Chapter 3
Conventional and Biotechnological Approaches for Targeted Trait Improvement in Lentil

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Abbreviations

AFLP	Amplified fragment length polymorphism
APX	Ascorbate peroxidase
ASAP	Allele specific amplified primer
AUD	Australian dollar
BA	Benzyladenine
BAC	Bacterial active chromosome
BAC	Bacterial artificial chromosome
BARI	Bangladesh Agricultural Research Institute
BC	Backcross
BIBAC	Binary bacterial artificial chromosome
BLAST	Basic local alignment search tool
CAPS	Cleaved amplified polymorphic sequences
cDNA	Complimentary DNA

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S. S. Gosal, S. H. Wani (eds.), *Accelerated Plant Breeding, Volume 3*,
https://doi.org/10.1007/978-3-030-47306-8_3

Cl−	Chloride ion
CMS	Cell membrane stability
CS	Climate smart
CTD	Canopy temperature depression
CWSI	Crop water stress index
DArT	Diverse array technology
dCAPS	Derived CAPS
DH	Double haploid
DNA	Deoxyribonucleic acid
DS	Dormant seeding
DTI	Drought tolerance index
DUS	Distinctiveness, uniformity and stability test
EC	Electrical conductivity
EMBL-EBI	European Molecular Biology Laboratory
eQTL	Expression QTL
EST-SSR	Expressed sequence tag-derived simple sequence repeats
Fe	Iron
FISH	Fluorescence in situ hybridization
GA3	Gibberellic acid
GABA	γ-Aminobutyric acid
GBS	Genotyping by sequencing
GEO	Gene Expression Omnibus
GMP	Geometric mean productivity
GSI	Germination stress index
GUS	Transient β-glucuronidase
HI	Harvest index
HM	Harmonic mean
HMM	Hidden Markov model
ICARDA	International Center for Agriculture Research in the Dry Areas
IIPR	Indian Institute of Pulses Research
IPCC	Intergovernmental Panel on Climate Change
IRLC	Inverted repeat-lacking clade
ISSR	Inter-simple sequence repeats
ITAP	Intron targeted amplified polymorphism
ITS	Internal transcribed spacer
K	Potassium
K+	Potassium ions
KEGG	Kyoto Encyclopedia of Genes and Genomes
LIS	Legume Information System
MAB	Marker-assisted breeding
MABC	Marker-assisted backcrossing
MABCB	Marker-assisted backcross breeding
MARS	Marker-assisted recurrent selection
MAS	Marker-assisted selection

Mg	Magnesium
Mha	Million hectare
miRNA	MicroRNAs
MP	Mean productivity
Mt	Million tons
Na+	Sodium ions
Na+/K+	Sodium to potassium ratio
NBPGR	National Bureau of Plant Genetic Resources
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NILs	Near-isogenic lines
NUE	Nutrient use efficiency
PAR	Photosynthetically active radiation
PBA	Pulse Breeding Australia
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PLANEX	Plant co-expression database
QTL	Quantitative trait loci
R/FR	Red/far red
RAPD	Random amplified polymorphic DNA
RGA	Resistance gene analogues
RIL	Recombinant inbred line
RNA	Ribonucleic acid
RNAi	RNA interference
RS ratio	Root-shoot ratio
RWC	Relative water content
SCAR	Sequence characterized amplified region
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide *gel* electrophoresis
SNP	Single-nucleotide polymorphism
SPLAT	Specific polymorphic locus amplification test
SRAP	Sequence-related amplified polymorphism
SSD	Single seed descent
SSI	Stress susceptibility index
SSR	Simple sequence repeats
STI	Stress tolerance index
STS	Sequence tagged site
Super-SAGE	Serial analysis of gene expression
Tc	Canopy temperature
TI	Heat tolerance index
VIGS	Virus-induced gene silencing
VNTR	Microsatellite variable number tandem repeats
VPD	Water pressure deficits
WUE	Water use efficiency
YAC	Yeast active chromosome
Zn	Zinc

3.1 Introduction

Lentil is a true diploid ($2n = 2x = 14$) annual plant with 4 Gbp genome size (Arumuganathan and Earle 1991). Lentil is an important legume crop which offers paddock to plate health benefits by enriching soil through N-fixing symbiotic relations with rhizobium (Jarpa-Parra 2018) and is embedded in cereal legume-based farming system as a high-value cash crop. It is one of the nutritious grain legumes being rich in dietary protein (20.6–31.4%), vitamins, minerals and many essential amino acids (lysine and tryptophan) (Erskine et al. 1990; Faris et al. 2013; Johnson et al. 2013; Ray et al. 2014; Jarpa-Parra 2018) and also having other benefits such as high fibre and low glycaemic index (Srivastava and Vasishtha 2012; Moravek et al. 2018). Among various food legume crops, the lentil has not seen tremendous adoption primarily in developing countries despite crop's ability to thrive under limited water conditions. Its high protein content makes it the best alternative to animal-based protein for vegetarian people. Pulses are grown over 95.2 Mha area and lentil covers 6.6 Mha area worldwide. Lentil among less privileged crops has not seen immense improvement in its productivity over the past few decades – 0.8 (1997) to 1.2 ton/ha (2017) – at the world level (FAO 2020).

To meet the food demand of increasing human population, by 2050 we need to produce double the amount of food from half of the available resources by facing the vagaries of climate change. Crop yields around the world will significantly be affected due to climate pressures and narrow genetic base of staple crops. Biotic and abiotic stresses will not only affect quantity but also the quality of the produce. Legumes which play a significant role in crop cycle not only fall second to cereals but have been neglected and grown mostly on marginal lands especially in developing countries and have lost genes of importance (Bejiga and Degago 2000). Along with cultivation on marginal lands which generally have low soil fertility, lentil crop is mostly grown as rainfed and is subjected to mainly terminal drought and heat stress (abiotic) and various fungal and bacterial diseases – ascochyta blight, rust, stemphylium blight, collar rot, root rot, white mould, fusarium wilt and anthracnose (Kumar et al. 2013; Sharpe et al. 2013). Currently, cultivated lentil cultivars do possess tolerance/resistance to some abiotic and biotic stresses; still, the breeding focus of these cultivars primarily has been for higher yields. Therefore, changing climate has threatened scumming of most of the cultivars to various biotic and abiotic stresses over a short period of time after their release. Looking at the significance of legumes including lentil in current crop production system around the world, targeted trait improvements for resistance to various stresses, improved quality and higher yields will ascertain sustained quality production over the years to meet the growing demand for healthy food alongside facing the challenges of drastic climatic events. The narrow genetic base of lentil cultivars due to their reliance on few improved cultivated germplasm is certainly a great concern (Singh et al. 2014), though there is a hidden wealth of wild and distant lentil relatives which possess untapped genes of interest to be targeted for further desired improvements of existing and development of new lentil cultivars (Ford et al. 1997; Duran et al. 2004;

Gupta and Sharma 2007; Gupta et al. 2019). Therefore, this chapter will focus on targeted trait improvements accomplished using conventional and biotechnological approaches in lentil as well as genetic resources explored for traits from close and distant wild and cultivated sources not included in existing cultivars around the world.

3.2 Pre-breeding for Targeted Trait Improvement in Lentil

Cultivated lentil has been categorized in two major groups based on its seed size – small-seeded, 'microsperma' (2–6 mm), and large-seeded, 'macrosperma' (6–9 mm). Wild species *L. orientalis* is considered the wild progenitor of cultivated lentil (Zohary 1972) as ascertained by higher percentage of crossing ability of these two which mostly leads to fertile hybrids. Wong et al. in 2015 classified genus *Lens* into primary (cultivated lentil, *L. culinaris*; wild lentil species, *L. orientalis* and *L. tomentosus*), secondary (*L. lamotte* and *L. odomensis*), tertiary (*L. ervoides*) and quaternary (*L. nigricans*) distinct gene pools and related species mentioned in brackets. Various researchers have demonstrated that primary and secondary gene pools harbour compatible species and majority of the genotypes can be crossed through conventional breeding techniques and with or without any exogenous application of growth hormones or assist via tissue culture techniques (Ahmad et al. 1995; Fratini et al. 2004; Gupta and Sharma 2005). However, pre- and postfertilization barriers hamper successful introgression of genes of interest primarily from tertiary and quaternary gene pools into cultivated lentil (Gupta and Sharma 2007; Singh et al. 2013).

Long-term sustainability of lentil cultivars to mitigate stresses and sustain higher-quality yields will hugely depend upon their ability to harbour many genes of agronomical importance as well as biotic and abiotic stress-resistant/tolerant genes, which are mostly of quantitative nature. As without stable resistance/tolerance in existing cultivars, huge yield penalties are experienced by growers around the world. Because of low or nil resistance to biotic stresses in most of the existing cultivars, reliance on chemical control has increased so does the crop production cost.

