5 Lentil

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5.1 Introduction

5.1.1 Origin, Domestication and Taxonomy

Lentil, *Lens culinaris* ssp. *culinaris* Medic., was domesticated with wheat, barley and other pulses in the Fertile Crescent of the Near East and spread through southern Europe, the Middle East, North Africa and across the Indo-Gangetic plain before 1000 BC (Cubero 1981). The current distribution of lentil ranges over subtropical and temperate cropping regions of Europe, Asia, northern Africa and Ethiopia, the Indian subcontinent, North and South America, southern Africa and Australia, grown either as a spring crop or a winter crop (Blain 1988).

The earliest evidence of lentil cropping is in association with wheat and barley at Mureybit in Syria 8500–7500 BC, Hacilar and Cayonu in Turkey 7500-6500 BC, and other sites from western Iran to Palestine before 7000 BC (Cubero 1981). In many of these sites, wild lentil is rare and it is likely that the seed remains are of cultivated lentil, especially in association with cultivated wheat. The migration of lentil to Europe and Asia matched the initial spread of cereal and other legume crops (Hancock 2004).

Domestication of lentil led to the evolution of two major seed groups: macrosperma (6 to 9 mm diameter) in the Mediterranean Europe and Africa plus Asia minor and microsperma (2 to 6 mm diameter) in western Asia and in northern Africa (Cubero 1981). Macrosperma and microsperma lentils are useful functional groups within cultivated lentil as they relate to processing and consumer end uses that are different for each type (Barulina 1930; Erskine 1996).

According to the United States Department of Agriculture (USDA) and the International Legume Database and Information Service (ILDIS), the genus *Lens* comprises the cultivated *Lens culinaris* ssp. *culinaris* and the wild relative subspecies and species; *L. c.* ssp. *orientalis, L. c.* ssp. *odemensis, L. c.* ssp. *tomentosus, L. c* ssp. *nigricans, L. ervoides, L. montbretii,* and *L. lamottei.* The USDA also classifies *L. nigricans* as a separate species and places all the above species in the family Fabaceae, but with the family Papilionaceae as an alternate. Historically the *Lens* genus has also been placed in the genus *Ervum*, and some species have been placed in genus *Vicia* as botanically there is a continuum between the *Vicia* and *Lens* genera (Cubero 1981).

The *Lens* genus is primarily self-pollinated, with a chromosome number of $2n = 14$. Wide intraspecific variations in chromosomal rearrangements occur within the wild species, which affects the success of both intraspecific and interspecific crossing (Ladizinsky 1979). Hancock (2004) listed three groups: Group 1 comprises *L. c.* ssp. *culinaris* and *L. c.* ssp. *orientalis* with overlapping ranges, similar morphology and a high percentage of shared molecular markers. These species are intercrossable and also crossable with *L. c.* ssp. *odemensis*, but the interspecific hybrids are only partially fertile (Ladizinsky et al. 1984). Group 2 contains the intercrossable species *L. nigricans, L. ervoides* and *L. lamottei.* Hybrids of *L. culinaris, L. orientalis* and *L. odemensis* with *L. nigricans* have irregular meiosis and do not produce viable seed (Ladizinsky et al. 1984, 1985). However, hybrids of *L. culinaris* with *L. ervoides* can produce viable seed but only with the use of embryo rescue culture (Ladizinsky et al. 1985). Group 3 contains *L. tomentosus* as a single species group. Between these groups no viable crosses have been achieved.

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The probable progenitor species for domesticated lentil is *Lens c.* ssp. *orientalis*, which has a center of origin in the Middle East Fertile Crescent (Zohary 1972). Although many endemic and rare forms of lentil occur in the Hindu-Kush region, the geographic distribution of *L. c.* ssp. *orientalis* is centered in southern Turkey – Lebanon. *L. c.* ssp. *orientalis* is able to form interspecific hybrids with the domesticated subspecies, which undergo normal meiosis resulting in seven bivalents (Ladizinsky 1979). Morphologically, *L. c.* ssp. *orientalis* has the appearance of miniaturized *L. c.* ssp. *culinaris*; however, some accessions differ from the cultivated subspecies by one or two chromosomal inversions and were found to be cross incompatible (Ladizinsky 1979).

5.1.2 Botany and Ecology

Domesticated lentil is an annual bushy herb, has very branched with slender, soft-haired angular stems, 25 to 75 cm tall, has compound pinnate leaves often ending in a tendril, one to four flowers per raceme with colors of white to blue and purple, one- to two-seeded oblong flattened pods, and biconvex rounded seed 4 to 8 mm in diameter and 2 to 3 mm thick (Duke 1981). The plant may exhibit considerable variation in the growth habit: low and bushy, suberect or erect. Flowering and branching are indeterminate. The 100-seed weight ranges from 2 to 8 g, with the seed coat varying in color from green, green-brown, to light red with black speckles (Duke 1981). Cotyledons may be red/orange, yellow or green.

Seed protein content varies from 22 to 35%, with relatively high levels of lysine, leucine and sulphur-amino acids (Muehlbauer and Tullu 1997). Anti-nutritional factors include trypsin inhibitors, haemoglutinins and oligosaccharides. Williams et al. (1994) suggested that seed nutritional quality might be improved by increasing both protein content and amino acid balance and by reducing the levels of anti-nutritional factors.

Lentil is produced as a winter crop in the semiarid tropics, mild temperate and Mediterranean regions but is spring grown in climates with very cold winters. Lentil grows best on neutral to alkaline well-drained clay to sandy loam soils. Lentil is a quantitative long-day plant with a flowering response pattern that is independently controlled by photoperiod and temperature. A wide genetic variation for these traits was detected within a world collection (Erskine et al. 1990).

5.1.3 Genetic Resources

The largest and most representative collection of lentil landraces is maintained by the International Center for Agricultural Research in the Dry Areas (ICARDA), Syria (Erskine et al. 1989), currently at 3351 accessions. Large collections are also held in the USA (2807), Russia (2857), Australia (2847) and Turkey (1435) (European Genetic Resources Database 2004: www.ecpgr.cgiar.org).

The first systematic analysis of genetic diversity in lentil was performed by Barulina (1930) with the Vavilov collection, defining the macrosperma and microsperma types as subspecies and describing regional groups (*grex*) according to geographic distribution of qualitative traits, chiefly seed characteristics, pubescence and size of vegetative organs, phenology, height and pod shape. Landraces held at the ICARDA were later characterized and four major regional groups were identified through analysis of variability in quantitative and qualitative morphological traits (Erskine and Witcombe 1984; Erskine et al. 1989). These were the Levantine group (Egypt, Jordan, Lebanon, Syria), northern group (Greece, Iran, Turkey, USSR, Chile), Indian subcontinent group and Ethiopian group. The most discriminating traits were in time to maturity, lowest pod height and 100-seed weight, but seed color was also important. The ecological environment was a major evolutionary force in the spread of cultivated lentil (Erskine et al. 1989). For example, the importance of flowering response for adaptation in south Asia caused a bottleneck in the introduction of lentil and subsequently low genetic variability among landraces into this region (Erskine 1997; Erskine et al. 1998). Extensive variation within landraces and between landraces within a region was found for both morphological traits and allelic variation of isozymes, indicating a complex organisation of lentil populations with co-adapted multilocus allelic combinations mediated by a very low frequency of outcrossing (Erskine and Choudhary 1986; Erskine and Muehlbauer 1991).

