Euphytica (2006) 147: 81–103
DOI: 10.1007/s10681-006-4261-4 © Springer 2006

Chickpea molecular breeding: New tools and concepts

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Received 15 December 2004; accepted 18 October 2005

Key words: chickpea breeding, functional genomics, genetic map, pathogen resistance, stress tolerance

Summary

Chickpea is a cool season grain legume of exceptionally high nutritive value and most versatile food use. It is mostly grown under rain fed conditions in arid and semi-arid areas around the world. Despite growing demand and high yield potential, chickpea yield is unstable and productivity is stagnant at unacceptably low levels. Major yield increases could be achieved by development and use of cultivars that resist/tolerate abiotic and biotic stresses. In recent years the wide use of early maturing cultivars that escape drought stress led to significant increases in chickpea productivity. In the Mediterranean region, yield could be increased by shifting the sowing date from spring to winter. However, this is hampered by the sensitivity of the crop to low temperatures and the fungal pathogen *Ascochyta rabiei*. Drought, pod borer (*Helicoverpa* spp.) and the fungus *Fusarium oxysporum* additionally reduce harvests there and in other parts of the world. Tolerance to rising salinity will be a future advantage in many regions. Therefore, chickpea breeding focuses on increasing yield by pyramiding genes for resistance/tolerance to the fungi, to pod borer, salinity, cold and drought into elite germplasm. Progress in breeding necessitates a better understanding of the genetics underlying these traits. Marker-assisted selection (MAS) would allow a better targeting of the desired genes. Genetic mapping in chickpea, for a long time hampered by the little variability in chickpea's genome, is today facilitated by highly polymorphic, co-dominant microsatellite-based markers. Their application for the genetic mapping of traits led to inter-laboratory comparable maps. This paper reviews the current situation of chickpea genome mapping, tagging of genes for ascochyta blight, fusarium wilt resistance and other traits, and requirements for MAS. Conventional breeding strategies to tolerate/avoid drought and chilling effects at flowering time, essential for changing from spring to winter sowing, are described. Recent approaches and future prospects for functional genomics of chickpea are discussed.

Chickpea: A valuable grain legume

Chickpea (*Cicer arietinum* L.) is the only cultivated species within the genus *Cicer*. The crop is a self-pollinated diploid ($2n = 2x = 16$) with a relatively small genome (740 Mb, Arumuganathan & Earle, 1991). It is cultivated in arid and semi-arid areas around the world. With over 10 million ha under cultivation, chickpea is second only to common bean (*Phaseolus vulgaris*) and third in production among the legumes. The major producers India, Pakistan and Turkey contribute 65%, 9.5% and 6.7% respectively,

to the world harvest (FAOSTAT, 2005). Despite its high morphological variability, genetic variation is low, probably a consequence of its monophyletic descendence from its wild progenitor *C. reticulatum* in the Fertile Crescent (Ladizinsky & Adler, 1976; Lev-Yadun et al., 2000; Abbo et al., 2003). Chickpea seeds contain 20–30% protein, approximately 40% carbohydrates, and only 3–6% oil (Gil et al., 1996); and moreover, they are a good source of calcium, magnesium, potassium, phosphorus, iron, zinc and manganese (Ibrikci et al., 2003). Chickpeas do not contain as high amounts of isoflavones as soybeans do (USDA-ARS, 2004), but provide more beneficial carotenoids such as β-carotene than genetically engineered "Golden Rice". Compared to other grain legumes, anti-nutritive components are nearly absent (Williams & Singh, 1987). Thus, chickpea is considered a functional food or nutraceutical (Agharkar, 1991; McIntosh & Topping, 2000; Charles et al., 2002). While it is a cheap source of protein and energy in the developing world, it is also an important food to the affluent populations to alleviate major food-related health problems. However, more research is necessary to elucidate and extend the food and nutraceutical benefit of this important food legume through breeding.

Commercially, the species is grouped into desi and kabuli types: desi chickpeas generally have small, coloured seeds, whereas kabulis produce large, cream-coloured ones. To a certain extent this classification overlaps with the macrosperma and microsperma races proposed by Moreno and Cubero (1978). Classification also reflects utilisation: whereas kabulis are usually utilised as whole grains, desis are decorticated and processed into flour. Differing also in other agronomic characteristics, kabuli chickpeas probably evolved in the Mediterranean basin from desi type (Moreno & Cubero, 1978; Gil & Cubero, 1993; Jana & Singh, 1993). Kabuli × desi crosses are used in many breeding programs to combine genes for cold tolerance, resistance to ascochyta blight and long vegetative growth more frequently found in kabuli types, with genes for heat and drought tolerance, resistance to fusarium wilt and early flowering prevalent in desi types (Singh, 1987).