Therefore, the trait targeted approach in breeding programmes needs a focus on tapping germplasm with multiple traits/genes of interests or common defence mechanisms (Gupta et al. 2019). Agronomical traits of importance, resistance to biotic stresses and tolerance to abiotic stresses have been identified among wild and distant lentil genotypes with superior expression to the popular cultivated lentil cultivars in various studies (Table 3.1).

In-depth understanding of underlying mechanisms of stress tolerance/resistance and ability of wild genotypes to thrive under such stresses provides insight into tapping right species and desirable traits of interest for introgression into the cultivated background. Among various stresses, drought management operates either through drought avoidance or tolerance. Presence of dense leaf hairiness, closure of stomata

Table 3.1 Pre-breeding identification of various genes of interest from cultivated and wild lentil germplasm

Trait of interest	Germplasm	References
Agronomical traits		
Early flowering and maturity, leaf area, higher number of leaves, pods and seeds per plant	Wild and cultivated germplasm	Hamdi et al. (1991), Ferguson and Robertson (1999), Gupta and Sharma (2006), Singh et al. (2014)
Abiotic stresses		
Drought avoidance		
Early vigour, root traits, rapid root growth, root-shoot ratio, nodulation, flowering and maturity, desired canopy structure, leaf surface, stem length, stomatal traits, high yield	Wild, cultivated and mutant germplasm	Erskine and Saxena (1993), Silim et al. (1993a, b), Salam and Islam (1994), Erskine et al. (1994), Shrestha et al. (2005), Idrissi et al. (2016), Biju et al. (2017), Gorim and Vandenberg (2017a, b)
Drought tolerance		
Seedling survival and vigour, root traits (root length, lateral roots number, root weight), root-shoot ratio, plant height, pod and seed number, grain yield and harvest index, early flowering and maturity, germination stress index, cell membrane stability, electrolyte leakage, water use efficiency, relative water content, osmotic regulation, drought susceptibility index, crop water stress index, canopy temperature depression, drought tolerance efficiency	Wild and cultivated germplasm	Hamdi and Erskine (1996), Mia et al. (1996), Sarker et al. (2005), Gupta and Sharma (2006), Shrestha et al. (2006), Stoddard et al. (2006), Salehi et al. (2008a, b), Chakherchaman et al. (2009), Aswaf and Blair (2012), Kumar et al. (2012a), Idrissi et al. (2015), Mishra et al. (2014, 2016, 2018), Singh et al. (2017), Biju et al. (2018)
Heat tolerance		
Higher antioxidant activities, pollen germination and viability, nodulation, heat tolerance index (TI) and cell membrane thermostability, number of filled pods, seed weight and yield	Cultivated germplasm	Chakraborty and Pradhan (2010, 2011), Choudhury et al. (2012), Barghi et al. (2013), Delahunty et al. (2015), Gaur et al. (2015), Kumar et al. (2016, 2017), Bhandari et al. (2016), Sita et al. (2017)
Cold and frost tolerance		
Early vigour, controlled freezing test, winter hardiness and survival rate	Wild and cultivated germplasm	Hamdi et al. (1996), Ali et al. (1999), Sarker et al. (2002), Kahraman et al. (2004)
Salinity tolerance		

(continued)

Table 3.1 (continued)

Trait of interest	Germplasm	References
Seed germination, nodulation, root-shoot length and weight, water use efficiency, sodium-potassium ratio, soluble sugars, proline, antioxidant activity, salt tolerance percentage, salinity scores, stress indices, biomass yield	Wild and cultivated germplasm	Rai and Singh (1999), Hamdi et al. (2000), Yasin et al. (2002), Maher et al. (2003), Cicerali (2004), Sidari et al. (2007), Kokten et al. (2010), Siddique et al. (2013), Ouji et al. (2015), AL-Quraan and AL-Omari (2017), Kumawat et al. (2017), Aslam et al. (2017)
Biotic stresses		
Ascochyta blight	Wild and cultivated germplasm	Gurdip et al. (1982), Cromey et al. (1987), Iqbal et al. (1990), Abi-Antoun et al. (1990), Sugha et al. (1991), Ahmed and Beniwal (1991), Andrahennadi (1994), Bayaa et al. (1994), Erskine et al. (1996), Ahmad et al. (1997), Nasir and Bretag (1998), Tullu et al. (2006, 2010a, b), Iqbal et al. (2010), Dadu et al. (2017, 2018)
Anthracnose	Wild and cultivated germplasm	Buchwaldt et al. (2004), Tullu et al. (2006), Fiala et al. (2009), Shaikh et al. (2012), Vail et al. (2012)
Botrytis grey mould	Cultivated germplasm	Karki et al. (1993), Bretag and Materne (1999), Kuchuran et al. (2003), Lindbeck et al. (2008)
Fusarium wilt	Wild and cultivated germplasm	Bayaa et al. (1995), Erskine et al. (1996), Nasir (1998), Gupta and Sharma (2006)
Powdery mildew	Wild germplasm	Gupta and Sharma (2006)
Rust	Wild and cultivated germplasm	Singh et al. (1994), Negussie et al. (1998), Sarker et al. (1999, 2004), Gupta and Sharma (2006), Fikru et al. (2007), Peñaloza et al. (2007), Sadiq et al. (2008)
Stemphylium blight	Cultivated germplasm	Kant et al. (2017)
Viral disease resistance		Makkouk and Kumari (1990), Kumari and Makkouk (1995), Makkouk et al. (2001), Latham and Jones (2001), Rana et al. (2016)
Insect resistance		
Aphids	Cultivated germplasm	Kumari et al. (2007)
Sitona weevils	Wild germplasm	El-Bouhssini et al. (2008)

Partially adapted from Gupta et al. (2019)

in a regulated manner, enhanced antioxidant levels, osmotic adjustment, and yield are related to drought tolerance in lentil. Recent studies by Gorim and Vandenberg (2017a, b) suggested that wild lentils and climatic conditions of their place of origin must have evolved them to cope with drought stress through different mechanisms of escape, avoidance or tolerance. These mechanisms operate through the expression of various traits such as late onset of flowering, less water loss through transpiration, putting less biomass through reduced plant height and letting roots grow deeper. As expected, some genotypes did express more than one drought stress management strategies.

So far various researchers have identified many useful genes from cultivated and wild germplasm (Table 3.1) which either have been transferred into existing lentil cultivars or are part of current breeding programmes or still need to be considered for their inclusion in lentil breeding programmes.

3.3 Important Traits of Interest for Breeding Strategy

The first challenge in breeding for multiple traits is to determine and prioritize traits which are most important for the target environment and market. Several traits of importance can simultaneously be targeted for genetic improvement of lentil cultivars. However, prioritization of traits is very important, as there is a cost for every trait the plant expresses in the final phenotype. Breeders should focus on the identification of genotypes with desired adaptation to biotic and abiotic stresses, superior grain quality, nutritional attributes and appropriate phenology to match with the environment. An overview of region-wise targeted traits across the globe and traits that can be used for genetic improvement of lentil is summarized in Table 3.2 and Fig. 3.1, respectively. For multiple trait selections and integration, a breeding programme must focus on the traits that are associated genetically.

3.4 Conventional Breeding Approaches for Targeted Trait Improvement in Lentil

Lentil is the oldest domesticated self-pollinating crop (Erskine 1997) with less than 0.8% of natural cross-pollination (Wilson and Law 1972). Unlike the other major oldest domesticated cereals or pulses, the history of lentil spread indicates that the crop improvement in lentil has largely been achieved through natural and artificial selection within landraces (Erskine 1997). A survey in 1979 reported that landraces occupied greater than 80% of the area under cultivation in the major countries (Solh and Erskine 1981). Later, with the commencement of the International Center for Agriculture Research in the Dry Areas (ICARDA) in 1977, lentil improvement programmes received valuable assistance, and subsequently, different breeding

Table 3.2 List of region-wise targeted traits in lentils for improvement across the globe

Region	Targeted trait
Africa	Yield and related traits, nutritional enhancement, heat and drought tolerance, resistance to ascochyta blight, anthracnose and rust
Eastern Europe	Yield and related traits; nutritional enhancement; tolerance to heat, cold and drought; resistance to ascochyta blight, anthracnose and fusarium wilt
North Africa	Yield and related traits; nutritional enhancement; tolerance to salinity, heat, cold and drought; resistance to ascochyta blight, anthracnose, fusarium wilt, botrytis grey mould, rust and stemphylium blight
North America	Yield and related traits; nutritional enhancement; tolerance to salinity, heat, cold and drought; resistance to ascochyta blight, anthracnose, botrytis grey mould, rust and stemphylium blight
Oceania	Yield and related traits; nutritional enhancement; tolerance to heat, drought and boron; resistance to ascochyta blight, botrytis grey mould
Russia	Yield and related traits, nutritional enhancement, tolerance to heat and drought, resistance to ascochyta blight
South America	Yield and yield-related traits; nutritional enhancement; tolerance to heat, drought and cold; resistance to ascochyta blight, fusarium wilt, botrytis grey mould and rust
South Asia	Yield and related traits; nutritional enhancement; tolerance to salinity, boron, heat, drought and cold; resistance to ascochyta blight, fusarium wilt, anthracnose, botrytis grey mould, rust and stemphylium blight
Western Asia	Yield and related traits; nutritional enhancement; tolerance to heat, drought, cold, salinity, boron; resistance to ascochyta blight and fusarium wilt
Western Europe	Yield and related traits, nutritional enhancement, tolerance to heat and drought, resistance to ascochyta blight, fusarium wilt, botrytis grey mould and rust