5.1.4 Economic Importance and Current Breeding Objectives

World production of lentil was predicted to reach 3.3 million metric tons in 2004 (Agriculture Canada Market Analysis). Lentil is currently grown in the Indian subcontinent, Middle East, northern Africa, southern Europe, North and South America, Canada, Australia and New Zealand. India is the leading producer of lentil, producing about one third of the total world production, predominantly for its own domestic usage. With the exception of Chile (Barulina 1930), lentil production in the Americas is a relatively recent event (Muehlbauer and McPhee 2002). However, Canada has since become a major world producer and the largest exporter of lentil. There has also been a considerable increase in the area (125,000 ha in 2002) under lentil in Australia since 1991 (293 ha) (Carter and Materne 1997).

Water Availability and Waterlogging. Lentil yields are very dependent on available soil moisture during the growing season (Erskine and Saxena 1993; Erskine et al. 1994 a). Although mechanisms such as good early vigour and early flowering and maturity (Erskine et al. 1994 a), higher osmotic adjustment (Leport et al. 1998), deeper rooting (Buddenhagen and Richards 1988; Turner et al. 2001) or tolerance to subsoils constraints (Materne et al. 2002) have been advocated as ways of increasing legume productivity under moisture limiting conditions, they are unproven over seasons. Selection is typically based on grain yield under variable rainfed conditions to increase water use efficiency (Erskine and Saxena 1993). Furthermore, drought may occur during plant establishment, intermittently during the vegetative growth period, terminally or progressively during the season depending on the environment (Rahman and Mallick 1988; Erskine et al. 1994 a).

Excess water and waterlogging during winter and late spring to summer can reduce lentil yields in Mediterranean and subtropical environments respectively. The sensitivity of lentil to waterlogging and anaerobic conditions is thought to account for the poor response of the crop to irrigation, although responsive genotypes have been identified with larger parenchyma in their roots (Erskine and Saxena 1993; Erskine et al. 1994 a; Bejiga and Anbessa 1995).

Diseases. A wide range of pathogens infect lentil crops worldwide (Brouwer et al. 2000). The diseases rust, caused by *Uromyces viciae-fabae* (Pers.), vascular wilt, caused by *Fusarium oxysporum* f.sp. lentis Vasd. and Srin. and ascochyta blight, caused by *Ascochyta lentis* Vassilievsky, are the key fungal diseases of lentil worldwide (Erskine and Saxena 1993; Erskine et al. 1994 c). Other important fungal diseases are powdery mildew, caused by *Erysiphe polygoni* D.C. and downy mildew, caused by *Peronospora lentis* Gauman (Khare et al. 1993). Anthracnose, caused by *Colletotrichum truncatum* (Schwein.) Andrus and Moore, and stemphylium blight, caused by *Stemphylium botrysum* Wallr., are locally of major economic importance in Canada and Bangladesh respectively. *Botrytis cinerea* is regarded as a major problem in Pakistan (Brouwer et al. 2000) and *Botrytis fabae* is the major Botrytis pathogen on lentil in Australia (Materne et al. 2002). Lentil also has viral pathogens such as pea seed-borne mosaic virus (PSbMV), bean leaf roll virus (BLRV) and pea enation mosaic virus (PEMV) as well as several bacteria, nematodes, insects and parasitic weeds (Erskine et al. 1994 a). Tolerance to many viruses has been identified at ICARDA and in Australia (ICARDA 1994; Latham and Jones 2001; Latham et al. 2001).

Research, including breeding, has been initiated for many diseases in lentil. The highest level of understanding of these diseases in terms of the host-pathogen relationships and genetic improvement can be demonstrated using ascochyta blight as an example. Many sources of foliar resistance to ascochyta blight have been recorded, but resistance to seed infection is more limited (Andrahennadi et al. 1996; Nasir and Bretag 1997 a). Pathogen variability also exists (Ahmed et al. 1996; Ahmed and Morrall 1996) and of most significance was the identification of an isolate that was virulent on accession ILL5588 (Nasir and Bretag 1997 b), a major source of resistance used in breeding programs around the world. Importantly, the accession ILL7537 was shown to be highly resistant to this isolate (Nasir and Bretag 1997 b; Nguyen et al. 2001) and, with ILL5588 and Indianhead, is a key source of resistance genes for breeding. Breeding is well advanced for ascochyta blight, and resistant cultivars have been released in many countries. Individually these genes can be selected for in well-designed field screening nurseries; however, differentiating genotypes with a combination of these genes is more

difficult. Resistance to anthracnose was recently found in accession PI320937 and is being selected for in elite genotypes along with resistance to ascochyta blight (Tullu et al. 2003).

Temperature Stress. Hot or dry weather during flowering and pod fill causes severe constraints on the productivity of lentil crops in many regions of the world, including the Mediterranean (Erskine 1985 b). In colder areas such as in the USA and Turkey, large yield increases can be achieved by sowing lentil in winter rather than spring (Muehlbauer and McPhee 2002). However, problems associated with a lack of winter hardiness, increased incidence of diseases and weed control issues must be addressed before winter sowing becomes widespread. Kahraman et al. (2004) identified the most winter-hardy accessions in the USA and Turkey as WA8649041, WA8649090, ILL1878 and ILL669. Inheritance was shown to be quantitative, indicating that several genes of varying effect may be required for survival. Frost at flowering is a major limitation to lentil production in Australia, but genetic variation has not been identified.

Flowering. The timing of flowering is a particularly important event as it determines the duration of the vegetative phase, which establishes the potential of the crop and determines the climatic conditions to which the crop will be exposed during reproductive growth. The optimal flowering response differs between regions and is simple to measure within a target region (Erskine et al. 1994 b). Presently, the understanding of the genetic control of flowering is limited (Sarker et al. 1999).

Nutrient Imbalances. Boron toxicity is increasingly being recognised as a problem in the arid areas of west Asia and Australia, where lentil is widely grown (Yau and Erskine 2000). Tolerance to high soil boron was identified in lentil (Yau and Erskine 2000; Hobson et al. 2003), and breeding was initiated in Australia using soil-based screening methods. Conversely, boron deficiency has been identified as a limitation to production in Nepal. However, genetic variability was identified and field-based selection is currently under way (Srivastava et al. 1999). There is increasing evidence that the same genetic mechanisms are likely to control tolerance to both boron deficiency and toxicity, predominantly boron exclusion (Yau and Erskine 2000; Dannel et al. 2002).