Currently, productivity of chickpea is very low (world average ~0.8 t/ha, FAOSTAT, 2005) and has stagnated in recent years. Reasons for only marginal improvements are a series of biotic and abiotic stresses that reduce yield and yield stability. Especially ascochyta blight and fusarium wilt, pod borer, drought and cold are major constraints to yield improvement and adoption of the crop by farmers. Therefore, improving resistance to biotic and tolerance to abiotic stresses as well as a general increase in dry matter are major aims of chickpea breeders around the world. Recent years have seen tremendous progress in the development of novel genetic tools such as DNA molecular markers, dense genetic maps, and whole-genome transcription profiling techniques to identify genomic regions and genes underlying plant stress responses. Although fully applied only to a few model species, these new technologies are dramatically improving our understanding of basic principles of plant metabolism in general. Now, it is up to researchers and breeders to capitalise on these new opportunities to improve and stabilise chickpea yield for the benefit of farmers and consumers. The aim of this review is to provide an update on progress in the development and application of molecular breeding approaches to the improvement of chickpea's resistance to biotic and abiotic stresses.

Genome mapping in chickpea: Merging maps is a major prerequisite for molecular breeding

Knowledge of the inheritance of agronomic characters is a basic requirement to identify and integrate interesting genes in linkage maps and to utilise these maps for marker-assisted selection (MAS) of these characters to accelerate the development of new cultivars. In chickpea, genetics of resistance to ascochyta blight (Singh & Reddy, 1983; Tewari & Pandey, 1986; Dey & Singh, 1993; Tekeoglu et al., 2000a), fusarium wilt (Muehlbauer & Singh, 1987; Gumber et al., 1995; Kumar, 1998; Tullu et al., 1998; Tekeoglu et al., 2000b; Rubio et al., 2003), chilling tolerance at flowering (Clarke & Siddique, 2003), and flowering time (Or et al., 1999) have been extensively analysed. A comprehensive overview of previous genetic mapping efforts in chickpea covering the period until 2001 is available (Winter et al., 2003) and thus, we will focus only on new developments here. Most chickpea geneticists agree that the generation of an integrated genetic map of the crop, comprising loci of both economic and scientific importance, presently is a central goal of chickpea genetics. Until recently, the low level of polymorphism in the chickpea genome and the scarcity of co-dominant DNA-based markers were serious constraints to achieving this goal. The advent of sequence-tagged microsatellite sites (STMS) markers (Hüttel et al., 1999; Winter et al., 1999), however, provided the opportunity to integrate the different available maps.

Here, we summarise some of the progress achieved using these markers. In recent years, STMS markers were indeed applied for the generation of almost all published genetic maps of chickpea developed employing populations from crosses between *C. arietinum* and *C. reticulatum* (Tekeoglu et al., 2002; Benko-Iseppon et al., 2003; Rakshit et al., 2003; Pfaff & Kahl, 2003; Abbo et al., 2005), *C. arietinum* × *C. echinospermum* (Collard et al., 2003) and intraspecific populations (Cho et al., 2002; Flandez-Galvez et al., 2003a, 2003b; Udupa & Baum, 2003; Cho et al., 2004; Sharma et al., 2004; Cobos et al., 2005). Nevertheless, most genomic regions harbouring genes for important traits are not yet sufficiently saturated with co-dominant markers to apply MAS in plant breeding programs.

Genetic mapping mostly focussed on tagging agronomically relevant genes such as ascochyta (Tekeoglu et al., 2002; Udupa & Baum, 2003; Collard et al., 2003; Flandez-Galvez et al., 2003a; Cho et al., 2004) and fusarium resistance genes (Benko-Iseppon et al., 2003; Sharma et al., 2004), and yield-influencing characters such as double podding and other morphological characters (Cho et al., 2002; Rajesh et al., 2002). Most of the authors compared their maps to the most extended genetic map of chickpea (Winter et al., 2000). Though this map, which currently comprises more than 470 markers, was based on an interspecific cross between the cultigen and a *C. reticulatum* accession, linkages of STMS markers is consistent with most other maps, irrespective of whether they were generated for an inter- or intraspecific population. However, distances between the markers differed considerably in some cases (Tekeoglu et al., 2002; Collard et al., 2003; Cho et al., 2004; Cobos et al., 2005). Contrary to the results obtained with STMS markers, Collard et al. (2003) could not detect similarities between the order of RAPD and ISSR markers in their map as compared to previous studies. The emerging body of data now allows us to draw three conclusions: (i) STMS markers are indeed elite anchor markers for merging genetic maps in chickpea, (ii) dominant markers are transferable between populations only in rare cases, and their identity needs to be confirmed by either linkages to other markers co-segregating in at least two populations, or sequencing and conversion into e.g. a sequence characterised amplified region (SCAR) marker, (iii) the map of Winter et al. (2000) together with its amendments developed on the same population may be employed as a reference map for genetic mapping in chickpea and comparative mapping between chickpea and other legumes, at least until a comprehensive integrated map becomes available. A current map integrating much of the information available in the literature is presented in Figure 1.