Adapted and modified from Rana et al. (2019)

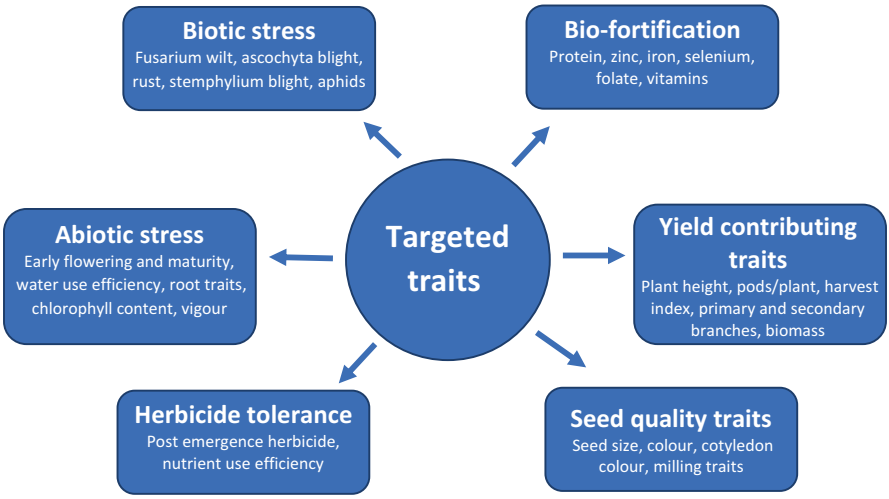

Fig. 3.1 Targeted traits used in lentil breeding strategies

strategies were laid out. Soon after its inception, as one of its mandate, ICARDA in collaboration with other national institutes emphasized collection of genetic resources in the view of unanticipated future needs. Successive efforts resulted in the accumulation of large collection of germplasm at various gene banks around the world including ICARDA, which hosts around 10,800 wild and cultivated lentil genotypes (Global Crop Diversity Trust 2008). The variability for various economically important traits within these conserved germplasms has been characterized to some extent as given in Sect. 3.2 and is revealed useful in breeding and selection programmes (Tullu et al. 2010a, b; Singh et al. 2018).

Like any other self-pollinating crop, breeding methods for incorporation of target traits employed in lentil majorly included pure line selection, hybridization, backcross, bulk, pedigree and single seed descent (SSD) method (Rahman et al. 2009). As a result of these methodologies, a total of 146 cultivars have been released until 2017 across major lentil-producing countries with targeted traits (Table 3.3). During early adaptation of lentil, pure line selection was extensively used to release cultivars with adaptability to wider areas and superior performance in terms of yield and disease resistance for ascochyta blight, rust and fusarium wilt. A few popular cultivars to release through pure line selection include Pant L 406, Pant L 639, L 830, L 4076, B 77, etc. in India, Barimasur 1 in Bangladesh, Shital in Nepal and Masoor 85 in Pakistan (Rahman et al. 2009). Cultivars that performed well in a country were often introduced in another country with similar climatic conditions.

Table 3.3 Lentil cultivars released from national programmes using ICARDA-supplied genetic material during 1977–2017

Region	Country	Number of cultivars	Targeted traits
Asia	Bangladesh, India, Nepal, Pakistan, China, Afghanistan, Iran, Iraq, Syria, Lebanon, Jordan, Yemen, Turkey	80	High yield; seed traits; micronutrient enrichment; short duration; suitability to machine harvesting; resistance to ascochyta blight, rust, stemphylium and fusarium wilt; tolerance to drought, frost and cold
Africa	Ethiopia, Egypt, Morocco, Libya, Tunisia, Algeria, Lesotho, Sudan, Eritrea	39	High yield and seed quality, early maturity, seed traits, adaptation to new environments, suitability to machine harvesting, resistance to wilt, rust and powdery mildew
The Americas	Argentina, Chile, Canada, Ecuador, USA	7	High yield and biomass, erect, resistance to ascochyta and rust, drought tolerance
Oceania	Australia, New Zealand	12	High yield, early maturing, resistance to ascochyta blight and botrytis grey mould
Europe	Portugal	2	High yield, large-seeded, tall
Central Asia and the Caucasus	Georgia, Uzbekistan, Azerbaijan	6	High yield, tall and erect, suitability for machine harvesting, high protein content, lodging resistance, rust resistance

Adapted from Sarker et al. (2009) and the data is sourced from ICARDA's website https://indms.icarda.org/pages/12

Some of the successful introductions include Vipasha and VL 507 in India and Mansehra 89 and Shiraz 96 in Pakistan (Rahman et al. 2009). Likewise, several other introductions have enriched local gene pools and led to the development of cultivars with greater yield stability in major lentil-producing countries (Laskar et al. 2019).

Cross-breeding is the widely chosen method in the recent past by breeders particularly to introgress special traits from exotic or other popular germplasm to the locally adapted cultivars (Laskar et al. 2019). The crosses have not been just limited to single crosses between two parents but involved double, three-way and multiple crosses. In a successful hybridization, selection of parents, as well as selection post crossing in resultant generations, is crucial to produce cultivars with desirable traits (Sarker et al. 2009). Selection procedures often varied with the objective of the breeding programme though the aim is to retain the best lines towards the end of selection cycle. Some of the methods used in the lentil breeding programme included pedigree, bulk population, recombinant-derived family and SSD. Furthermore, few modifications existed to bulk method with single pod selection being employed at F2 and F3 and single plant selections at F4 (Muehlbauer et al. 2009). These populations were screened for various traits including ascochyta blight resistance, seed shape, seed colour, pod drop, shattering and biomass. Selected lines were then evaluated for yield and quality in target environments. Popular cultivars in Australia such as PBA Ace, PBA Bolt and Nipper were developed using this method (Pulse Australia 2019).

SSD in lentil has often been used to produce recombinant inbred line populations (RILs) for use in constructing linkage maps (Eujayl et al. 1998; Tullu et al. 2008; Saha et al. 2010; De la Puente et al. 2012; Gupta et al. 2012a, b; Fedoruk et al. 2013; Kaur et al. 2014; Temel et al. 2015; Ates et al. 2016, 2018; Sudheesh et al. 2016a, b; Aldemir et al. 2017; Polanco et al. 2019) and identification of quantitative trait loci (QTL) controlling traits of interest such as resistance to ascochyta blight, anthracnose and fusarium wilt, tolerance to frost and winter hardiness and several other economically important traits (Ford et al. 1999; Rubeena et al. 2006; Tullu et al. 2008; Gupta et al. 2012a, b; Ates et al. 2016, 2018; Sudheesh et al. 2016a, b; Aldemir et al. 2017; Bhadauria et al. 2017; Polanco et al. 2019). To further accelerate the generation of a new cultivar, speed breeding integrated with SSD (Watson et al. 2017) has been employed in lentil, and an F_7 RIL population of cross *L. culinaris* × *L. ervoides* targeting aphanomyces root rot resistance has been developed in less than 300 days (Lulsdorf and Banniza 2018).

Mutation techniques have been tested in lentil as a complementary breeding strategy to introduce a desirable trait which is absent in the available germplasm (Muehlbauer et al. 2009). Some popular cultivars with different traits of interest have been developed and released worldwide using irradiation and ethyl methanesulfonate (EMS) as a source of mutagens. Majority of cultivars developed through mutation breeding registered in the Indian subcontinent have a variety of improved attributes such as high yield, resistance to rust and blight, tolerance to cold and early maturity (Laskar et al. 2019). Cultivars bred through mutation breeding outside the Indian subcontinent possessed some useful traits such as high yield, high protein

content, suitability to machine harvesting, resistance to fusarium wilt, blight, botrytis and anthracnose, tolerance to drought and herbicide resistance (Laskar et al. 2019). In Canada and Australia, EMS treatment was used to produce lentil mutants tolerant to imidazolinone herbicides (Slinkard et al. 2007; Mao et al. 2016). This trait is now integrated into all the lentil cultivars that are released in these countries, and some popular cultivars include CDC Impala, CDC Imperial, CDC Imigreen, CDC Peridot, etc. in Canada and PBA Herald XT and PBA Hurricane XT in Australia.