Lentil is sensitive to zinc and iron deficiency and poor nitrogen fixation due to factors that affect the host, rhizobia and the symbiosis between the two. Interactions between rhizobia and host have been identified with the potential for future selection of genotypes with improved nitrogen fixation (Slattery, personal communication, 2004).

The major lentil growing areas of the world are regions with a high frequency of saline or sodic soils (Saxena 1993). Variation in tolerance to a range of salts was initially identified in the USA (Jana and Slinkard 1979), and NaCl-tolerant accessions have been identified by others (Rai et al. 1985; Ashraf and Waheed 1990). The inheritance of salt tolerance has been investigated and recessive genes were implicated (Ashraf and Waheed 1993).

Harvestability. Hand harvesting is considered a major constraint to lentil production in the Middle East and northern Africa (Erskine and Goodrich 1988; Erskine et al. 1991). The development of cultivars for mechanised harvesting is a prime research goal in these countries and in countries where lentil is machine harvested, such as Australia and North America (Erskine and Goodrich 1988; Erskine et al. 1991; Materne et al. 2002). Plant breeding can assist harvest mechanisation through the development of taller, non-lodging cultivars that retain their pods and seeds at maturity and mature uniformly. Genetic variability exists for height (Erskine and Witcombe 1984; Muehlbauer et al. 1995), lodging resistance (Muehlbauer et al. 1995), pod dehiscence (Erskine 1985 a) and uniformity of maturity.

Quality. Lentil quality is largely defined by visual characteristics such as size, shape and color and contamination of the sample. The preferred appearance is most often that which mimics the local product. Lentil seeds are susceptible to mechanical damage due to their thin shape and sharp edges. Therefore, the development of rounder seeded cultivars offers potential for reduced mechanical damage, while these types are also the preferred quality in many markets.

5.1.5
Limitations of Classical Breeding Approaches and the Need for Molecular Breeding

Although lentils are an old crop species, genetic improvement other than farmer selection has only occurred relatively recently, led by establishment of an international breeding program at ICARDA during the late 1970s. In the last 20 years limitations to lentil production have been defined in many countries, genetic variability sought for economically important traits and breeding initiated to overcome these limitations. Lentil is also a relatively small crop compared to crops such as wheat, rice, maize and soybean, and consequently funding for research is more limited and often directed at applied research to address the large number of limitations to production. Also, much of the crop is grown in economically poor countries where research funding and expertise in novel molecular breeding approaches such as biotechnology and genomics have often been limited. This lack of funding and expertise, the short period of breeding history and the large number of limitations to be addressed have meant that molecular research for lentil has been much less extensive than for many other crop species. However, even with these restrictions, some substantial advances have been made towards understanding the lentil genome and the development and application of molecular markers in breeding.

Much of the focus has been on developing molecular markers for resistance to a few major diseases, especially where research was complementary between countries, for example ascochyta blight. Molecular markers have also been invaluable in understanding the inheritance of resistance and in identifying novel genes (Nguyen et al. 2001). In the near term, potential exists to develop markers for many traits where genetic variability exists, for example boron and salinity tolerance, and resistance to botrytis grey mould, anthracnose, rust, Fusarium and stemphylium diseases. Where classical breeding attempts have been limited, markers will become invaluable if genetic variability for difficult-to-measure traits, such as frost and drought tolerance, is identified. The devastating effect that the introduction of ascochyta blight had on chickpea production in Australia and North America highlighted the potential effect of exotic diseases on lentil cultivation. Molecular markers offer potential to improve pre-emptive breeding strategies and overcome the difficulties associated with screening in distant localities.

The implementation of markers for routine use in lentil breeding programs is currently very limited, often because the traits investigated can be phenotyped relatively cost effectively. The key to both research and implementation of markers for lentil lies in the integration of the markers within the breeding program to ensure that cost-effective utilisation of the technology is achieved.

5.2
Genetic Markers and Lentil Genome Mapping

5.2.1
Morphological and Biochemical Markers

Morphological and biological markers have been used by lentil researchers and breeders as useful tools for the purposes of diversity analysis, taxonomy and trait selection (Barulina 1930; Ladizinsky and Sarkar 1982; Erskine and Choudhary 1986; Erskine et al. 1989; Ferguson and Robertson 1999). Cotyledon color has been used to estimate the percentage of natural outcrossing (Wilson and Law 1972). Other useful morphological markers include number of days to flowering (*Sn*), seed coat pattern (*Scp*) and pubescent peduncle (*Pep*). These were found to be linked together and mapped in linkage group (LG) 5 of the lentil genome, whereas tendrilled leaf (*Tnl*) was linked with colored stem (*Gs*) in LG 1 (Sarker et al. 1999). Linkage was also reported between spreading-erect growth habit, brown-green stem and brown green leaf (Emami and Sharma 1999).

Similar to morphological markers, biochemical markers have also demonstrated allelic variation of gene expression products, and isozyme markers have been used for lentil genome mapping and trait association (Vaillancourt and Slinkard 1993; Tahir and Muehlbauer 1994; Rodriguez et al. 1997). However, accuracy of linkage with these markers may be limited due to the relatively small number of morphological and isozyme loci which may be assessed and potential restriction of their expression to a specific development stage or tissue type.

5.2.2 DNA-Based Markers

Many types of DNA-based markers, arising from point mutations, insertions or deletions or errors in replications of tandem-repeated DNA, have been developed for interrogating the *Lens* genome. Restriction fragment length polymorphism (RFLP) markers, developed from cutting genomic DNA with restriction enzymes and electrophoretic separation of the resulting DNA fragments, were the first type of molecular markers used in the construction of a *Lens* genome linkage map (Havey and Muehlbauer 1989).

More recently, arbitrarily produced polymerase chain reaction (PCR)-based markers, such as random amplified polymorphic DNA (RAPD) markers, have been used to study the diversity, phylogeny and taxonomy of *Lens* (Sharma et al. 1996; Ford et al. 1997; Ferguson et al. 2000), to develop linkage maps (Eujayl et al. 1997; Rubeena et al. 2003), to tag genes of interest (Eujayl et al. 1998 b, 1999; Ford et al. 1999; Chowdhury et al. 2001; Tullu et al. 2003) and to determine pathogen population structure (Ford et al. 2000). Amplified fragment length polymorphism (AFLP) markers have also been used in *Lens* linkage mapping (Eujayl et al. 1998 a; Durán et al. 2004; Kahraman et al. 2004) and to study genetic diversity (Sharma et al. 1996), differentiate cultivars (Závodná et al. 2000) and identify markers linked to a specific trait (Tullu et al. 2003).

Simple sequence repeats (SSR), also known as microsatellites, consist of tandem repeats of two to five nucleotide DNA core sequences spread throughout the genome. The DNA sequences flanking microsatellites are generally conserved within individuals of a given species, allowing the design of PCR primers that amplify the intervening SSR. Variation in the number of tandem repeats results in PCR products of different lengths. A library of lentil-specific microsatellite markers was previously developed by Závodná et al. (2000) and more recently at ICARDA by Hamwieh et al. (2004). The ICARDA SSR library was developed from the genome of the Northfield cultivar (ILL5588) and was found to have $(CA)_n$ as the most abundant repeat type (Hamwieh et al. 2004).

