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Application of biotechnology in breeding lentil for resistance to biotic and abiotic stress

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Summary

Lentil is a self-pollinating diploid ($2n = 14$ chromosomes) annual cool season legume crop that is produced throughout the world and is highly valued as a high protein food. Several abiotic stresses are important to lentil yields world wide and include drought, heat, salt susceptibility and iron deficiency. The biotic stresses are numerous and include: susceptibility to Ascochyta blight, caused by *Ascochyta lentis*; Anthracnose, caused by *Colletotrichum truncatum*; Fusarium wilt, caused by *Fusarium oxysporum*; Sclerotinia white mold, caused by *Sclerotinia sclerotiorum*; rust, caused by *Uromyces fabae*; and numerous aphid transmitted viruses. Lentil is also highly susceptible to several species of *Orabanche* prevalent in the Mediterranean region, for which there does not appear to be much resistance in the germplasm. Plant breeders and geneticists have addressed these stresses by identifying resistant/tolerant germplasm, determining the genetics involved and the genetic map positions of the resistant genes. To this end progress has been made in mapping the lentil genome and several genetic maps are available that eventually will lead to the development of a consensus map for lentil. Marker density has been limited in the published genetic maps and there is a distinct lack of co-dominant markers that would facilitate comparisons of the available genetic maps and efficient identification of markers closely linked to genes of interest. Molecular breeding of lentil for disease resistance genes using marker assisted selection, particularly for resistance to Ascochyta blight and Anthracnose, is underway in Australia and Canada and promising results have been obtained. Comparative genomics and synteny analyses with closely related legumes promises to further advance the knowledge of the lentil genome and provide lentil breeders with additional genes and selectable markers for use in marker assisted selection. Genomic tools such as macro and micro arrays, reverse genetics and genetic transformation are emerging technologies that may eventually be available for use in lentil crop improvement.

Introduction

Lentil (*Lens culinaris* Medik.) is a highly valued annual food legume crop that coevolved with wheat, barley and other cool season pulses in the Near East arc about 8000 years ago (Cubero, 1981; Ladizinsky, 1979). World Production of lentil is estimated at 3.3 million metric tons from an estimated 3.8 million hectares with

an average yield of 850 kg/ha FAOSTAT, 2005). These yields seem small, but generally lentil is produced on marginal lands that are relatively dry and without the benefit of fertilizer inputs or irrigation. Attempts to improve yields through breeding are underway throughout the world and especially at the International Center for Agricultural Research in the Dry Areas (ICARDA), USA, Canada, Australia, Turkey, South Asia and many countries of the West Asia-North Africa (WANA) region. Major producing countries are India, Turkey, Canada, Australia, Ethiopia, Morocco, Spain, Chile and Argentina. Of these, the major exporting countries are Canada, Australia and the U.S. The countries of the Middle East and North Africa are major consumers.

The genus *Lens* comprises seven taxa in four species (Ferguson & Erskine, 2001; Ferguson et al., 2000). *Lens orientalis* is the presumed progenitor of cultivated *L. culinaris* and the two species are fully crossable and produce fully fertile progenies. However, except for *L. odemensis*, there is difficulty in crossing the other wild species to the cultigen. From the stand point of crossability for use in breeding, the *Lens* species can be divided into two groups (Ladizinsky, 1999): *L. culinaris–L. odemensis* and *L. ervoides–L. nigricans* that for convenience can be assigned to primary and secondary gene pools. Crosses between members of different groups fail because of hybrid embryo abortion; however, embryo rescue has been used successfully to obtain viable hybrids between groups (Ladizinsky et al., 1985).

Several abiotic stresses such as cold, drought, heat, salinity, nutrient deficiency and nutrient toxicity adversely affect lentil yields world wide (Monti et al., 1994; Saxena, 1993; Slindard et al., 1994). Numerous biotic stresses that adversely affect lentil include: susceptibility to Ascochyta blight, caused by *Ascochyta lentis*; Anthracnose, caused by *Colletotrichum truncatum*; Fusarium wilt, caused by *Fusarium oxysporum*; root rots caused by *Fusarium solani, Aphanomyces eutieches, Pythium ultimum, Rhizoctonia solani* and possibly other pathogens; Sclerotinia white mold, caused by *Sclerotinia sclerotiorum*; rust, caused by *Uromyces fabae*; and numerous aphid transmitted viruses. Lentil is also highly susceptible to several species of *Orabanche* prevalent in the Mediterranean region, for which there does not appear to be much resistance in the germplasm. Resistance to some of these stresses has been found in germplasm collections. See Erskine et al. (1994b) for a review of strategies for breeding lentil for resistance to biotic and abiotic stresses.

Molecular markers and gene "tagging" has provided plant breeders with a means to accelerate breeding programs and an efficient tool for indirect selection for traits that would otherwise be difficult to select for using standard procedures. Biotechnological techniques such as Microarrays, TILLING, Genetic transformation and others hold promise for improving our knowledge of the lentil genome and accelerating progress through breeding. Our objectives were to review the status of available and emerging biotechnologies and their current and potential application for overcoming major biotic and abiotic stresses of lentil crops.