The upgraded cultivars produced using the above methodologies provided better stability, wide adaptation and extended yielding capacity as an outcome of the collective effect of genes transferred from close and distant germplasm. However, improvement in yields has been only marginal but not significant (Singh et al. 2013). This phenomenon in lentil as suggested before is largely attributed to the loss of valuable alleles for high productivity and low genetic variation within the cultivated species (Muench et al. 1991; Alveraz et al. 1997; Ford et al. 1997; Ferguson et al. 2000; Duran et al. 2004). To potentially recreate the genetic variability and maximize the lentil productivity, several attempts (discussed below) have been made to domesticate wild lentils that are known to house several desirable genes (Cohen et al. 1984; Ladizinsky et al. 1988; Muehlbauer et al. 1989; Vandenberg and Slinkard 1989; Fratini et al. 2004; Fratini and Ruiz 2006; Gupta and Sharma 2007; Fiala et al. 2009; Tullu et al. 2013; Kumari et al. 2018; Polanco et al. 2019).

The gene pool structure suggested by Wong et al. (2015) and as discussed previously in this chapter also reflects the crossability between cultivated and wild lentils, which varies with the percentage of chromosomal similarities between the species (Ladizinsky et al. 1988; Fratini et al. 2004; Gupta and Sharma 2007). Conventional crossing techniques have been used to produce hybrids between cultivated and wild lentils. Although sufficient success has been realized for the crosses between *L. culinaris* × *L. orientalis/L. odemensis* (Muehlbauer et al. 1989; Vandenberg and Slinkard 1989; Fratini et al. 2004; Gupta and Sharma 2007; Singh et al. 2013), the efforts were futile in obtaining fertile interspecific hybrids between *L. culinaris* and *L. ervoides* and *L. nigricans* species (Abbo and Ladizinsky 1991, 1994; Gupta and Sharma 2007). Pre- and postfertilization barriers owing to pollen-stigma incompatibility and embryo abortion, respectively, are identified as the key reasons for such unsuccessful hybridizations in lentil (Gupta and Sharma 2005).

Nonetheless, to overcome these pre- and postfertilization barriers and unlock the breeding potential of wild lentils, various methods termed as wide hybridization protocols have been developed (Cohen et al. 1984; Liu et al. 2005; Van de Wiel et al. 2010). A few of these methods including tissue culture techniques such as embryo and ovule rescue, exogenous use of growth hormones (gibberellic acid-GA_3) and micrografting have been tested in lentil to produce viable hybrid plants (Cohen et al. 1984; Ahmad et al. 1995; Gupta and Sharma 2005; Yuan et al. 2011; Saha and Muehlbauer 2014). Utilizing the success of the wide hybridization protocols, interspecific hybrids derived from crosses between *L. culinaris* and *L. orientalis*, *L. odemensis*, *L. ervoides* and *L. lamottei*, have been advanced to produce several RIL populations to detect lines with useful traits. Evaluation of these populations

revealed useful variations for various traits including agronomical traits (plant height, days to flowering and maturity), yield and yield-related traits (number of branches/plant, number of pods/plant, seed yield/plant, biological yield/plant and harvest index) and resistance to anthracnose, rust and ascochyta blight (Ye et al. 2000; Gupta and Sharma 2007; Fiala et al. 2009; Vail et al. 2012; Singh et al. 2013; Tullu et al. 2013; Kumari et al. 2018; Dadu et al. 2019; Polanco et al. 2019).

Although promising variations have been reported through wide crosses, cultivars developed with wild species in the pedigree are yet to be registered worldwide. One of the major reasons for avoidance of wild lentils in breeding programmes is the linkage drag effect, which may result in deriving unwanted/lethal genes along with desired genes (Singh et al. 2018). However, with the availability of high-throughput phenotyping and genotyping methods, utilization of wild lentils in breeding programmes is expected to increase circumventing the linkage drag effect (Wang et al. 2017).

3.5 Biotechnological Approaches

3.5.1 Tissue Culture for Targeted Trait Improvement in Lentil

The main purpose of employing tissue culture techniques in lentil was to reduce the genetic distance between wild and cultivated germplasm as described in Sect. 3.4. Upon the success achieved in wide crosses, the objective of the tissue culture in lentil has been upgraded to transfer desirable traits. This needed construction of large F_1 populations to produce large numbers of F_2 seeds useful for assessing the trait introgression. Through consistent efforts, several fertile plants from wide crosses have been produced using tissue culture methods with different explants (Table 3.4).

Cohen et al. (1984) produced viable hybrids from interspecific crosses between *L. culinaris* × *L. ervoides* and *L. Nigricans* using embryo rescue protocol. Following Cohen et al.'s (1984) embryo rescue protocols and some minor modifications, several other successful interspecific crosses for traits such as anthracnose resistance have been produced between cultivated lentil and wild lentil species (Fratini and Ruiz 2006; Fiala et al. 2009). Ovule rescue method was applied to obtain interspecific hybrids from crosses between *L. culinaris* and *L. Tomentosus* (Suvorova 2014). Although these methods helped to rescue embryos from aborting, difficulties existed in transforming the embryos into a viable plant. Gupta and Sharma (2005) were successful in rescuing ovules of interspecific crosses involving cultivated lentil and *L. ervoides* and *L. nigricans*, but the embryos did not differentiate to form root organ. To overcome this research gap, Yuan et al. (2011) proposed a fusion of embryo rescue and micrografting methods. They used shoot regenerations of six wild lentil species as scions and grafted them onto the rootstocks of faba bean which helped the lentil shoots to establish and develop into functional plants. The method

Table 3.4 Explants chosen for tissue culture experiments in lentils

Explant of choice	Reference
Cotyledonary tissue	Chhabra et al. (2008)
Cotyledonary node	Sevimay et al. (2005), Chhabra et al. (2008), Bermejo et al. (2012), Özdemir and Türker (2014)
Cotyledons with a small part of the embryo axis	Tavallaie et al. (2011)
Decapitated embryo	Omran et al. (2008), Bagheri et al. (2012), Das et al. (2012), Sarker et al. (2003b)
Ovule-embryo	Cohen et al. (1984), Ahmad et al. (1995), Gupta and Sharma (2005), Fratini and Ruiz (2006), Fiala et al. (2009), Galina (2014)
Hypo- and epicotyl-derived callus	Williams and McHugen (1986)
Embryonic axis	Saxena and King (1987)
Meristem tip	Bajaj and Dhanju (1979)
Shoot	Khentry et al. (2014)
Seed	Chopra et al. (2011a, b)

Adapted and modified from Laskar et al. (2019)

given by Yuan et al. (2011) showed a remarkable survival rate. Saha and Muehlbauer 2014 following a similar approach produced large numbers of F_2 seed from interspecific crosses between *L. culinaris* and *L. tomentosus*, *L. odemensis* and *L. lamottei.* Readers are further encouraged to read the detailed review by Gupta et al. (2018) on cytogenetic manipulations in lentil using tissue culture methods.

3.5.2 Embryo Rescue Assisted Breeding

The embryo rescue method has been used for shortening the breeding cycle in lentil to produce elite cultivars in short span of time. Ochatt et al. (2002) also described shortening of the life cycle for lentil based on in vitro culture of embryos. Bermejo et al. (2016) have studied an efficient in vitro assisted single seed descent technique where seeds were obtained in about 78–80 days for macrosperma and 107–110 days for microsperma genotypes. This method significantly reduced the days to flowering up to 13–15 days in macrosperma and 42–45 days in microsperma with a possibility to have four generations in a year.

Various researchers have attempted double haploid (DH) technology (Croser et al. 2006; Wędzony et al. 2008; Germanà 2011) which has the potential to simplify crop breeding through the production of haploid plants in a single generation. Legumes, being recalcitrant, have not seen much success through in vitro techniques; however efforts were made in lentil for DH production to obtain pure homozygous plants, though it couldn't produce plantlets (Keller and Ferrie 2002). Croser and Lulsdorf (2004) also reported the same difficulty with the regeneration of

embryos through in vitro techniques. Later abiotic stress pretreatments, such as centrifugation, electroporation and osmotic shock, were shown to have a positive effect on induction of androgenesis in a number of species including legumes (Hosp et al. 2007; Ribalta et al. 2012). Deswal (2018) stated that various compositions of hormones and different stress treatments also became effortless in lentil to get homozygous plants. Till today, none of the attempts for lentil DH protocols are successful.

Somatic embryogenesis, adventitious shoot production comprising de novo meristem formation (organogenesis) and axillary shoot production using pre-existing axillary buds and meristems are the techniques under micropropagation to provide large materials in less time (Ahmed et al. 2001). Cotyledonary nodes have been used to obtain multiple shoots (Mallick and Rashid 1989; Polanco and Ruiz 2001). Polanco and Ruiz 2001 achieved 5–20 shoots per immature seed of 4 lentil genotypes on media supplemented with BAP. They obtained a higher frequency of shoot regeneration from the cotyledonary node of wild lentil explants using thidiazuron (TDZ). In order to conserve wild resources of lentil, Sevimay et al. (2005) used the micropropagation technique to provide disease-free material for lentil improvement with TDZ in culture medium and cotyledonary node as an explant. Recalcitrant nature of lentil limits many in vitro approaches by affecting the root initiation process. To overcome this limitation, slight modifications in culture medium such as replacing IAA with chlorinated IAA or adjusting the concentration of NAA from 1 to 1.5 mgL^{-1} reported higher rooting efficiency in lentil (Polanco and Ruiz 2001; Ye et al. 2002; Saha et al. 2015). Sarker et al. (2012) approached a new way of in vitro flowering with cotyledonary nodes. They decapitated macrosperma cultivar embryos with one cotyledon attached as explants. While attempting gene transformation in lentil microsperma cultivars, Das et al. (2012) developed a protocol for and witnessed in vitro flowering and pod formation. Although there is still a huge gap to ascertain successful in vitro regeneration protocols in lentil, successful transfer of traits particularly resistance to diseases (anthracnose, ascochyta blight, stemphylium blight) from distant cultivars using tissue culture techniques has been achieved (Fiala et al. 2009; Tullu et al. 2013; Saha et al. 2015; Polanco et al. 2019; Dadu et al. 2019).