Inter simple sequence repeat (ISSR) markers are detected using repeat-anchored primers that amplify between SSR, and these have been used in lentil genome mapping (Durán et al. 2004; Rubeena et al. 2003). Resistance gene analogue (RGA) markers are developed from degenerate PCR primers based on conserved regions of cloned plant resistance genes. RGA markers have also recently been used in lentil genome mapping (Rubeena et al. 2003) and in the future will be used to aid in the localisation of disease resistance genes via a candidate-gene approach. This approach has been used to identify pathogen resistance genes in other plant genomes (Kanazin et al. 1996; Leister et al. 1996; Feuillet et al. 1997).

5.2.3 *Lens* Genome Mapping

Genome mapping is the act of putting genomic markers in order, indicating the relative genetic distances between them and assigning them to LGs that represent chromosomes (Jones et al. 1997). This technique was pioneered by Morgan (1911), who stated that Mendelian genetic factors, which lie close together on a chromosome, are usually cotransmitted from parent to progeny. Although some markers are physically linked on LGs, they are sometimes split during recombination. The amount of recombination between markers is taken as the measure of distance separating them (Winter and Kahl 1995). Lentil ($2n = 2x = 14$) has a genome size of 4063 Mbp/C (Arumuganathan and Earle 1991), which is about four and ten times larger than the genomes of tomato and rice respectively.

Maps are developed using genetic markers, which segregate in patterns or sequences among progeny of a single genetic cross. The choice of parents for use in constructing a mapping population is crucial. Parents that are homozygous but highly variable from each other in the traits mapped are preferable. Due to limited polymorphism, mapping in inbreeding species often requires the selection of parents that are distantly related or belong to different subspecies or even species. To achieve this, the progeny of crosses between wild progenitors and cultivars have been employed as mapping populations in *Lens* (Havey and Muehlbauer 1989; Muehlbauer et al. 1989; Weeden et al. 1992; Tahir et al. 1993; Vaillancourt and Slinkard 1993; Tahir and Muehlbauer 1994; Eujayl et al. 1997, 1998 a; Durán et al. 2004). However, the use of more divergent parents often results in lower recombination rates and, therefore, smaller map sizes (Tadmor et al. 1987). Indeed, the first *Lens* maps comprised relatively small marker numbers and

spanned relatively small amounts of the genome (Havey and Muehlbauer 1989; Weeden et al. 1992; Eujayl et al. 1997).

Segregation distortion due to irregular chromosome pairing is also thought to cause bias estimates of marker distances in wide interspecific populations (Tadmor et al. 1987; Lorieux et al. 1995; Collard et al. 2003). Also, maps based on distantly related parents are less useful in breeding applications, as polymorphic markers linked to traits of interest may not be present within the cultivated gene pool. Therefore, intraspecific *L. c.* ssp. *culinaris* genome maps have recently been constructed using PCR-based markers (Rubeena et al. 2003; Kahraman et al. 2004).

Various types of populations may be used to study the inheritance and segregation of genetic markers, to enable determination of recombination and hence localisation within LGs. Factors that should be considered include the mating system of the organism, the kind of traits to be analysed, the time available, and the cost and technical demands to develop the population. To date, published *Lens* maps have been produced using F_2 and recombinant inbred line (RIL) populations (Eujayl et al. 1998 a; Rubeena et al. 2003; Durán et al. 2004; Kahraman et al. 2004). In the case of an F_2 population, each individual represents a set of unique recombination events. In addition, all possible combinations of parental alleles are assumed to be present within the population. Therefore, the size of such a mapping population will greatly impact on the ultimate resolution of a map (Young 1994).

The major drawback in using F_2 populations is that they are ephemeral and determinate, unlike an RI population. Doubled-haploid (DH) populations, produced by regenerating plants from single pollen grains and inducing chromosome doubling, may

Table 1. Published *Lens* linkage maps

Population type[a]	Population size	Number of markers	Marker type	Length (cM)	Reference
(*L. c.* ssp. *c.*×*L. c.* ssp. *o.*) F_2	94	5	Allozymes and morphological	2 LGs	Zamir and Ladizinsky (1984)
(*L. c.* ssp. *c.*×*L. e.*) F_2	107	18	Isozymes, morphological and translocation	258	Tadmor et al. (1987)
(*L. c.* ssp. c.×*L. c.* ssp. *o.*) F_2	66	34	Morphological, isozymes and RFLP	333	Havey and Muehlbauer (1989)
(*L. c.* ssp. *c.*×*L. c.* ssp. *o.*) F_2	n.i.	14	Allozymes and morphological	6 LGs	Muehlbauer et al. (1989)
(*L. e.*×*L. c.* ssp. *c*) F_2, F_3	100	64	Morphological, isozymes and RFLP	560	Weeden et al. (1992)
L. c. ssp. *c.*×(*L. c.* ssp. *o.*×*L. c.* ssp. *od.*,×*L. n.*×*L. e.*)	n.i.	76	Morphological, isozyme, RFLP and seed protein	10 LGs	Tahir et al. (1993)
(*L. c.* ssp. *c.*×*L. c.* ssp. *o.*)	n.i.	18	Isozymes and morphological	4 LGs	Vaillancourt and Slinkard (1993)
(*L. c.* ssp. *c.*×*L. c.* ssp. *o.*) F_2	40	33	RAPD, RFLP, morphological and oligonucleotides	206	Eujayl et al. (1997)
(*L. c.* ssp. *c.*×*L. c.* ssp. *o.*) RIL	86	177	RAPD, AFLP, RFLP and morphological	1073	Eujayl et al. (1998a)
(*L. c.* ssp. *c.*×*L. c.* ssp. *c.*) F_2	150	114	RAPD, ISSR and RGA	784.1	Rubeena et al. (2003)
(*L. c.* ssp. *c.*×*L. c.* ssp. *o.*) F_2	113	161	RAPD, AFLP, ISSR, SSR and morphological	2172.4	Durán et al. (2004)
(*L. c.* ssp. *c*×*L. c.* ssp. *c.*) RIL	106	130	RAPD, ISSR, AFLP and morphological	1192	Kahraman et al. (2004)
(*L. c.* ssp. *c.*×*L. c.* ssp. *o.*) RIL	86	283	SSR, RAPD, AFLP, RFLP and morphological	715	Hamwieh et al. (2005)

[a] Population types: *L. c.* ssp. *c.*=*Lens culinaris* ssp. *culinaris*, *L. c.* ssp. *o.*=*Lens culinaris* ssp. *orientalis*, *L. e.*=*Lens erviodes*, *L. n.*=*Lens nigricans*, *L. c.* ssp. *od.*=*Lens culinaris* ssp. *odemensis*

[b] n.i.=not indicated

represent a far better solution for reproducible and multiple environment lentil trait mapping, where, after recombination, each locus is fixed and self-pollination can create an infinite amount of genetically identical individuals in a relatively short period of time. However, the production of a lentil DH population is dependent on amenability to another culture, and in general grain legumes are more recalcitrant to in vitro manipulation than many other species (reviewed by Christou 1997).