The step from genetic to physical mapping

One of the logical spin-offs of a genetic map, the construction of a complete physical map of a genome, still represents a challenge for chickpea genomics. However, a physical map is fundamental to any progress towards a more complete understanding of the structure, composition and function of the genome. This cannot be achieved by mere recombination mapping. More so, the isolation of genes of agronomic importance (e.g. genes encoding receptor kinases, proteins of signal transmission, transcription factors, regulatory proteins or small regulatory RNAs, or enzymes of defence pathways) inevitably necessitates a physical map. In essence, the era of physical mapping in chickpea is beginning now. It will, and has to be succeeded by an era of DNA sequence analysis. And the first steps towards this goal have already been made: at least four bacterial artificial chromosome (BAC) libraries are available, but definitely under-used, and a cytogenetic map of the chickpea chromosomes is close to completion.

One of the BAC libraries has been described in detail (Rajesh et al., 2004). A second one, derived from the fusarium-resistant chickpea cultivar (ICC 4958) was established in the binary vector V41 with a 5× coverage of the genome. The library has been spotted onto high-density nylon filters (close to 14,000 clones/filter) and used for hybridisation experiments. These experiments clearly proved, that some markers, that were located on the integrated genetic map (Winter et al., 2000; Benko-Iseppon et al., 2003), and later on sequenced, are either low-copy (e.g. the thaumatin [PRP5]-encoding gene), middle-repetitive (e.g. the gene encoding *N*-hydroxycinnamoyl-benzoyl transferase, a protein catalysing a particular step in the phytoalexin synthesis pathway), or highly repetitive (marker CS 27, a *Ty*3-gypsy-like LTR retrotransposable element CaRep; Staginnus et al., 1999, 2001). Also, a series of 141 resistance gene analogues (RGAs) have been identified in this BAC library. Clustering of the various R-genes was neither observed in the BACs nor suggested by genetic mapping of RGAs (Hüttel et al., 2002). Recently, Lichtenzveig et al. (2005) also constructed a BAC and a BIBAC library for chickpea; the two libraries contain a total of 38,016 clones and are