Constraints to production of lentil

Abiotic stresses

The major abiotic stresses affecting cool season food legumes were the subject of a comprehensive review (Singh & Saxena, 1993). Stresses that affect lentil were listed as cold, drought, heat, salinity, nutrient deficiency and nutrient toxicity. Of these stresses, drought and heat are considered the most important world wide (Turner et al., 2001). Cold stress was considered important in the West Asia-North Africa (WANA) region. Salinity is an important stress factor in the Indian sub-continent and to some extent in WANA. Nutrient deficiency and nutrient toxicity is of lesser importance world wide but important in localized regions. For breeding approaches for crop improvement in stress environments see the review by Buddenhagen and Richards (1998).

Susceptibility to cold temperatures has limited production of lentil in cold highland areas of the world. However, germplasm is available that has useful degree of tolerance to cold temperatures which makes it possible to breed winter hardy cultivars that can be planted in the fall with a reasonable expectation of surviving the winter (Erskine et al., 1981; Spaeth & Muehlbauer, 1991). Kahraman et al. (2004a) reported that winter hardiness in lentil is conferred by several genes. In a genetic analysis of winter hardiness, Kahraman et al. (2004b) showed that the combined effects of several quantitative trait loci accounted for 42% of the variation in winter survival. Molecular markers associated with those QTL have potential use in a marker assisted selection program for winter hardiness, but first must undergo validation.

Lentil is able to produce something of value in many of the semi-arid regions primarily through drought

avoidance (Erskine & Saxena, 1993; Turner et al., 2001). Early senescence and crop maturity forced by drought conditions that are often more severe due to the usually associated high temperatures. Rainfall accounted for 41% and 55% of the variation in yields of two lentil cultivars, respectively, in Syria. Various procedures have been attempted for screening lentil germplasm and breeding material for drought tolerance. Field screening using line source irrigation to develop a moisture gradient has been attempted at ICARDA. Also, covering field plot areas to restrict moisture to form moisture differential areas, the use of late planting to increase exposure of the plants to higher temperatures and greater moisture stress have all been attempted with inconclusive results. Increased rooting depth as a means of drought avoidance has been suggested; however, that approach may be at considerable expense to succeeding crops, most likely wheat. Molecular approaches such as marker assisted selection may have merit; however, considerable work is needed to identify the important regions of the genome, most likely through a QTL analysis, and validation of associated molecular markers.

Heat stress often accompanies drought causing difficulties in separating the two stresses and their effects on lentil growth and yield. There is general agreement that heat affects the distribution of dry matter to reproductive growth and that high temperatures have an adverse effect on lentil yields. Research is needed to critically define the effects of heat on the reproductive stages of lentil and the effects on yield. Evaluation of the world collection of lentil germplasm has indicated useful genetic variation is available for improving adaptation to environmental extremes (Erskine et al., 1990, 1994b).

Salinity problems with lentil are not wide spread but can be acute in certain regions of South Asia, the Nile delta of Egypt and in some areas of Turkey. Canada also has some difficulty in high salinity areas of Saskatchewan. Of the legumes, lentil is more salt sensitive when compared to faba bean and soybean (Katerji et al., 2001, 2003). Salt stress can adversely affect nodulation and N_2 fixation (Rai & Singh, 1999; Rai et al., 1985) presumably by restricting growth of the root hairs and the potential sites of infection by *Rhizobium*. Some germplasm with tolerance to salt stress has been identified (Ashraf & Waheed, 1993; Ashraf et al., 1990; Jana & Slinkard, 1979).

Nutrient deficiencies and toxicities are important in specific situations. Iron deficiency symptoms appear in germplasm introduced to other areas from the Indian subcontinent and Ethiopia and has been shown to have a genetic basis (Erskine, 1997; Erskine et al., 1993). The acute yellowing symptomatic of iron deficiency can be alleviated by germplasm efficient in the utilization of iron (Erskine, 1997; Erskine et al., 1993). Boron toxicity has also been problematic and there appears to be some tolerant germplasm (Yau & Erskine, 2000).

Biotic stresses

Foliar diseases are the most serious biotic stresses affecting lentil crops. Ascochyta blight caused by *A. lentis* is problematic to various degrees in all lentil growing regions of the world, but especially damaging in Canada (Ahmed & Morrall, 1996; Ahmed et al., 1996), Australia and Middle Eastern countries (Johansen et al., 1994). Symptoms of Ascochyta blight include lesions on all above ground parts of the plant, stem girdling, pod and seed lesions and resistance has been found in the germplasm (Andrahennadi et al., 1996). Other major biotic stresses of lentil include Anthracnose caused by *C. truncatum*, Botrytis grey mold caused by *Botrytis fabae* and *B. cinerea*, Stemphylium blight caused by *Stemphylium botryosum*, lentil rust caused by *U. fabae* and Sclerotinia white mold caused by *S. sclerotiorum.* See Tivoli et al. this volume for a description of Ascochyta blight, Anthracnose and Botrytis grey mold.

Stemphylium blight is a major threat to lentil in South Asia and North America (ICARDA, 2004; Vandenberg: Personal communication). The pathogen causes a leaf blight, plant defoliation and death. The disease is poorly understood but apparently there is some resistance available in the germplasm; however, the genetics of resistance is still to be determined.

Lentil rust caused by *U. fabae* is widespread in South Asia, Morocco and Ethiopia and is characterized by lesions on the stems and leaves, leaf drop and premature plant death (Ahmed & Morral, 1996; Ahmed et al., 1996). Losses from the disease, estimated at up to 70%, have been reported (Erskine and Sarker, 1997; Negussie et al., 1998). Resistance to the disease has been identified in germplasm line ILL5588 and is currently being used as a source of resistance in breeding programs and in studies to determine the inheritance of resistance and to map the important genes (Ahmed & Morral, 1996; Ahmed et al., 1996).