Many *Lens* genome linkage maps have been produced over the past 20 years (Table 1). Historically, *Lens* maps comprise a small number of markers, covering a relatively small portion of the lentil genome. The first map of *Lens* was constructed using morphological and isozyme markers (Zamir and Ladizinsky 1984; Tadmor et al. 1987) and the first map with DNA-based markers (RFLP) was developed by Havey and Muehlbauer (1989). PCR-based markers subsequently revolutionized the construction of linkage maps, and the first extensive linkage map of *Lens* comprised 177 markers (RAPD, AFLP, RFLP and morphological markers) and was developed from an RIL population created from an intersubspecific cross (Eujayl et al. 1998 a). The first intraspecific linkage map of lentil was constructed with 114 RAPD, ISSR and RGA markers (Rubeena et al. 2003, Fig. 1). Recently, two more extensive molecular linkage maps have been reported, one using an intraspecific population (Kahraman et al. 2004) and the other based on an intersubspecific population (Durán et al. 2004). The lentil linkage map, produced by Durán et al. (2004), contained 62 RAPD, 29 ISSR, 65 AFLP, 4 morphological and 1 SSR markers and spanned a distance of 2172 cM within 10 LGs. This was the first time a lentil-specific SSR marker was mapped. The map of Kahraman et al. (2004) covered 1192

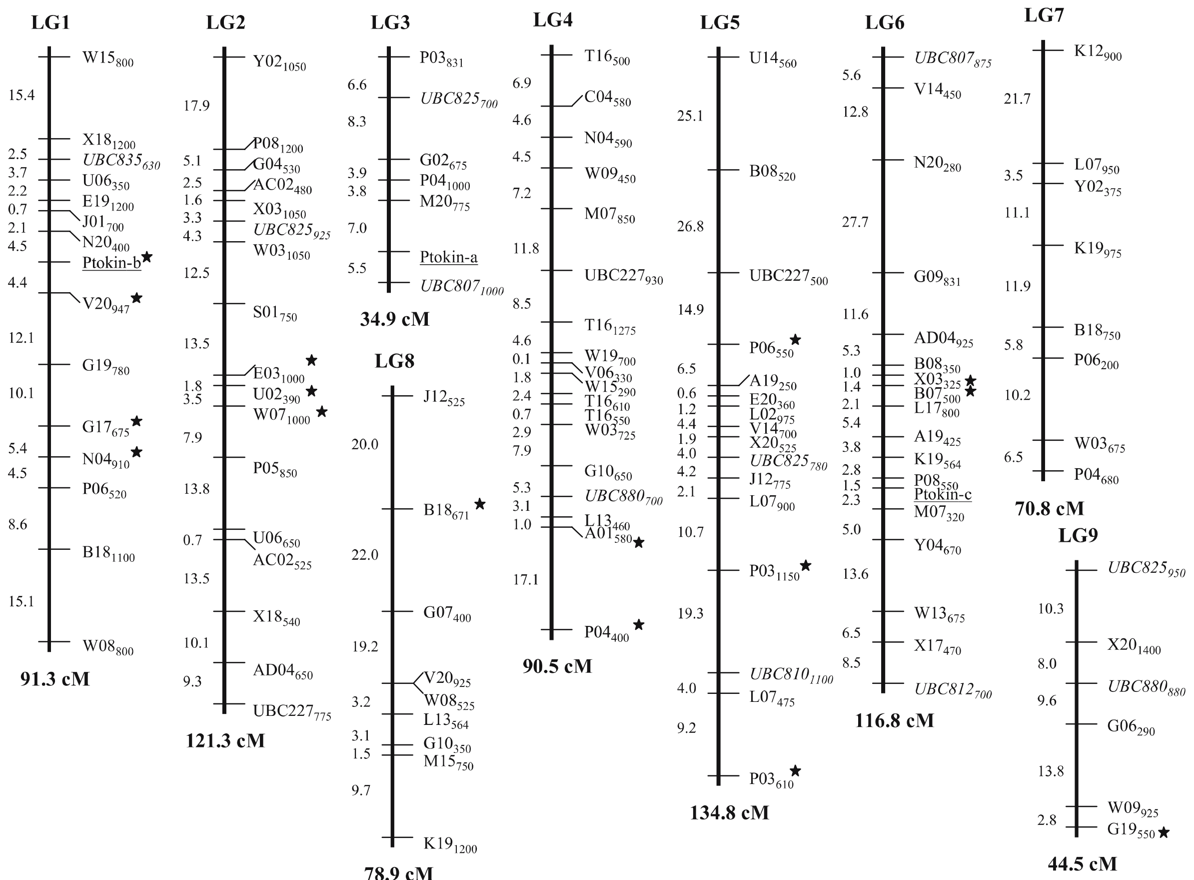

Fig. 1. Intraspecific map of lentil (ILL 5588×ILL 7537) at a LOD score of 4.0 (Rubeena et al. 2003). The LGs are numbered from LG1 to LG9. Loci names are indicated on the *right side* of the *vertical lines* and genetic distances (cM) are on the *left side* of the *vertical lines*. ISSR markers are *italicized*, RGA markers are *underlined* and RAPD markers are in *normal type*. Distorted markers are indicated with *stars*

cM within 9 LGs and comprised a total of 130 arbitrarily produced (RAPD, ISSR and AFLP) markers. Most recently a comprehensive intersubspecific *Lens* map was developed by enriching the previous map of Eujayl et al. (1998 a) with 39 new lentil-specific SSR and 50 new AFLP markers (Hamwieh et al. 2005). The map comprised a total of 283 markers spanning 751 cM within 14 LGs (8 with more than 3 markers).

To date, all *Lens* and lentil genome maps developed have more LGs than the species haploid chromosome number (n = 7). The amount of the genome mapped varies from 751 to 2172 cM with an average marker density of 2.7 to 15.87 cM (Table 1). The expected full genome length is as yet unknown. However, given the close phylogeny among the species, perhaps the expected length would be close to that of field pea, which is 700 to 800 cM as determined by cytological studies (Hall et al. 1997 a, b).

Other important characteristics of the current maps include the clustering of markers at various regions and the inclusion of distorted markers. Clustering may be indicative of centromeric and telomeric regions, which experience up to ten-fold less recombination than other areas of the genome (Tanksley et al. 1992). This was also observed in *Pisum* and *Cicer* maps (Laucou et al. 1998 and Winter et al. 2000 respectively). Segregation distortion is the consequence of unequal inheritance of parts of chromosomes, which may affect the ordering of markers within a LG (Lorieux et al. 1995). Factors that contribute to marker distortion include recessive alleles, structural rearrangements or differences in DNA content, abortion of male and female gametes and the selective fertilization of a particular gametic genotype (Tadmor et al. 1987; Barzen et al. 1995; Berry et al. 1995; Quillet et al. 1995; Jenczewski et al. 1997; Xu et al. 1997).