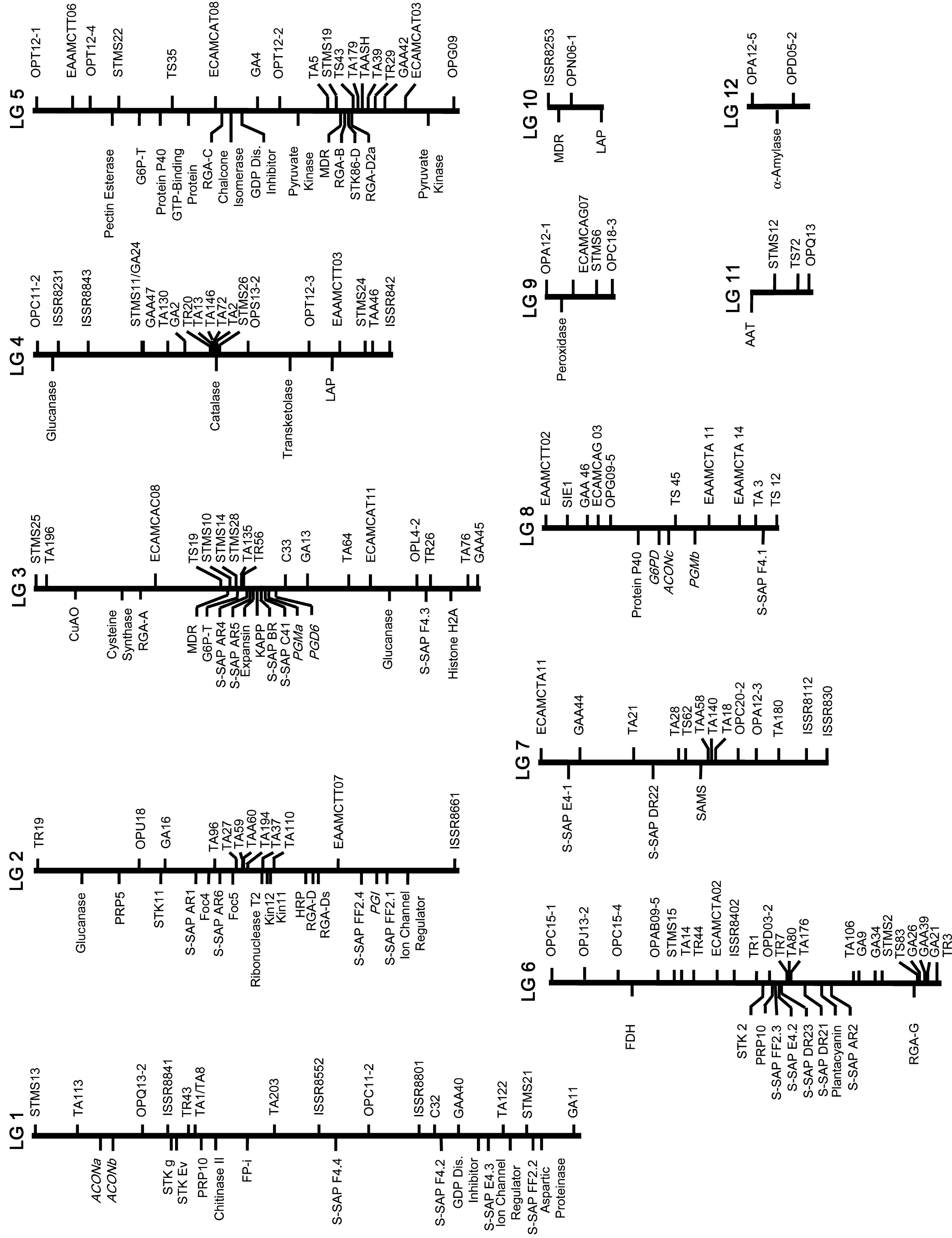

Figure 1. Integrated genetic map of chickpea based on 131 RILs from the cross between chickpea variety ICC 4958 × *C. reticulatum* P.I. 489777 summarizing data from Winter et al. (2000), Hüttel et al. (2002) and Pfaff and Kahl (2003). Abbreviations for the different genetic markers are given in these papers. Markers on the left of the vertical bar are derived from genes, those on the right are STMS or dominant framework markers. Of the more than 460 available markers, only a few necessary for demonstrating the context within the linkage groups are shown. See text for more detail.

equivalent to ca. 7.0× genomes of chickpea. Thus, the available BAC libraries could be employed for the generation of a physical map and as potential resources for whole genome sequencing, which should be a future perspective in chickpea genomics.

An alternative route to physical mapping has already started in collaboration between the laboratory of J. Dolezel (Olomouc, Czech Republic) and the University of Frankfurt with the aim to bridge the gap between the recombination-based genetic map and the chromosome-based map. The chromosomes were isolated from root tip cells synchronised for their mitosis, separated by fluorescent cell sorting and identified by their size. As a proof of principle the localization of 5S-rDNA on chromosomes 2 (B) and 7 (G), that had already been shown by fluorescent *in situ* hybridizations (Gortner et al., 1998; Staginnus et al., 1999) was confirmed. Moreover, the smallest LG 8, identified by the STMS GAA46, corresponds to the smallest chromosome 8 (H). None of the other chromosome fractions contain the sequence of this marker (Vlácilová et al., 2002).

Exploiting this technology, linkage group (LG) 1 has already been identified as chromosome F (or G), LG 2 as chromosome F (or G), LG 3 as chromosome C (or D), LG 4 as chromosome B, LG 5 as chromosome C (or D), LG 6 as chromosome E, LG 7 as chromosome A, and LG 8 as chromosome H, respectively. At present, the separation is brought to perfection, and packages of at least 10 different linkage-group-specific markers address the precise identification of linkage group–chromosome relationships. The resulting map then will allow the identification of the most interesting chromosomes carrying a particular trait (or gene), opening an avenue for the isolation and characterisation of the underlying sequence, its transcription and regulation, and mechanism of action of the encoded protein. These features are still missing in all chickpea research, but they are badly needed for an understanding of basic plant properties as, for example, yield, resistances towards abiotic and biotic stresses, growth and development, and seed quality, to name only few.