3.5.3 Transgenic Approaches for Targeted Trait Improvement

The methodological developments in lentil tissue culture made the elementary way for genetic transformation. Transgenic approaches have been evolved as a reassuring methodology to work with elite traits, which are not transferable through conventional breeding (Gardner 1993). *Agrobacterium*-mediated gene transfer has gained commercial importance when it succeeded in transferring insect resistance and herbicide tolerance traits. Horizontal gene transfer mediated by *Agrobacterium tumefaciens* has been limited in legumes being non-hosts. Different *Agrobacterium tumefaciens* strains such as *C58*, *Achh5*, *GV3111* and *A281* have been evaluated to ascertain lentil explant's susceptibility for the transformation process (Warkentin

and McHughen 1991). All the strains are capable of inducing tumours with high frequency, which highlights the possibility of horizontal gene transformation in lentil. Another strain – *A281* – has shown the capability to introduce heavy tumours on different explants of 21 lentil genotypes, where transgene GUS expression was low with such tumours (Khawar and Ozcan 2002). Successful herbicide resistance gene *acetolactate synthase (ALS)* transfer was achieved through vertical gene transformation via biolistic method using highly regenerable lentil cotyledonary node meristems. This led to successful transgene expression in putative transformants (Gulati et al. 2002). Transgenic lentil shoots were produced with an overall frequency of 1.01%. To develop an efficient, rapid, reproducible and genotype-neutral in vitro regeneration system for lentil, SAAT (sonication-assisted *Agrobacterium tumefaciens* transformation) method was used with a super virulent *Agrobacterium tumefaciens* strain *EHA105* to transfer T-DNA containing *nptII* and *uidA* genes into whole lentil seeds. Further transfected shoots could differentiate into roots and shoots on a medium with IBA and kanamycin (Chopra et al. 2011a, b). Many explants, including shoot and root apices, epicotyls, cotyledonary nodes, nodal segments and embryonic axes, were used as explants for *Agrobacterium*-mediated genetic transformation (Warkentin and McHughen 1991, 1992, 1993; Lurquin et al. 1998; Öktem et al. 1999; Mahmoudian et al. 2002; Akcay et al. 2009). Multiple explants were studied by Sarker et al. (2003a) for their regeneration ability followed by gene transformation through *Agrobacterium tumefaciens*. Histochemical staining experiments showed that epicotyl explants exhibited highest transgene expression followed by decapitated embryos, which were found to be more effective in the formation of multiple shoots and were thus suggested as suitable explants for lentil transformation.

Among abiotic stresses, drought and salinity are two important stresses; to improve tolerance to these stresses, *DREB1A* gene with *rd29A* promoter has been introduced into lentils via decapitated embryo with *Agrobacterium*-mediated transformation (Khatib et al. 2011). Expression analysis proved gene function in putative transformants by RT-PCR analysis. This was the first reported abiotic tolerance transformant in lentil. Hashem (2007) developed the first marker free transformation system in legumes and improved fungal resistance in lentil by transferring *Ripgip* gene through decapitated embryos with one cotyledon and achieved 35% of transformation efficiency; further rooting was achieved by micrografting. Though there are successful reports for rooting, still, its application is limited as the substantial successful protocols are not available; therefore, there is still an immense need to develop alternative stable protocols for root regeneration in lentil.

3.5.4 High-Throughput Sequencing for Targeted Trait Improvement in Lentil

Improvement of desired traits in plants is based on breeding and selection for individuals that harbour the genetic components that will consistently produce the expected crop qualities. Conventionally this process had been performed by selecting the best performing individuals in each generation and expecting that their performance would be indicative of their genetic potential or introducing new genetic material to gain the advantages of hybrid heterosis. However, these methods have relied on the plant's phenotype alone, often influenced by environmental effects and interactions with its genotype and were not indicative of its true genetic potential to inherit its performance to the next generation.

Current biotechnological approaches for breeding and selection for the trait improvement in lentil rely on comprehensive knowledge of the lentil genome and the genetic variations within different landraces and cultivars. Molecular markers, such as microsatellites or simple sequence repeats (SSRs) and single-nucleotide polymorphism (SNP), provide an accurate way to track down the presence and transfer of specific genetic alleles between individuals and generations. Traits that are strongly linked to these markers could be improved by selection for individuals that possess the high-performing alleles in these markers in an approach that was named marker-assisted selection (MAS) (Nadeem et al. 2018). For example, identification of quantitative trait loci (QTL) that are linked to desired traits requires high-density linkage maps developed from molecular markers, such as microsatellites or SSR and SNP, so they can be incorporated in marker-assisted selection programmes (Kumar et al. 2015; Nadeem et al. 2018). Furthermore, functional annotation of transcripts and expressed sequence tags (ESTs) to identify target genes that are involved in the molecular pathways governing the trait of interest depends on existing gene databases of lentil and other closely related legume and plant species.

During the last decade of the twenty-first century, the discovery of gene sequences and molecular markers for lentil and other crops were based on a low-throughput, labour-intensive and time-consuming workflow consisting of cloning sheared DNA fragments, followed by polymerase chain reaction (PCR) amplification and Sanger 'shotgun' sequencing (Sharpe et al. 2013). This resulted in a modest number of molecular markers available for QTL studies and genetic map construction in lentil, limiting the analysis resolution to wide genomic regions and posing a major difficulty to accurately identify and annotate the responsible genes in the loci of interest. For example, two consecutive studies aiming at developing new SSR markers for lentil to construct the genetic map and determine genetic variation in wild and cultivated lines resulted in just over 40 microsatellite markers (Hamwieh et al. 2005, 2009). Furthermore, the large size of the lentil genome, approximately 4 billion base pairs (Gb) long, made it impractical to fully sequence using the shotgun sequencing method that was available at the time (Kumar et al. 2015).

High-throughput sequencing (HTS), or next-generation sequencing (NGS) as it is often referred to, was introduced in the mid-2000s and revolutionized genomic

research by offering massively parallel sequencing of short nucleic acid molecules at high accuracy, affordable prices and within a short timeframe. Early HTS technologies included Solexa's (now Illumina) sequencing-by-synthesis, Roche pyrosequencing and Ion Torrent's Ion Proton which differed by their method of library preparation protocols, amplification substrate (silica flowcell in Solexa vs. emulsion of microbeads in the latter two), signal detection mode (fluorescence signal, light emission and pH change, respectively) and read lengths produced (Varshney et al. 2009). These technologies were rapidly adopted and applied to produce large volumes of sequence data from a wide range of model and non-model species, as well as commercially important crops, including legumes.

Once introduced, NGS was employed for whole-genome sequencing (WGS) of lentil cultivars in an effort to sequence, assemble and annotate the entire genome. These efforts led to the release of draft *L. culinaris* genomes, covering roughly two-thirds of its length (2.7–2.9 Gb out of the expected 4 Gb). These drafts enabled the discovery of thousands of SNPs to be used by MAS breeding programmes and annotation of genes and functional markers, but despite substantial resources invested in these sequencing projects, the full-length genome is yet to be assembled (Bett et al. 2014, 2016).

Though assembling the entire lentil genome proved extremely challenging with short-read NGS technologies, they were found to be well suited for RNA sequencing (RNA-seq), capable of capturing the entire transcriptome of multiple samples in a single run. Transcriptome sequencing in lentil tissues was then used to identify genes that are functionally associated with a trait of interest, such as resistance to fungal diseases (Khorramdelazad et al. 2018) and drought tolerance (Singh et al. 2017). RNA-seq was also utilized in lentil to identify SNP markers within the transcribed regions of the genome, focusing on variants that are more likely to be associated with phenotypic changes (Kaur et al. 2011; Temel et al. 2015).