Until recently, a major limitation to *Lens* mapping has been the unavailability of locus-specific PCR-based and co-dominant markers such as expressed sequence tag (EST), cleaved amplified polymorphic sequences (CAPS), single nucleotide polymorphism (SNP) or SSR microsatellite markers, which are more robust and informative than arbitrary DNA markers. The lack of such markers has largely hampered the ability to compare various published linkage maps. However, the development of SSR markers for lentil was recently achieved by researchers at the ICARDA (Hamwieh et al. 2004, 2005). At present, one SSR marker has been mapped by Durán et al. (2004), and a further 39 SSR loci have been mapped by Hamwieh et al. (2005).

5.2.4 Towards a *Lens* Consensus Map

The existing maps have not been well linked to each other due to the lack of common markers. However, morphological markers and the recently developed lentil SSR markers (Hamwieh et al. 2005) should prove useful in assigning common LGs. Of the seven morphological markers already mapped, cotyledon color (orange vs. yellow; *Yc*), presence or absence of anthocyanin in the stem (*Gs*), seed coat pattern or spotting (*Scp*), pod dehiscence-indehiscence (*Pi*), ground color (brown vs. tan) of the seed (*Ggc*), erect or prostrate growth habit (*Gh*) and presence or absence of anthocyanin in the pod (*Pdp*), four have been placed on multiple maps (*Yc*, *Gs*, *Scp* and *Pi*) (Table 2).

Other markers that may be useful for consensus mapping include the repetitive DNA sequences that have been localised by fluorescent in situ hybridization (FISH) (Patil et al. 1995) and other gene-specific markers such as expressed sequence tag (EST) markers. Also, gene-specific markers transferable from related model legume crop species such as *Medicago truncatula* and *Lotus japonicus* and converted to SNP or cleaved amplified polymorphism (CAP) type markers. Such markers will also be useful for comparative mapping across species, to assign genetic LGs to specific *Lens* chromosomes and for integrating information from both physical and genetic maps (Galasso et al. 2001).

Table 2. Morphological markers mapped on different LGs in *Lens* genome may be useful as anchor markers

Marker locus	Linkage group in different studies		
	Tahir et al. 1993	Eujayl et al. 1998	Durán et al. 2004
Scp	V	III	I
Yc	II	–	II
Gs	I	–	IV
Pi	IV	II	–

5.3 Marker-Assisted Trait Mapping and Selection

5.3.1 Trait Mapping

Many simply inherited traits have been placed upon *Lens* genome maps. By knowing the map position of a gene, one can diagnose the presence of the gene using flanking DNA markers without waiting for the gene effect to be present in the phenotype (Paterson et al. 1991).

Bulked segregant analysis (BSA), first described by Michelmore et al. (1991), is one method used for linking molecular markers to phenotypic traits controlled by single major genes. This method relies on the availability of two bulked DNA samples collected from individuals that segregate for an extreme phenotype within a single population. One bulk contains the DNA of the trait being targeted, while the other contains DNA from individuals lacking the trait. DNA polymorphisms between the bulks are therefore likely to be linked to genes that govern the trait. In lentil, this method has been used to identify markers that are tightly linked to genes for resistance to Fusarium vascular wilt and ascochyta blight (Eujayl et al. 1998 b; Ford et al. 1999; Chowdhury et al. 2001).

Eujayl et al. (1998 b) used an RIL mapping population to identify linked molecular markers to the single dominant gene conditioning Fusarium vascular wilt resistance (*Fw*). They subsequently identified a RAPD marker ($OPS16_{750}$) that was 9.1 cM from the radiation-frost tolerance locus (*Frt*) (Eujayl et al. 1999). However, most probably due to insufficient genome map coverage, the *Frt* locus and the linked RAPD marker were unable to be placed on the existing linkage map developed by Eujayl et al. (1998 a).

Ford et al. (1999) identified RAPD markers, RV01 and RB18, approximately 6 and 14 cM, respectively away from and flanking the foliar ascochyta blight resistance locus *Ral*1 (AbR_1) in ILL5588. These were subsequently converted to locus-specific sequence characterized amplified region (SCAR) markers and screened for applicability across parental lines in the Australian breeding program. Although the linkage was not maintained across all parental genotypes, great potential exists for the targeted use of these markers in breeding and the pyramiding of resistance genes in ILL5588-derived genetic backgrounds. Subsequently, two RAPD markers, $UBC227_{1290}$ and $OPD\text{-}10_{870}$, were identified that flanked and were linked in repulsion phase to the resistance gene *ral*2 in the cultivar Indianhead at 12 and 16 cM respectively (Chowdhury et al. 2001). Most recently, molecular markers were developed linked to the complementary dominant resistance genes in ILL7537 (Rubeena, unpubl.). The resistance sources within these genotypes were shown to be novel using pathogenicity tests (Nguyen et al. 2001). Thus the potential exists to use markers to pyramid ascochyta blight resistance genes to develop durably resistant varieties.

Two RAPD markers, $OPE06_{1250}$ and $UBC704_{700}$, linked at 6.4 cM (in repulsion) and 10.5 cM (in coupling) respectively, were recently identified for selecting the anthracnose resistance locus *LCt-2* in accession PI 320937 (Tullu et al. 2003). Three AFLP markers were also identified linked in repulsion phase to *LCt-2*; however, the closest of these was 21.5 cM. Most recently, an SSR marker and an AFLP marker were identified flanking the *Fw* locus in ILL5588 at 8.0 and 3.5 cM respectively (Hamwieh et al. 2005).

5.3.2 Quantitative Trait Loci Mapping

When a trait is governed by multiple and quantitative trait loci (QTL) and/or co-dominantly inherited genes, a more holistic genome mapping approach may be undertaken to identify relative genome loci location, interaction and subsequent molecular markers for accurate trait selection.