Sclerotinia white mold caused by *S. sclerotiorum* has been responsible for extensive damage to lentil crops in areas that are relatively moist and humid. Dense crop canopies also contribute to the severity

of the disease. The pathogen affects 148 known plant genera and there is little resistance to the disease. Research is underway by the USDA-ARS Grain Legume Breeding program at Pullman to determine the genetics of resistance/tolerance found in lentil cultivars and germplasm. Research is currently underway towards conducting a Quantitative Trait Loci analysis using recombinant inbred lines from crosses of presumed resistant cultivars with highly susceptible cultivars.

Root rots and wilts usually attack lentil plants in the seedling stage and cause seed rot, damping off, wilt, destruction of the root system and rotting of lower stems. These disease problems have been extensively reviewed (Khare, 1981; Kraft et al., 1988, 1994). Fusarium wilt caused by *F. oxysporum* is considered to be the most damaging soil borne disease problem facing lentil world wide (Khare, 1981) and several races of the pathogen can cause the disease. Hamwieh et al. (2005) developed a genetic map of lentil that comprises over 300 molecular markers and used the map to determine the location of the gene for resistance to Fusarium wilt (Figure 1).

Molecular markers and marker assisted selection

The current state of molecular breeding in lentil

Lentil is a relatively small crop compared to wheat, rice, maize and soybean and ranks third among the cool season food legumes in area harvested (4.08 million hectares) annually (http://apps.fao.org/faostat), and behind pea and chickpea. Much of the crop is grown in economically poor countries where research funding and expertise in novel molecular breeding approaches is limited. Consequently, molecular breeding research has often been directed to address major production limitations that are relevant to developed countries and where resources for equipment and consumables are available. However, even with these restrictions, some substantial advances have been made towards understanding the lentil genome and the development and application of molecular markers to advance breeding strategies.

Much of the focus has been on developing molecular markers for selecting resistance to a few major diseases, such as Ascochyta blight, especially where research is complementary between countries and research groups. In the near future, the potential exists to develop markers for many other highly sought traits that are difficult to breed for conventionally. Molecular markers aim 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, sometimes because the traits to be selected can already 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 utilization of the technology is achieved. Globally, trait selection that would greatly benefit from the availability of robust and accurate markers includes; drought, frost, boron (Hobson et al., 2003; Yau & Erskine, 2000) and salinity tolerance, and resistances to Ascochyta blight, Botrytis gray mold, Anthracnose, rust, Fusarium and Stemphylium blight.

Molecular markers and genome mapping

Morphological and isozyme markers were used to identify the first genetic linkages in lentil (Muehlbauer et al., 1989; Tadmor et al., 1987; Vaillancourt & Slinkard, 1993; Zamir & Ladizinsky, 1984). Soon thereafter many types of DNA-based molecular markers, arising from point mutations, insertions or deletions or errors in replications of tandem-repeated DNA, were identified and used for mapping the lentil genome. Restriction fragment length polymorphism (RFLP) markers, developed from cutting genomic DNA with restriction enzymes and separating the resulting DNA fragments with electrophoresis, were the first type of molecular marker used in the construction of a lentil genetic linkage map (Havey & Muehlbauer, 1989). Subsequently, arbitrarily produced polymerase chain reaction (PCR)-based markers, such as random amplified polymorphic DNA (RAPD) were used to study diversity, phylogeny and taxonomy of *Lens* (Ford et al., 1997; Ferguson et al., 2000; Sharma et al., 1996), to develop linkage maps (Eujayl et al., 1997, 1998a; Rubeena et al., 2003), to tag genes of interest (Chowdhury et al., 2001; Eujayl et al., 1998b, 1999; Ford et al., 1999; Tullu et al., 2003) and to determine pathogen population structure (Ford et al., 2000). Arbitrarily produced amplified fragment length polymorphism (AFLP) markers have also been used in lentil linkage mapping (Durán et al., 2004; Eujayl et al., 1998a; Hamwieh et al., 2005; Kahraman et al., 2004) and to study genetic diversity (Sharma et al., 1996), differentiate cultivars (Závodná et al., 2000) and identify markers linked to specific traits (Tullu et al., 2003). Comparisons of the lentil