Another strategy to utilize NGS-derived short reads to accurately identify thousands of SNPs throughout the genome is by using a combination of restriction enzymes to fragment the DNA and subsequently sequence just the DNA fragments that are flanked by the enzymes' cut sites. By doing so, it is possible to call SNP variants from a partial, reduced-complexity representation of the genome, enabling genotyping of multiple samples at a fraction of the cost of whole-genome sequencing (WGS). The methods, like genotyping-by-sequencing (GBS) and its variants, restriction site-associated DNA sequencing (RAD-seq) and diversity array technology sequencing (DArT-seq) have been applied recently as cost-effective methods to call SNP variants and assign genotypes in a large number of samples such as RIL segregating families (Elshire et al. 2011; He et al. 2014). The acquired SNPs are then applied to construct genetic maps and identify QTL for traits, such as iron content in seed (Aldemir et al. 2017), fungal disease resistance (Bhadauria et al. 2017) and root and shoot drought tolerance (Idrissi et al. 2016), and assess genetic diversity and population structure in a diverse collection of global genotypes (Pavan et al. 2019).

Despite the continuous development of NGS platforms, led by Illumina, and major improvements in the bioinformatics software used in the analysis of the

produced data, these short-read technologies proved limited in certain tasks, such as assembling genomes with large repetitive regions and transposable elements. To fill that need, new third-generation sequencing platforms were developed by Pacific Biosciences (PacBio) and Oxford Nanopore that perform single-molecule real-time sequencing and produce reads up to 1 Mbp long (van Dijk et al. 2018). The long reads produced by these technologies are currently more expensive to sequence and inferior in their base calling accuracy in comparison with Illumina's established short-read platforms, which makes them less suitable for SNP calling applications in large sample experiments. However, a hybrid approach, using PacBio or Oxford Nanopore long reads to allow better scaffolding across gaps and repetitive regions and high coverage of Illumina's proven cost-efficient accurate short reads to settle inaccuracies, is a promising strategy in genome assembly applications (Madoui et al. 2015; Jung et al. 2019).

The unprecedented wealth of sequences produced by NGS technologies introduced new challenges for data storage, annotation, access and sharing. Online databases, such as the American National Center for Biotechnology Information (NCBI), GenBank and Short Read Archive (SRA) collections, provide public access to annotated and raw sequences (Cochrane et al. 2016). In addition to species-specific genes and sequences, NCBI offers homology search tools, mainly within the BLAST suite, for annotation of unknown sequences and other comparative genomics applications, that are particularly useful in non-model crops such as lentil, which lack the genomic resources available for other well-studied model organisms (Camacho et al. 2009; Bhat et al. 2018).

The Cool Season Food Legume Crop Database (https://www.coolseasonfoodlegume.org/, Washington State University) is an online portal that provides comparative genomics and genetics tools for legumes such as chickpea, pea, lentil and faba bean, though it only includes the full genome of chickpea. KnowPulse (http://knowpulse.usask.ca/portal/), from the University of Saskatchewan Pulse Crop Research Group, currently hosts the only annotated draft genome of lentil, as well as established genetic maps and a large collection of SNP markers from known cultivars, though the access to these resources is restricted and requires preapproval (Sanderson et al. 2019). These resources are under continuous development to follow advancements in sequencing technologies and equip lentil researchers and breeders with tools and genomic resources required for molecular-based breeding and trait improvement.

3.5.5 Transcriptomics for Targeted Trait Improvement in Lentil

RNA sequencing has recently been applied to transcriptome profiling in order to enable profound insight into the genome functions that occur in response to different conditions simultaneously (Wang et al. 2009). Lentil possesses a very large

nuclear genome with non-coding and repetitive DNA components which contribute the majority of nuclear DNA content (Ford et al. 1999). Transcriptome profiling is a powerful and the most popular tool providing a cost-effective in-depth analysis of the transcribed portions of the lentil genome (Kaur et al. 2011). This method has been applied widely to investigate the genes responsible to improve lentil production including crop productivity and quality, defence to biotic stresses and tolerance to abiotic stresses. In January 2020, there are about 10,352 ESTs available for lentil (NCBI, 2020, https://www.ncbi.nlm.nih.gov/nuccore).

Second-generation transcriptome profiling of six lentil genotypes (Northfield, ILL2024, Indianhead, Digger, ILL6788 and ILL7537) using Roche 454 GS-FLX Titanium enabled large-scale unigene assembly and SSR marker discovery (Kaur et al. 2011). Of 3470 SNPs and EST-SSRs, a set of 2393 EST-SSR markers have been developed and validated in lentil (Kaur et al. 2011). In 2013, using 454 pyrosequencing technology, transcriptome sequencing of lentil could develop 3′-cDNA reads from 9 *L. culinaris* and 2 *L. ervoides* genotypes (Sharpe et al. 2013). A 1536 SNP Illumina GG array was developed and used to construct an SNP-based genetic map of *L. culinaris* (Sharpe et al. 2013). Illumina GAII technology and de novo transcriptome assemblies identified lentil SSR markers to utilize in diversity analysis (Verma et al. 2013). Further success has been achieved to identify 50,960 putative SNP markers with transcriptome profiling of 2 lentil cultivars (Precoz and WA8649041) and 101 F_7 RILs (Temel et al. 2015). These SNP markers were successfully utilized to generate an SNP-based linkage map using Illumina CASAVA (Temel et al. 2015). The following year, seven RNA-seq libraries were generated and sequenced from a variety of tissue types of lentil cultivar *Cassab* (Sudheesh et al. 2016a, b). A unigene set comprising 58,986 contigs and scaffolds was developed for further genomic exploration (Sudheesh et al. 2016a, b). Several transcriptomic studies were conducted for targeted trait improvement in lentil such as crop quality, defence response and stress tolerance, and the results added more details to the genomic resources of lentil.

Lentil sensitivity to the water scarcity and drought period could highly affect lentil growth and productivity (Morgil et al. 2019). Thus, to address this issue of lentil drought tolerance at the seedling stage, Illumina HiSeq 2500 platform for transcriptome profiling of drought-tolerant (PDL2) and drought-sensitive (JL3) lentil cultivars was performed among different physiological and biochemical parameters (Singh et al. 2017). A recent study focused on a drought-sensitive lentil cultivar (Sultan from Turkey) and specifically on its root, leaf and stem under short- and long-term water deficits, utilizing Illumina HiSeq 4000 sequencing platform with de novo RNA-seq-based transcriptome analysis (Morgil et al. 2019). Results led to the detection of the root as the most sensitive plant organ to the period of drought stress in lentil as transcriptional changes during a long-term drought stress have been over six times more than short-term stress in the root system (18,327 compared to 2915 differentially expressed genes (DEGs), respectively (Morgil et al. 2019)). Moreover, gene ontology analysis depicted the differences in transcriptional regulation of biological processes such as protein phosphorylation, embryo development, seed dormancy, DNA replication and maintenance of root meristem as a response to

long-term drought stress (Morgil et al. 2019). Heat-responsive genes and their role in regulatory mechanisms of lentil through genome-wide transcriptomic study on PDL2 (tolerant) and JL3 (sensitive) mutants revealed many high-quality SNPs, microsatellites and insertion-deletions (indels) (194,178, 141,050 and 7388) (Singh et al. 2019). Furthermore, DEGs analysis revealed that PDL-2 has higher membrane stability index (MSI) and pollen germination under heat shock compared to JL3 and that the cell wall and secondary metabolite pathways are mostly exposed to heat stress effects (Singh et al. 2019).

Transcriptomic studies also helped with understanding of disease defence mechanisms in lentil. Transcriptional changes during early stages of *A. lentis* infection causing devastating ascochyta blight disease were comprehensively profiled in a resistant (ILL 7537) and a susceptible (ILL 6002) lentil genotype using Ion Proton sequencing system along with de novo RNA-seq-based transcriptome analysis (Khorramdelazad et al. 2018). Differential expression analysis helped with identification of 24 genes involved in the 3 main defence-response stages within the first 24 hours of pathogen attack (Khorramdelazad et al. 2018). Some detected protein kinases are involved in pathogen recognition and defence signalling pathways. Also, PR2, PR4 and PR10 from pathogenesis-related (PR) protein families have been characterized with a role in biochemical defence against the pathogen, and many structural and hypersensitive-response (HR) related genes which play a part in systemic acquired resistance (SAR) and cell death were detected (Khorramdelazad et al. 2018).

Application of high-throughput sequencing approaches facilitates generation and characterization of reference transcriptome datasets that leads to gene-based marker discovery, which in turn can be useful in genetic map construction among other purposes.

3.5.6 Linkage Mapping and QTLs for Targeted Trait Improvement in Lentil

Prior to the determination of the chromosomal location of a desirable gene, it is pivotal to develop linkage map that may be thought of as a ‘road map’ of the chromosomes (Collard et al. 2005). Identification of quantitative trait loci for important agronomic traits has been made possible in several plant species with the availability of polymorphic markers and linkage maps (Verma et al. 2015). In the recent past years, molecular markers have helped to know the gene networks underlying the quantitatively inherited traits and linked to genomic regions (QTLs/genes) controlling such traits have been identified in several crops including lentil (Kumar et al. 2017).