A few QTL studies have been reported thus far for lentil. The first one employed a genetic linkage map developed from an intersubspecific population (*L.c.* ssp. *culinaris*×*L.c.* ssp. *orientalis*). A total of 22 QTLs were placed upon the map including five for height of the first ramification, three for plant height, five for flowering, seven for pod dehiscence, one for shoot number and one for F_3 seed diameter (Durán et al. 2002). QTLs governing winter hardiness were recently mapped using an F_6-derived RIL population of 106 individuals of a cross between WA8649090 and Precoz (Kahraman et al. 2004). For this, a framework map was produced with 9 LGs comprising a total of 130 markers and spanning 1192 cM. Quantitative survival and injury data were

Table 3. Putative QTL for winter hardiness in lentil (Kahraman et al. 2004)

Location	Linkage group	QTL position (cM) [a]	LOD	R^2 (%)
Haymana 1997–98	3	28	2.3	10.9
	4	118	7.3	28.8
	6	80	3.2	17.7
Total				**33.4**
Pullman 1998–99	4	110	2.5	11.5
Haymana 1999–2000	1	38	2.3	9.5
	1	146	2.2	10.1
	4	132	2.0	9.5
Total				**22.9**
Combined	4	116	6.9	28.8
	6	80	3.1	14.2
Total				**31.5**

[a] QTL position from bottom of LG

collected at two locations in 1997 (Pullman, WA, USA and Haymana, Turkey) and three locations in 1998 and 1999 (Pullman, WA, USA, Haymana and Sivas, Turkey). Five independent QTLs were detected to account for survival with a LOD score >2. These were located on LGs 1, 3, 4 and 6 with two QTLs on LG 1 (Table 3). One QTL on LG 4 was common among locations, although the effect and position differed. The maximum of the winter survival phenotype variation that these accounted for was 33.4%. One ISSR marker, ubc808-12, was identified that may be useful for MAS of winter survival. A further four QTLs were reported to influence winter injury in the USA location and together accounted for 42.7% of the trait variation.

Preliminary QTL analysis of the ascochyta blight resistance in ILL7537 was conducted using a population comprising 153 F_2 individuals [ILL 7537 (R)×ILL 6002 (S)] and a linkage map comprising 72 markers spanning 412.5 cM anchored to a pre-existing map (Rubeena et al. 2003). The disease reaction was scored using a 1–9 scale on each of the F_2 individuals at 14, 21 and 28 d after inoculation, and three QTL peaks (two on LGI and one on LGII) were observed using composite interval mapping (CIM) (Fig. 2). Two QTLs (QTL-1 and QTL-2) were observed on LG I in close proximity; since these were

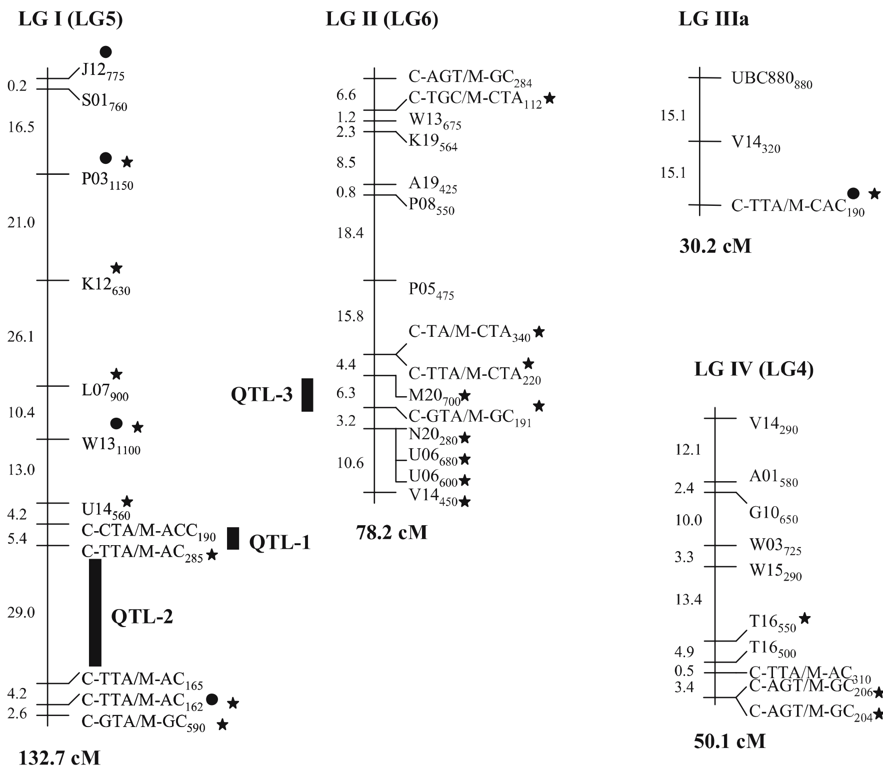

Fig. 2. Four LGs with significant markers shown by *stars* and distorted markers by *dots*. QTL regions are shown as *filled vertical bars* and named QTL-1, QTL-2 and QTL-3. All three QTL were observed by CIM, whereas only QTL-2 and QTL-3 were detected by MIM

Table 4. Putative QTL for ascochyta blight resistance identified in the F_2 population (ILL 7537/ILL 6002) by composite interval mapping (CIM)

Test parameter	QTL	Linkage group	Interval length (cM)[a]	Flanking markers	QTL position (cM)[b]	LR[c]	Additive effect	R^2[d] (%)
14 DAI	QTL-1	LG I	5.4	C-CTA/M-ACC_{190} – C-TTA/M-AC_{285}	2.0	12.78	–0.48	7.82
	QTL-2	LG I	29.0	C-TTA/M-AC_{285} – C-TTA/M-AC_{165}	14.0	13.83	–1.03	26.80
	QTL-3	LG II	6.3	$M20_{700}$ – C-GTA/M-GC_{191}	0.0	10.49	–0.59	6.19
Total								**40.81**
21 DAI	QTL-1	LG I	5.4	C-CTA/M-ACC_{190} – C-TTA/M-AC_{285}	4.0	20.35	–0.65	11.02
	QTL-2	LG I	29.0	C-TTA/M-AC_{285} – C-TTA/M-AC_{165}	12.0	26.36	–0.95	33.62
	QTL-3	LG II	6.3	$M20_{700}$ – C-GTA/M-GC_{191}	0.0	17.38	–0.72	9.25
Total								**53.89**
28 DAI	QTL-1	LG I	5.4	C-CTA/M-ACC_{190} – C-TTA/M-AC_{285}	2.0	31.19	–0.89	16.41
	QTL-2	LG I	29.0	C-TTA/M-AC_{285} – C-TTA/M-AC_{165}	8.0	34.70	–1.06	30.70
	QTL-3	LG II	6.3	$M20_{700}$ – C-GTA/M-GC_{191}	0.0	20.80	–0.73	10.25
Total								**57.36**
MDS	QTL-1	LG I	5.4	C-CTA/M-ACC_{190} – C-TTA/M-AC_{285}	2.0	24.05	–0.74	13.76
	QTL-2	LG I	29.0	C-TTA/M-AC_{285} – C-TTA/M-AC_{165}	12.0	29.12	–0.98	33.80
	QTL-3	LG II	6.3	$M20_{700}$ – C-GTA/M-GC_{191}	0.0	18.73	–0.70	10.10
Total								**57.66**

[a] Interval between the two flanking markers (cM)
[b] QTL position from left flanking marker (cM)
[c] Peak value of maximum likelihood ratio (LR) test statistic observed for QTL
[d] Proportion of phenotypic variance explained by QTL

>10 cM apart, they were considered to be separate QTLs (Table 4). They accounted for ca. 47%, whereas QTL-3 on LG II accounted for ca. 10% of the variance of the trait. The position of the QTL changed slightly over the different scoring periods after inoculation. The AFLP marker C-TTA/M-AC_{285} was found to be 3.4 cM away from QTL-1 and 12 cM away from QTL-2. The RAPD marker $M20_{700}$ was located at the same position as QTL-3. When multiple interval mapping (MIM) was performed, only two significant QTLs (QTL-2 and QTL-3) were identified. These two QTLs may potentially be the major effects of the two co-dominant resistant genes previously identified to govern resistance in ILL 7537 (Nguyen et al. 2001). However, the QTLs identified must be validated in different genetic backgrounds and populations before incorporation into breeding programs.