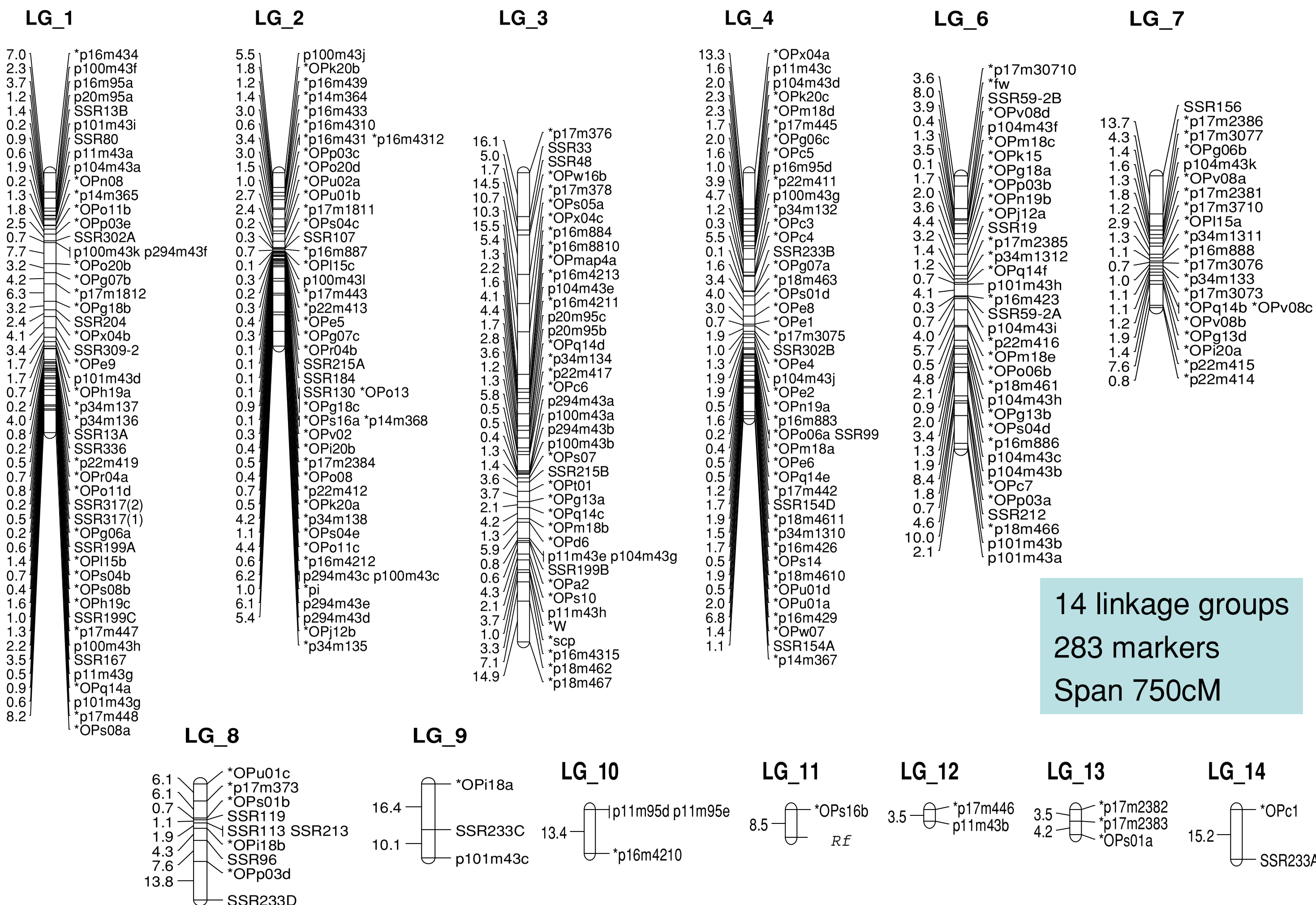

Figure 1. A genetic map of lentil (from Hamweih et al., 2005).

linkage map with that of chickpea and pea indicate interesting similarities (Simon & Muehlbauer, 1995).

More recently, simple sequence repeat (SSR) or microsatellite markers, which consist of tandem repeats of two to five nucleotide DNA core sequences that are spread throughout the genome, were used to construct lentil linkage maps (Durán et al., 2004; Hamwieh et al., 2005). The DNA sequences flanking microsatellite repeats are generally conserved within individuals of a given species, allowing the design of highly specific and robust PCR primers that amplify the intervening SSR. Two sets of microsatellite markers were developed from lentil by Závodná et al. (2000) and at ICARDA by Hamwieh et al. (2005). 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).

Other marker types such as inter-simple sequence repeat (ISSR) markers, amplified with SSR-anchored primers, and resistance gene analogue (RGA) markers, amplified with degenerate primers designed from conserved regions of cloned plant resistance genes were also used in lentil genome mapping (Durán et al., 2004; Rubeena et al., 2003). Positional cloning of RGA markers will be potentially aid in the localization of disease resistance genes via the candidate-gene approach (Kanazin et al., 1996; Leister et al., 1996).

Lens mapping populations

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 to be 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. The initial mapping populations used in lentil were F_2 progenies from crosses between wild species and cultivars (Havey & Muehlbauer, 1989; Muehlbauer et al., 1989; Tahir et al., 1993; Vaillancourt & Slinkard, 1993; Weeden et al., 1992). However, it is known that the use of such divergent parents often results in lower recombination rates and smaller map sizes (Tadmor et al., 1987). Indeed, the first lentil maps comprised relatively small marker numbers and spanned relatively small segments of the genome (Eujayl et al., 1997; Havey & Muehlbauer, 1989; Weeden et al., 1992). More recently, linkage maps have been based on populations of recombinant inbred lines (RILs) (Eujayl et al., 1997, 1998a; Hamwieh et al., 2005; Kahraman et al., 2004).

The major drawback in using F_2 populations is that they are ephemeral and determinate, unlike RIL populations derived in F_6 or later generations and homozygous at most of the loci (Tahir & Muehlbauer, 1994). RIL populations can have multiple uses for mapping and can easily be maintained for future genomic research. It is also noted that the size of a mapping population can greatly impact the ultimate resolution of a map (Young, 1994).

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

Doubled haploid (DH) populations, produced by regenerating plants from single pollen grains and inducing chromosome doubling, may 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 anther culture and in general, grain legumes are more recalcitrant to in vitro manipulation than many other species (reviewed by Christou, 1997).