In lentil, the availability of molecular markers associated with agronomically important traits is limiting the use of the biotechnological tool in breeding programmes. However, the use of molecular markers has been accelerated due to the

enrichment of genomic resources in the recent years (Kumar et al. 2015), and molecular markers including SNP, SSR, inter-simple sequence repeat (ISSR) and direct amplification of minisatellite DNA (DAMD) have been developed (Hamwieh et al. 2005, 2009; Kaur et al. 2011; Temel et al. 2015; Verma et al. 2015; Khazaei et al. 2016).

3.5.6.1 Linkage Mapping from Single Mapping Populations

Linkage mapping based on single mapping populations was mainly constructed based on F_2 populations. Havey and Muehlbauer (1989) developed the first DNA-based marker genomic map of lentil from 20 restriction fragment length polymorphisms (RFLP), 8 isozymes and 6 morphological markers segregating in a single interspecific cross (*L. culinaris* × *L. orientalis*). Later, several interspecific crosses were utilized to create linkage maps in lentil (Weeden et al. 1992; Tahir et al. 1993; Vaillancourt and Slinkard 1993; Tahir and Muehlbauer 1994). Linkage mapping across the *Lens* genome became very popular with the introduction of PCR-based markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and RFLP (Kumar et al. 2012b, 2014, 2015). Subsequently, the first extensive linkage map of lentil was constructed in the late 1990s from RAPD, AFLP, RFLP and morphological markers using $F_{6:8}$ of RIL population from *L. culinaris* and *L. orientalis* (Eujayl et al. 1998). The map covered 1073 cM of the lentil genome with an average distance of 6.0 cM between adjacent markers.

Intraspecific mapping populations found to be more informative for QTL identification and to tag desirable genes than interspecific mapping populations (Kumar et al. 2015). Thus, in the early 2000s, the first intraspecific lentil map was developed using 100 RAPD, 11 ISSR and 3 resistance gene analogue (RGA) markers using F_2 population of lentil cultivars ILL5588 and ILL7537 with different resistance backgrounds against *A. lentis* causing ascochyta blight (Rubeena et al. 2003). The resulted intraspecific map spanned a total length of 784.1 cM comprising nine linkage groups. Another intraspecific linkage map, comprising 38 RAPD, 30 AFLP, 3 ISSR and 1 morphological marker, was constructed using a F_2 population from a cross between ILL6002 (ascochyta blight-susceptible) and ILL7537 (ascochyta blight-resistant) cultivars (Rubeena et al. 2006).

The first lentil map with short sequence repeat (SSR) markers was developed based on the segregation analysis of 5 different types of molecular and morphological genetic markers in 113 F_2 plants obtained from a cross of *L. culinaris* and *L. orientalis* (Duran et al. 2004). This map contained a total of 200 markers including 71 RAPDs, 39 ISSRs, 83 AFLPs, 2 SSRs and 5 morphological loci. Markers (161) were grouped into ten linkage groups covering 2172.4 cM of the genome, with an average distance between markers of 15.87 cM at a LOD score of 3.0. Phan et al. (2007) constructed another linkage map (928.4 cM) containing 18 SSR markers and 79 intron-targeted amplified polymorphic (ITAP) gene-based markers, using a F_5 RIL population from a cross between ILL5588 and ILL5722. This map was

constructed to develop a gene-based genetic map of lentil and to characterize syntenic relationships with *Medicago truncatula* as well as to integrate the resulting genic and comparative map with the other comprehensive genetic map of lentil. This map contained seven linkage groups comprised of 5–25 markers that varied in length from 80.2 to 274.6 cM. Gupta et al. (2012b) constructed a genetic linkage map using 114 F_2s derived from the interspecific cross of *L. culinaris* and *L. orientalis*. F_2 population used for this linkage map exhibited sufficient polymorphism for DNA markers, including variation for rust resistance and other agro-morphological traits.

Abiotic obstacles like water deficit and boron toxicity could also affect lentil growth and productivity to a large extent (Idrissi et al. 2016; Rodda et al. 2018). Kaur et al. (2014) performed large-scale SNP discovery and dense genetic mapping in a lentil intraspecific cross and identified a single chromosomal region controlling tolerance to boron toxicity. Another intraspecific linkage map constructed using a RIL population derived from a cross of Precoz × WA8649041 identified QTLs for flowering time in lentil (Kahriman et al. 2015). In order to detect the QTLs conferring drought tolerance in lentil, a total of 252 codominant and dominant markers were used to create a genome map from a population of 132 RILs developed from a cross between two contrasting parents, ILL6002 (drought tolerant) and ILL5888 (drought sensitive) (Idrissi et al. 2016). Markers were mapped on 9 linkage groups, and 18 QTLs regulating a total of 14 root and shoot traits were identified. A population of F_6 178 RILs (boron-tolerant line ILL2024 × susceptible line ILL6788) was studied to characterize genomic sources of tolerance to elevated soil boron toxicity in lentil (Rodda et al. 2018). A high-quality genetic linkage map was established with 758 markers that cover 1057 cM of lentil genome, and a single boron tolerance genomic region was identified which accounted for up to 76% of phenotypic variation. Another intraspecific linkage map was constructed containing 12 LGs with a total length of 1868 cM and identified genome regions associated with earliness and plant height using RILs derived from a cross between Eston × PI 320937 (Tullu et al. 2008).

Polanco et al. (2019) constructed a high-density interspecific (*L. culinaris* × *L. odemensis*) genetic map based on functional markers for mapping morphological and agronomical traits and resistance to ascochyta blight in lentil. SNPs and short indels were used to construct this map from a F_7 RIL population derived from the interspecific cross between *L. culinaris* and *L. odemensis*. The genome regions corresponding to a QTL governing time of flowering (chromosome 6), three QTLs controlling seed size (chromosomes 1 and 5) and three QTLs for *Ascochyta blight* resistance (chromosome 6) were identified.

3.5.6.2 Linkage Mapping from Multiple Mapping Populations (Consensus Maps)

While the conventional genetic linkage maps were created from a single mapping population, 'consensus maps' were created from multiple mapping populations. Consensus map offers various advantages including (a) higher marker density in single map and better genome coverage, (b) detection of the position of common markers across different mapping populations, (c) better assignment of linkage groups to chromosomes, (d) detection of conserved marker locus position, (e) identification of chromosomal rearrangements and degree of gene duplication, (f) comparison of genes of interest or QTLs across the maps and (g) creation of a basis for comparing genomes between related species (Ford et al. 2007).

Hamwieh et al. (2005) reported a comprehensive *Lens* map covering 715 cM, comprising 283 genetic markers by reconstructing the linkage map created by Eujayl et al. (1998). This map was based on microsatellite and AFLP markers. A total of 41 microsatellite and 45 AFLP markers were mapped using 86 RILs of ILL5588 × L692-16-1(s) cross. The map contained 283 markers spanning over 751 cM, with an average marker distance of 2.6 cM. Furthermore, resistance to the fungal disease, fusarium vascular wilt, was localized on the linkage group. Rubeena et al. (2006) constructed a consensus map by anchoring seven linkage groups with those of a previously constructed map (Rubeena et al. 2003) for tagging ascochyta blight resistance from two F_2 populations, viz. ILL5588 × ILL7537 and ILL7537 × ILL6002. This study demonstrated the transferability of QTLs among populations as markers were closely linked to the major QTL with a potential to future marker-assisted selection for disease resistance. Phan et al. (2006) used 126 cross-species markers from *Medicago truncatula* to generate comparative genetic maps of lentil and white lupin. Eventually, they used 18 common SSR markers to connect the new map with another already constructed comprehensive map in lentil by Hamwieh et al. (2005). They compared ESTs from the phylogenetically distant species, *M. truncatula*, *Lupinus albus* and *Glycine max*, and produced 500 ITAPs. The study reported 90%, 80% and 70% of the ITAP markers amplified genomic DNA in *M. truncatula*, *L. albus* and *L. culinaris*, respectively. The comparative map of *L. culinaris* was constructed based on 79 ITAP markers. The *L. albus* comparative map was developed from 105 gene-based markers together with 223 AFLP markers. Moderate chromosomal rearrangement was observed between *M. truncatula* and *L. culinaris* genomes, although a direct and simple syntenic relationship existed between the genomes. A population of 94 RILs at F_5 generation from a cross between ILL5588 × ILL5722 was used to construct a linkage map. The map clustered into 11 linkage groups covering 1156.4 cM of the genome, and 3 QTL regions were detected separately for each seedling and pod resistance that mapped to LG1 and LG9 and LG1, LG4 and LG5 linkage groups, respectively (Gupta et al. 2012a).

In another study on *L. ervoides* defence to few fungal pathogens, a population of 94 RILs at F_9 generation of a cross between two *L. ervoides* genotypes was used (Bhadauria et al. 2017). This high-density genetic linkage map developed from the comparative mapping between the genetic map of *L. ervoides* with *L. culinaris*

spanned 740.94 cM, and composite interval mapping detected five, six and three QTLs on chromosomes 1 and 5 controlling resistance to *Colletotrichum lentis* race 0, *C. lentis* race 1 and *Stemphylium botryosum*, respectively.

