistance to ascochyta blight, fusarium wilt, botrytis grey mould and virus resistance, flowering responses and adaptation (Materne 2003). Currently the political, social and regulatory environment is limiting the development of transgenic cultivars more than the capability of the scientists.

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