Lentil genetic linkage maps

The first linkage map of lentil using DNA-based markers (RFLP) was constructed by Havey and Muehlbauer (1989). PCR based markers were used to construct a more extensive linkage map comprising 177 markers (RAPD, AFLP, RFLP and morphological markers) and was based on a RIL population created from an inter-subspecific cross (Eujayl et al., 1998a). The first intraspecific linkage map of lentil was constructed with 114 RAPD, ISSR and RGA markers (Rubeena et al., 2003). Recently, two more extensive molecular linkage maps were reported, one using an intraspecific population (Kahraman et al., 2004) and the other based on an inter-subspecific population (Durán et al., 2004). The lentil linkage map, produced by Durán et al. (2004) contained 62 RAPD, 29 ISSR, 65 AFLP, four

morphological markers and one SSR, and spanned a distance of 2172 cM within 10 linkage groups. The lentil specific SSR marker was the first of its type to be mapped in lentil. The map of Kahraman et al. (2004) covered 1192 cM within nine linkage groups and comprised a total of 130 arbitrarily produced (RAPD, ISSR, and AFLP) markers. Most recently a comprehensive inter-sub specific lentil map was developed by enriching the previous map of Eujayl et al. (1998a) with 39 new lentil-specific SSRs and 50 new AFLP markers (Hamwieh et al., 2005). The map (Figure 1) comprises a total of 283 markers spanning 751 cM within 14 linkage groups (eight of the linkage groups had more than three markers).

To date, all lentil genetic maps have had more linkage groups than the species haploid chromosome number ($n = 7$). The estimated amount of the genome mapped currently varies from 751 to 2172 cM with an average marker density of 2.7–15.9 cM. 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 700–800 cM as determined by cytological studies (Hall et al., 1997a, 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 10-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; Winter et al., 2000, respectively). Segregation distortion is the consequence of unequal inheritance of parts of chromosomes and may affect the ordering of markers within a linkage group (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 (Barzen et al., 1995; Berry et al., 1995; Jenczewski et al., 1997; Quillet et al., 1995; Tadmor et al., 1987; Xu et al., 1997).

Until recently, a major limitation to lentil mapping has been the unavailability of locus-specific PCR based and co-dominant markers such as expressed sequence tags (EST), cleaved amplified polymorphic sequences (CAPS), single nucleotide polymorphism (SNP) or simple sequence repeat (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. The recently reported SSR markers placed on the lentil genetic map should facilitate the development of a consensus map.

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 linkage groups. Of the seven morphological markers already mapped, cotyledon color (orange versus yellow; *Yc*), presence or absence of anthocyanin in the stem (*Gs*), seed coat pattern or spotting (*Scp*), pod dehiscence-indehiscence (*Pi*), ground color (brown versus tan) of the seedcoat (*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*).

Other markers that may be useful for consensus mapping include the repetitive DNA sequences that have been localized by fluorescent in-situ hybridization (FISH) and used to assign genetic linkage groups to specific chromosomes and for integrating information from both physical and genetic maps (Galasso et al., 2001; 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 single nucleotide polymorphism (SNP) or cleaved amplified polymorphism (CAP) type markers. Such markers will also be useful for comparative mapping across species.

Trait mapping

Many simply inherited traits have been placed on lentil genetic maps. By knowing the map position of a gene, the presence of the gene can be diagnosed 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 a method used to identify molecular markers linked to phenotypic traits controlled by single major genes. This method relies on the availability of bulked DNA samples collected from individuals that segregate for the two extreme divergent phenotypes 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 (Chowdhury et al., 2001; Eujayl et al., 1998b; Ford et al., 1999).

Eujayl et al. (1998b) used an RIL mapping population to identify molecular markers linked to the single dominant gene conditioning Fusarium vascular wilt resistance (*Fw*). They also 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. (1998a).

Ford et al. (1999) identified RAPD markers, RV01 and RB18, approximately 6 and 14 cM, respectively, 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 that were linked to the complementary dominant resistance genes in ILL7537 (Rubeena, pers. comm.). The resistance sources within these genotypes were shown to be novel using pathogenicity tests (Nguyen et al., 2001). Thus there is potential for using markers to pyramid Ascochyta blight resistance genes to develop durably resistant varieties.

Molecular markers for resistance to Anthracnose and Fusarium wilt have also been developed for screening breeding material. In the case of anthracnose resistance, bulk segregant analysis of 147 F_5-derived F_6RILs from the cross of resistant PI320937 and susceptible cultivar Eston was used to identify markers linked to resistance (Tullu et al., 2003). RAPD markers $OPE06_{1250}$ and $UBC704_{700}$ were linked at 6.4 cM (in repulsion) and 10.5 cM (in coupling), respectively, to the resistance locus, *LCt-2,* (Tullu et al., 2003). Three AFLP markers were also identified as linked in repulsion phase to *LCt-2*; however, the nearest marker was 21.5 cM away. Tar'an et al. (2003) have demonstrated the usefulness of $OPE06_{1250}$ in marker assistant selection to pyramid genes for Ascochyta blight and Anthracnose resistance. Fusarium wilt is reportedly controlled by a single dominant gene, *Fw,* and linked to RAPD marker $OPK\text{-}15_{900}$ at distance of 10.8 cM (Eujayl et al., 1998b). More recently Hamwieh et al. (2005), identified an SSR marker and an AFLP marker that flanked the Fusarium wilt resistance gene by 8.0 and 3.5 cM, respectively.