Recently, Ates et al. (2018) employed diversity array technology (DArT) markers to construct a consensus linkage map of lentil using three different lentil RIL populations (CDC Redberry × ILL7502, ILL8006 × CDC Milestone and PI320937 × Eston). The map had 9793 markers, covering a total of 977.47 cM distance with an average distance of 0.10 cM between adjacent markers and contained 7 linkage groups representing 7 chromosomes of the lentil genome. The mentioned studies are examples of genomic research within the last two decades. The available genomic datasets for lentil provide a powerful tool for crop improvement in lentil. Partial *L. culinaris* reference genome (v1.2, KnowPulse, Bett and Cook 2006) is available, and the whole reference genome of lentil can speed up and facilitate the genomic studies in this crop.

Until now, classical plant breeding approaches utilizing selection-recombination and selection cycles have contributed successfully to improve lentil crops. However, these approaches are inaccurate and time-consuming, particularly for improving complex quantitative traits. As we understand, the recent developments in molecular marker technologies have made it possible to localize genomic regions and assess their phenotypic effects on various quantitative traits. In lentil, several agronomic traits such as plant height, days to flowering, winter hardiness, pod dehiscence, growth habit and yield have been genetically dissected using both inter- and intraspecific populations (Taran et al. 2003; Kahraman et al. 2004; Fratini et al. 2007; Tullu et al. 2008). Similarly, QTLs for resistance to diseases like ascochyta blight, rust, anthracnose and stemphylium blight have also been mapped (Ford et al. 1999; Rubeena et al. 2006; Tullu et al. 2006; Gupta et al. 2012a, b; Sudheesh et al. 2016a, b; Bhadauria et al. 2017). Seed weight (Verma et al. 2015) and seed-related morphological and quality traits have been genetically mapped in lentil (Fratini et al. 2007; Fedoruk et al. 2013; Saha et al. 2013; Khazaei et al. 2017, 2018). Molecular markers linked to the QTLs identified in lentil are presented in Table 3.5, and these markers can be aid in targeted trait selection and improvement.

As explained earlier, quantitative traits have been mapped in lentil for the purpose of associating molecular markers with phenotypic traits. However, very few molecular markers are used in lentil breeding because many of the molecular markers are not reproducible in multiple populations (Ford et al. 2009). QTLs affecting earliness and plant height were identified on LG1, LG2, LG4, LG5, LG9 and LG12 at Saskatoon and Floral evaluation locations (Tullu et al. 2008). For days to flowering, a QTL (DTF1-d) explained phenotypic variation of 56.9%. Plant height QTLs explained a gross phenotypic variation of 95%, and a compact genomic region consisting of six QTLs for plant height and early flowering was detected within a map distance of 17.10 cM (Pote 2013). Similarly, Saha et al. (2013) identified map positions of some important agro-morphological traits including days to 50% flowering, plant height, seed diameter, 100 seed weight, cotyledon colour and growth habit in lentil. Three major QTLs governing seed diameter were mapped in lentil by applying random amplified polymorphic DNA markers by Fratini et al. (2007). Fedoruk

Table 3.5 Marker-trait association studies conducted in lentil

Trait	Marker(s) linked with associated QTL(s)	Phenotypic variation reaction explained by the QTL (%)	Reference
Ascochyta blight resistance	RAPD	90	Ford et al. (1999)
	RAPD, AFLP, ISSR	Up to 50	Rubeena et al. (2006)
	RFLP, AFLP	41	Tullu et al. (2006)
	ITAP, SSR, ISSR	Up to 61	Gupta et al. (2012b)
Earliness	RAPD, AFLP, SSR	37–46	Tullu et al. (2008)
Plant height	RAPD, AFLP, SSR	31–40	Tullu et al. (2008)
	RAPD, ISSR, AFLP, SSR, morphological markers	38.2	Fratini et al. (2007)
Branches at the first node	RAPD, ISSR, AFLP, SSR, morphological markers	91.7	Fratini et al. (2007)
Total number of branches	RAPD, ISSR, AFLP, SSR, morphological markers	54	Fratini et al. (2007)
Height at the first node	RAPD, ISSR, AFLP, SSR, morphological markers	33.3	Fratini et al. (2007)
Flowering time	RAPD, ISSR, AFLP, SSR, morphological markers	90.4	Fratini et al. (2007)
Pod dehiscence	RAPD, ISSR, AFLP, SSR, morphological markers	81.3	Fratini et al. (2007)
Seed weight	RAPD, ISSR, AFLP, SSR, morphological markers	18.2	Fratini et al. (2007)
Seed diameter	RAPD, ISSR, AFLP, SSR, morphological markers	37	Fratini et al. (2007)
Winter hardiness	RAPD, ISSR, RFLP	20.45	Kahraman et al. (2010)
Cotyledon colour class (cy)	SNP, SSR, colour loci	23	Fedoruk et al. (2013)
Seed thickness	SNP, SSR, colour loci	8.4	Fedoruk et al. (2013)
Seed diameter	SNP, SSR, colour loci	Up to 60	Fedoruk et al. (2013)
Seed plumpness	SNP, SSR, colour loci	Up to 50	Fedoruk et al. (2013)
Days to 50% flowering	SNP, SSR, colour loci	Up to 34	Saha et al. (2013)
Hundred seed weight	SSR, SRAP, RAPD	17.5	Saha et al. (2013)

(continued)

Table 3.5 (continued)

Trait	Marker(s) linked with associated QTL(s)	Phenotypic variation reaction explained by the QTL (%)	Reference
Plant height	SSR, SRAP, RAPD	15.3	Saha et al. (2013)
Seed diameter	SSR, SRAP, RAPD	32.6	Saha et al. (2013)
Stemphylium blight resistance	SSR, SRAP, RAPD	46	Saha et al. (2010)
Boron tolerance	SNP	71	Kaur et al. (2014)
Flowering time	SSR	57	Kahriman et al. (2015)
Seed weight and size	SNP	27.5–48.4	Verma et al. (2015)
Selenium content	SNP	6.3–16.9	Ates et al. (2016)
Drought tolerance	SSR	69.7	Singh et al. (2016)
Root and shoot traits	SNP, SRAP	27.6–28.9	Idrissi et al. (2016)
Days to 50% flowering	SSR	58–97	Kumar et al. (2018)
Plant height	SSR	24.0–47.0	Kumar et al. (2018)
100 seed weight	SSR	1.6–5.5	Kumar et al. (2018)
Days to maturity	SSR	99–133	Kumar et al. (2018)
Seed coat spotting	SNP, short indels	85.07	Polanco et al. (2019)
Stem pigmentation	SNP, short indels	33.96	Polanco et al. (2019)
Seed size	SNP, short indels	28.26	Polanco et al. (2019)
Flower colour	SNP, short indels	84.20	Polanco et al. (2019)
Flowering time	SNP, short indels	55.73	Polanco et al. (2019)
Ascochyta blight resistance	SNP, short indels	27.14	Polanco et al. (2019)

Adapted and modified from Kumar et al. (2015) and Kumar et al. (2018)

et al. (2013) observed that loci 16 for seed coat colour and pattern mapped to linkage groups 2 (Ggc), 3 (Tgc) and 6 (Scp) while the cotyledon colour locus (Yc) mapped to linkage group 1 in lentil. Verma et al. (2015) identified QTLs for the seed weight and size traits by single marker analysis (SMA) followed by composite

interval mapping (CIM) which resulted in one QTL each for the two traits (qSW and qSS) that were co-localized on LG4 and explained 48.4% and 27.5% of the phenotypic variance, respectively. Multiple QTLs for lentil seed diameter, thickness and plumpness were mapped in lentil via single-nucleotide polymorphism (SNP) markers (Fedoruk et al. 2013). The most stable and significant QTLs for seed diameter and plumpness were detected near the cotyledon colour locus (Yc), which explained 60% and 50% of the phenotypic variation for these traits, respectively, in that population (Fedoruk et al. 2013). Recently, these genomic regions were validated with a cultivated lentil association mapping panel (Khazaei et al. 2018).

The created genomic tools with mapping technology are the keys to MAS breeding strategies resulting in crop productivity and quality improvement for any crop. Despite the huge success in identifying QTLs controlling a wide variety of traits in lentil and the identification of the functional variants underlying these QTLs, the success of marker-assisted selection (MAS) for major genes in large public breeding programmes requires more efforts following examples from other major crops.

3.6 Conclusion

Towards improvement in lentil breeding programme, it is necessary to breed for multiple traits including seed yield and quality as well as resistance to abiotic and biotic stresses in order to develop more durable cultivars with superior grain quality to meet market demands under challenging environmental conditions. In the last two decades, significant efforts have been made to understand the genetics and genomics of lentil from wild and cultivated sources. Genomics-assisted breeding is relatively a powerful and fast approach to develop high-yielding cultivars adapted to different environmental conditions. Recent developments in the molecular tools including marker-assisted -selection, backcrossing, gene pyramiding and recurrent selection, and genome-wide selection have the potential for accelerated improvement in the effectiveness of breeding strategies.

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