Recently, resistance gene analogues belonging to the nucleotide binding site gene families were isolated from the lentil genotype ILL5588 (Yaish et al. 2004). Mapping of RGA, together with the ascochyta blight resistance trait, may be useful to validate the location of genes that are functional in the resistance mechanism, a step towards map-based cloning of the active resistance genes.

5.3.3 Marker-Assisted Selection and Trait Pyramiding

Marker-assisted selection (MAS) is the ability to select for and breed for a desirable trait with a marker, or suit of markers, from within a plant genotype without the need to express the associated phenotype. Therefore, MAS offers great opportunity for improved efficiency and effectiveness in the selection of plant genotypes with a desired combination of traits. This approach relies upon the establishment of a tight linkage between a molecular marker and the chromosomal location of the gene(s) governing the trait to be selected in a particular environment. Once this has been achieved, selection can be conducted in the laboratory and does not require the expression of the associated phenotype. For example, using MAS, disease resistance can be evaluated in the absence of the disease and in the early stage of plant development.

Markers used for MAS are also termed sequence tagged sites (STSs). These are mapped loci for which all or part of the corresponding DNA sequences has been determined. This information can be used to design PCR primers for amplification of all or part

of the original sequence. They are more robust and reproducible than the arbitrary sequences they are designed from, such as RAPD markers, as they are developed from the known sequences and produce an amplicon from longer primers. Differences in the lengths of amplified fragments serve as genetic markers for the locus. If no length polymorphism is detected, the amplified fragments can be cleaved with restriction enzymes to observe subsequent length differences. This technique is often referred to as cleaved amplified polymorphic sequences or CAPS (Jarvis et al. 1994).

The use of converted locus-specific PCR markers is also referred to as a specific polymorphic locus amplification test (SPLAT), as well as sequence characterized amplified region (SCAR) markers and allele-specific associated primer (ASAP) markers. SPLAT markers are designed from sequencing the insert of a polymorphic RFLP marker (Gale and Witcombe 1992), whereas SCAR and ASAP markers are developed from sequencing specific RAPD markers (Paran and Michelmore 1993; Gu et al. 1995; Ford et al. 1999). The conversion of more technically demanding RFLP markers into PCR-based markers (e.g. SPLAT) may provide a more rapid, cost-effective and efficient tool in lentil breeding.

Nguyen et al. (2001) first converted an arbitrarily produced lentil sequence to a SCAR marker (SCARW19) for selecting resistance to ascochyta blight in lentil accession ILL5588. Tar'an et al. (2003) converted the $RB18_{680}$ RAPD marker, formerly also shown to be linked to the *AbR1* gene (Ford et al. 1999), into a robust SCAR marker. They subsequently used SCAR markers linked to the *AbR1* gene and the *ral2* gene (Chowdhury et al. 2001), together with a marker linked in repulsion to a gene for anthracnose resistance (*LCt2*), to pyramid the traits in an RIL population. Using the linked markers, 11 of 156 RILs were shown to retain all three resistance genes. Of these, 82%, which contained the markers linked to *AbR1* and *ral2*, were resistant to a highly virulent *A. lentis* isolate. Furthermore, 85% of the lines that did not contain the marker linked to the *LCt2* gene were resistant to the virulent 95B36 isolate of *C. truncatum*. This is the first evidence of validating the use of molecular markers for marker-assisted trait selection in lentil. Pyramiding of multiple resistance genes to foliar fungal pathogens should provide a broader and more durable resistance, as similarly shown in rice against bacterial blight (Singh et al. 2001).

5.4 Future Scope of Works

To date, no gene-specific, SSR or arbitrarily produced molecular marker has been identified in extremely close proximity (<1 cM) to any mapped lentil quality, disease resistance or stress tolerance gene locus. Hence, in order to increase the accuracy of MAS, the identification of tightly linked markers is an ongoing goal for researchers targeting many traits.

Of the specific traits for which molecular markers could provide considerable benefit to current breeding practices, water usage and drought tolerance are paramount. Furthermore, breeding for a flowering response that gives broad adaptation is currently a goal of the lentil breeding program in Australia, and markers that are proven stable across multiple environments have great potential in improving grain yield across variable locations and years in water-limited regions of the world.

To densely map the genomic areas surrounding the genes governing traits of interest, highly robust, unilocus, co-dominant and transferable markers are required. In particular, those that may be transferred among multiple different genetic backgrounds, and hence applicable across a broad range of breeding programs, would be most useful. Markers such as SSRs and ESTs are only now being developed for lentil by several groups. Alternatively, sequences from the model species *Medicago trancatula* are showing great promise for their transferability to *Lens* (R. Oliver, personal communication). Such sequences are particularly useful since many have already been assigned function and associated with desirable traits. Furthermore, the gene space of the *M. trunculata* genome will be fully sequenced using a bacterial artificial chromosome (BAC) library approach by the end of 2006 (Young et al. 2004), enabling the elucidation of syntenic relationships among the grain legumes.

Another requirement necessary for the future of fine genome mapping in lentil is the availability of large fixed mapping populations, such as single seed descent RILs and DHs. Such populations would allow for the identification and validation of trait-associated markers across different environments and at different plant growth stages. For map-based cloning of genes shown to be associated with desirable traits, a BAC library of lentil is required with good genome coverage (>5). Furthermore, the ability to

tile the lentil BAC clones along the *M. truncatula* sequenced genome will also negate the necessity to sequence the entire lentil genome. Naturally the choice of genotypes for mapping populations and BAC library construction will need to be carefully considered in order to produce tools that are compatible with as many breeding priorities and programs throughout the world as possible.

Once genes have been isolated from the lentil genome they will need to be validated for function using a differential or transgenic approach. Already differential array chips exist that were constructed from expressed sequences within related legume species such as soybean (Vodkin et al. 2004). Through gene conservation and intergeneric synteny, these chips may be useful for determining the orthologous genes up-regulated in lentil in response to similar stimuli, such as exposure to a fungal pathogen. However, correct gene identification is still very much reliant on the sequences already present within existing databases. Agrobacterium-mediated and particle bombardment gene transfer protocols have been developed for lentil (Sarker et al. 2003 and Gulati et al. 2002 respectively), and ultimately these will serve as a means to prove gene function via gene silencing and gene expression in alternate genetic backgrounds.

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