Successful winter cropping of lentil depends on seedling survival and an optimum plant population in the early developmental stages. Eujayl et al. (1999) observed that seedling frost tolerance was governed by a single dominant gene *Frt* in a cross between ILL 5588 × L 692-16-1(S) and that the *Frt* locus was linked to RAPD marker $OPS\text{-}16_{750}$ at 9.1 cM.

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 suite 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 early stages of plant development.

Sequence tagged sites (STS) are ideal markers for MAS. STS markers are mapped loci for which all or part of the corresponding DNA sequence has been determined. The sequence information is 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 & Witcombe, 1992), whereas SCAR and ASAP markers are developed from sequencing specific RAPD markers (Gu et al., 1995; Ford et al., 1999; Paran & Michelmore, 1993). 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 found 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 a RIL population. Using the linked markers, 11 of 156 RIL were shown to retain all three resistance genes. Of these, 82%, that 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 *Colletotricum 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).

Quantitative trait loci mapping and identification of genes

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 genomic locations, interaction and subsequent molecular markers for accurate trait selection.

Few QTL studies have been reported thus far for lentil. The first employed a genetic linkage map developed from an inter-sub specific population (*L. c. ssp. culinaris* × *L. c. ssp. orientalis*). A total of 22 QTL 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). QTL governing winter hardiness were recently mapped using an F_6 derived population of 106 RILs from a cross between WA8649090 and Precoz (Kahraman et al., 2004). That population was used to construct a framework map of nine linkage groups comprising a total of 130 markers and spanning 1192 cM. Winter survival and winter injury data were collected at two locations in 1997 (Pullman, USA and Haymana, Turkey) and three locations in 1998 and 1999 (Pullman, USA, Haymana and Sivas, Turkey). Five independent QTL were detected to account for survival with a LOD score >2.0. One QTL on LG 4 was common among locations although the effect and position differed somewhat. These QTL accounted for 33.4% of the variation in the winter survival scores in the RIL population. One ISSR marker, ubc808-12, was identified as potentially useful for predicting winter survival using MAS. Overall, four QTL accounted for 42.7% of the variation in winter injury scores at the USA location.

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 days after inoculation and three QTL peaks (two on LG I and one on LG II) were observed using composite interval mapping (CIM). Two QTL (QTL-1 and QTL-2) were observed on LG I in close proximity, since these were >10 cM apart, they were considered to be separate QTL. They accounted for approximately 47%, whereas QTL-3 on LG II, accounted for approximately 10% of the variance of the trait. The position of the QTL changed slightly over the different scoring periods after inoculation. The AFLP marker $C\text{-}TTA/M\text{-}AC_{285}$ 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 QTL (QTL-2 and QTL-3) were identified. These two QTL may potentially be the major effects of the two codominant resistant genes previously identified to govern resistance in ILL 7537 (Nguyen et al., 2001). However, the QTL 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 (NBS) 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.

Association mapping

Association or linkage disequilibrium (LD) mapping was used successfully to discover genetic determinants to traits initially in humans is now being used in plants (Flint-Garcia et al., 2003; Thornsbury et al., 2001). Using association mapping, entire genomes can be scanned for markers associated with qualitative and quantitative traits. The association mapping approach may allow plant breeders to break out of restrictive F_1-derived mapping populations and employ any plant population including those from breeding programs or germplasm collections to conduct marker-trait association studies (Flint-Garcia et al., 2003). Gebhardt et al. (2004) clearly summarized the four potential benefits of the association mapping approach: (1) it allows assessment of the genetic potential of specific genotypes before phenotypic evaluation; (2) it allows the identification of superior trait alleles in germplasm; (3) it can assist in high resolution QTL mapping; and (4) it can be used to validate candidate genes responsible for individual traits (Gebhardt et al., 2004).

The important issues to consider in designing and implementing any association mapping studies in plants are: (1) determination of the population structure (Pritchard et al., 2000); (2) estimation of nucleotide diversity (Zhu et al., 2003); (3) estimates of haplotype frequencies and LD (nonrandom association of alleles at different loci) (Flint-Garcia et al., 2003); and (4) precise evaluation of phenotypes (Neale & Savolainen, 2004). For lentil, the information regarding nucleotide diversity and LD may be inferred from the *Medicago truncatula* sequencing project (www.genome.ou.edu/medicago) in conjunction with comparative mapping between *M. truncatula* and lentil. The USDA-ARS lentil germplasm project proposes to determine the population structure of the lentil core collection (Simon & Hannan, 1995) using 30 mapped SSR markers. The evaluation of the core collection will be conducted in two environments (rainfed and irrigated) over two years. The genotypes and phenotypes will be available at www.ars-grin.gov.

Advanced genomic tools applicable to lentil genomics

Remarkable advancement of genomic tools enabled excavation of entire genome sequences in model plant species such as *Arabidopsis* (Bevan et al., 2001) and rice (Goff et al., 2002; Yu et al., 2002). However, the same approach does not seem to be applicable to most of the crop species including lentil not only due to large genome size compared to the model species but also due to limitation in financial and human resources. To avoid these issues, it is desirable to establish indirect but efficient ways to understand genome structure and to investigate genes in narrow but important genomic regions.

By virtue of advances in genome scanning techniques such as microarray (Blanchard & Friend, 1999; Kuhn, 2001), investigation of entire genomes for genes of interest has become feasible. However, because new techniques can also allow substantial degree of false positive detection, we need to be cautious in the application of high throughput techniques in current breeding programs. To successfully survey an entire genome to identify genes determining traits of interest, the proper choice of genetic materials is essential. Genetically defined plant materials such as near-isogenic lines or any other types of genetic stocks having deletions or additions within narrow regions of the genome are considered efficient for this purpose. However, there are no such genetic stocks available for genomic studies in lentil. Creation of suitable genetic stocks suitable for application of advanced genomic tools needs to be considered as part of the overall lentil breeding approach. Breeding strategies will have a significant impact on long-term application of advanced genomic tools and the results can be re-applied to traditional lentil breeding programs. In this section, we discuss feasible approaches to generate new genetic stocks and the use in high throughput techniques that may benefit lentil genomics and breeding.

Development of new genetic materials

(1) *Alien gene transformants*. One of the direct approaches to create genetic materials with enhanced trait values are transformation of foreign genes of which functions were validated. Although this

approach is ethnically controversial, transformation technology is valuable not only for validation of gene function but also for planning for future direction of traditional breeding based on information obtained from transformants. Genetic modification mediated by Agrobacterium (Mahmoudian et al., 2002; Sarker et al., 2003; Warkentin et al., 1991, 1992) or bombardment (Gulati et al., 2002) has been attempted in lentil. However, these studies were mostly to establish transformation techniques rather than the introduction of genes into improved varieties. Additional genomic information generated through comparative genomics with species phylogenetically related to lentil is needed to identify candidate genes for lentil improvement. For a comprehensive review of gene technology for grain legumes see Popelka et al. (2004).

(2) *Randomly mutagenized lines*. An indirect and efficient approach to investigate an entire genome is to generate randomly mutagenized genetic stocks followed by analysis to reveal genes directly controlling traits of interest. Mutagenesis of lentil has received considerable attention as a tool for developing genetic variation for use in crop improvement and several mutagenic chemicals and gamma rays have proved effective in lentil (Solanki & Sharma, 1994). The chemicals most often used are ethyl methane sulfonate (EMS), *N*-nitroso-*N*-ethyl urea, ethylene imine, and sodium azide. Recently, mutagenesis has received great attention for use in a promising new technique known as "targeted induced local lesions in genomes" (TILLING). The procedure is a high-throughput process of generating chemically mutagenized lines and examining mutants systematically by PCR (Brachmann et al., 2004; Colbert et al., 2001; Henikoff et al., 2004; Kempin et al., 1997). This method was first demonstrated in *Arabidopsis* (McCallum et al., 2000) and also in barley (Caldwell et al., 2004). In legumes, this method was applied in *Lotus japonicus* and generated a general TILLING population of 3697 independent M_2 plants (Perry et al., 2003). Using the same procedure, approximately 2000 individual germlines were generated in *Medicago truncatula* (VandenBosch & Stacey, 2003), a species phylogenetically related to lentil.

Because genetic transformation techniques have been established for lentil, insertional mutagenesis such as T-DNA insertion (Feldman, 1991) and transposon insertion (Tisser et al., 1999) can be attempted. Generation of mutants by T-DNA insertion has been successfully conducted in *Arabidopsis* (Krysan et al., 2002) and rice (Sha et al., 2004). Stability of the tDNA insertion and a low copy number in a diploid genome (1.4 inserts) are the main advantages of this method (Sha et al., 2004). However, there are a few disadvantages such as multiple inverted or tandem copies or truncated tDNA that complicate the analysis (Nacry et al., 1998).

Transposon mutagenesis is also an effective functional genomic tool to generate large numbers of mutants and to efficiently screen an entire genome to identify genes of interest. Tissier et al. (1999) generated 48,000 Arabidopsis transposon insertion lines and approximately 80% of them were found to have independent insertion events. Unlike T-DNA insertion mutants, inserted transposons can move and consequently a mutation can revert. Furthermore, depending on the type of transposon, multiple insertions can be generated (reviewed by Tissier et al., 1999). Although this nature of transposon insertion can be an advantage in functional analysis, this can also be a disadvantage in establishing straightforward genetic stocks.

Genome scanning by transcriptome profiling

Transcript profiling is one of the frequently used methods to investigate global gene regulation and eventually to identify genes associated with traits of interest. Investigation of expressed sequences has many advantages over investigation of entire genomic sequences not only because the area of the genome under investigation can be significantly minimized by considering only expressed sequences but also because they imply biological impact on traits of interest. Furthermore, a transcriptome that represents an entire genome can be examined regardless of the amount of available genomic information.

Transcript profile analysis can be classified into two categories, qualitative and quantitative, based on detection characteristics. For qualitative analysis, differential display RT-PCR and cDNA-AFLP are representative techniques. Differential display RT-PCR was introduced to screen large numbers of transcripts by PCR using arbitrary primers (Liang & Pardee, 1992). However, in spite of experimental convenience, lack of sensitivity and reproducibility were critical issues in applying this technique (reviewed by Donson et al., 2002), and as a result has been replaced by other advanced techniques such as cDNA-AFLP. cDNA-AFLP was developed by modifying the genomic DNA-based

AFLP technique (Bachem et al., 1996). Because this technique adopted selective PCR of adaptor-ligated cDNA fragments, specificity and reproducibility could be improved. The cDNA-AFLP technique was applied to chickpea to identify candidate genes for resistance to *Ascochyta rabiei* causing Ascochyta blight (Cho et al., 2005). However, in lentil, studies of differential gene expression are very limited (Abrecht et al., 2000) and no study has been done to investigate global transcript regulation.

Quantitative transcript profiling frequently refers to macroarray (cDNA membrane array) and microarray (cDNA- or oligonucleotide-based array) techniques (Chen et al., 1998). Macroarray analysis is conducted using denatured double-stranded DNA printed on nylon membranes and probed with sample sequences and the abundance of target transcripts in the RNA pool is digitally monitored (Chen et al., 1998). In legume species, soybean (Vodkin et al., 2004) and Medicago (Küster et al., 2003) were the pioneers in transcript profiling studies using macroarray technique. Compared to macroarray, microarray technology is a superior alternative approach (Thibaud-Nissen et al., 2003). Microarrays can be divided into two formats, cDNA microarray format (Schena et al., 1996) and oligonucleotide format (Pease et al., 1994) depending on the types of probe sequences on microarray chips and the method of probe hybridization. cDNA slide chips for legume species were constructed to examine transcriptomes of *Medicago truncatula* (Fedorova et al., 2002; Küster et al., 2003), soybean (Maguire et al., 2002; Thibaud-Nissen et al., 2003; Vodkin et al., 2004) and *Lotus japonicus* (Endo et al., 2002). Compared to cDNA slide chips, oligonucleotide format can technically hold up to 500,000 of 25-mer oligonucleotide probes photolithographically synthesized on a single chip. The first oligonucleotide Chip for legume species was developed for *M. truncatula* (Mitra et al., 2004).

Transcript analysis using macroarrays or microarrays, requires the accumulation of sequence information on the species involved. Because sequence information is currently lacking, the immediate application of microarrays to cool season grain legumes including lentil is not possible. Alternatively, macroarray filters and cDNA microarray chips developed for other legume species may be analyzed in the grain legumes. A high degree of sequence homology especially for expressed sequences among legume species might enable cross-species application of macro- and microarrays. This approach was used successfully in animals (Moody et al., 2002; Wang et al., 2004) and plants (Becher et al., 2003). However, GeneChips having short oligonucleotide probes was reportedly unsuitable for cross-species hybridization due to interspecific specificity of probes (Close et al., 2004).

Comparative genomics

Genomic information acquired from phylogenetically related species can be beneficial if traits of interest share similar genetic mechanisms among the species being compared. A plan for future comparative genomics in legume species has been proposed by Legume Crop Genome Initiative (see meeting report by Gepts et al., 2005), and cool season food legumes including lentil have been chosen to be investigated for their genome structure comparative to model legume species, *M. truncatula* and *Lotus japonicus*. A key point in this approach is to apply genomic information obtained from model species to cool season food legumes. The first step to successful application is to accumulate genomic information that can be used to connect related species. This has been attempted using markers generated from *M. truncatula* (Gutierrez et al., 2005), and showed that the markers were significantly transferable to cool season legumes such as chickpea, pea and faba bean. A similar approach should be attempted in lentil to improve genome coverage with common markers needed for comparative genomics across species.

In addition to the approach to reveal macrosynteny across species as discussed above, development of tools for microsynteny is needed. Because cool season food legumes have relatively larger genome sizes compared to model legumes, gene identification in food legumes based on the genomic information from *M. truncatula* may not be accomplished easily. Large-sized genomes can be segmented into small pieces, e.g., BAC libraries, and these sequences can be physically aligned to be used as a path to search for genes of interest by reciprocal comparison of physical and genetic information between model legumes and food legumes including lentil.

Future scope of lentil research

Future direction of lentil genomics can be summarized and includes (1) new marker development and fine mapping, (2) development of new genetic materials applicable to advanced genomics and (3) application of advanced genomic tools for lentil genomics.

Regarding development of new markers, no genetic markers either gene-specific or arbitrary markers

have been found to be closely linked within 1cM proximity of important genes in lentil. Therefore, to increase the efficiency of MAS, the identification of tightly linked markers and development of additional markers is needed. Traits for which molecular markers can provide immediate benefits are the traits related to drought tolerance and broad adaptation to environmental stresses. Genes that prove to be stable across multiple environments will have great potential for improving grain yield of lentil across variable locations and years in water-limited regions of the world. To increase marker density within regions of interest, markers that are sequence-specific and co-dominant are recommended. In addition, transferable markers across related species will facilitate comparative genomic study in lentil. Not only the SSR or EST derived markers being developed in lentil (Baum, personal communication), but also transferable markers from the model species are needed to increase map density.

Development of new genetic materials is a prerequisite for future lentil genomics. For fine mapping in lentil, large mapping populations of recombinant inbred lines are needed. Such populations will allow for the identification and validation of trait-associated markers across different environments and at different plant growth stages. In order to apply advanced genomic tools such as transcriptome profiling and map-based gene cloning to lentil, additional refined genetic materials are needed.

Lentil genome analysis may proceed through transcriptome profiling and comparative genomics using BAC libraries. In case of transcriptome profiling, the entire genome may be examined simultaneously by monitoring gene expression patterns (El Yahyaoui, 2004). This approach is available even without sequence information and can lead to the accumulation of information on expressed sequences and the identification of interesting genes. BAC libraries may facilitate gene cloning and physical mapping of the lentil genome. A physical map of the lentil genome, when compared to physical maps of other species, will provide valuable genomic information about lentil and other cool season legumes.

The future of lentil genomics is promising because of the extensive research conducted in related species that provides appropriate guidelines. Also, most of the important techniques required to pursue genomic research are available and are feasible for use in lentil. When all components discussed above are coordinated properly among lentil researchers, remarkable progress can be expected.

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