Breeding for resistance to biotic stresses

In the Mediterranean region, chickpea is traditionally sown in spring and, as a consequence of the low rainfall during the growth period in dry summers, this results in poor biomass development. Winter sowing expands the vegetative growth period and improves the seed yield up to 2 t/ha (Singh & Reddy, 1996; Singh et al., 1997), but is rarely adopted by the farmers because the cool and wet weather, typical for Mediterranean winters, favours the development of ascochyta blight. Blight, caused by the necrotrophic fungus *Ascochyta rabiei* (Pass.) Lab., affects all aerial parts of the plant. It is also a problem in North America, Pakistan, Northwest India and Australia. Sources of resistance have been identified (Singh & Reddy, 1983) and the development of stable blight-resistant lines would allow a shift to sowing into the rainy season. Since this is currently not the case, winter planting is yet illusory (Halila et al., 2000). More durable resistance could probably be achieved by pyramiding of resistance genes via MAS and is a major challenge for chickpea breeders.

The second most important fungal disease and another major constraint for increasing chickpea yield is fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* (Nene & Reddy, 1987). Many traditional Mediterranean kabuli cultivars are susceptible to the soil-borne disease, and sources of resistance (mainly desi cultivars) were included in breeding programs (Singh, 1987; Kaiser et al., 1994). However, development of resistant cultivars is hindered by the pathogenic variability of the fungus. To date, eight pathogenic races of Fusarium, discriminated on the basis of reactions they evoke in a differential set of cultivars, have been reported (Haware & Nene, 1982; Jiménez-Díaz et al., 1993).

Other diseases and pests have been reported (reviews by Nene & Reddy, 1987; Greco, 1987; Reed et al., 1987; Singh et al., 1994), but seem to be a serious problem only in certain regions of the world. One of the most problematic pests in India and Australia is the pod borer, *Helicoverpa armigera*. Some resistance against this pest has been found in wild *Cicer* species (Patankar et al., 1999; Sharma et al., 2005). Botrytis grey mould can become problematic under conditions favouring overgrowth and dense crop canopy (Kaiser et al., 2000), and root rots caused by *Sclerotium, Pythium* spp. and *Phytophthora* are important in the sub-tropics and tropics (Kraft et al., 2000; Knights & Siddique, 2002). Rust of chickpea, caused by *Uromices-ciceris-arietini*, can be a problem in cool and moist weather. It may appear almost simultaneously with ascohyta blight (Nene & Reddy, 1987), and especially occurs at high altitudes as e.g. in Central Mexico (Díaz-Franco & Pérez-García, 1995; Haware, 1998). Only moderate levels of incomplete and partial resistance against rust are available (Rubiales et al., 2001). Wide-spread application of winter sowing under Mediterranean conditions may also result in outbreaks of broomrape (*Orobanche*

crenata) (Rubiales et al., 1999). Although high levels of resistance are found in chickpea germplasm which can be exploited for resistance breeding (Singh, 1987; Rubiales et al., 2003), the spread of winter sowing may be counteracted by the evolution of new and more aggressive broomrape pathotypes. Though sources of resistances or tolerances to pests and minor diseases have been recently identified (Prajapati et al., 2003; Rubiales et al., 2003; Ansari et al., 2004; Pande et al., 2004), in most cases genetic studies or segregating populations for genetic mapping studies are not available.

Mapping QTL for resistance to Ascochyta rabiei: Current status

The genetics of resistance to ascochyta blight has been extensively analysed because the disease is of great agronomic and economic importance. However, the emerging picture is confusing: depending on the fungal isolate and the cultivar, either one dominant, one recessive and one dominant, or one recessive resistance gene was reported. Also, two complementary recessive, or two complementary dominant genes were detected. At present, it is not clear whether the reported resistance genes represent the same or different loci because allelic tests were not performed (for a recent review see Winter et al., 2003). To complicate the picture even more, other genes may modify the expression of resistance. Another drawback for mapping of ascochyta resistance genes in the field is that, in many chickpea growing regions, several patho- and genotypes of the fungus may coexist in the same field or even in the same lesion (Morjane et al., 1994; Jamil et al., 2001; Peever et al., 2004). Since random mating may occur between different pathotypes of the fungus carrying different mating type alleles (Barve et al., 2003), genetic recombination may contribute to genotypic diversity and provide the fungus with an additional means to adapt to newly introduced resistant germplasm (Peever et al., 2004).

Different methods are applied for assessment of disease severity. Testing under controlled glasshouse or growth chamber conditions (Singh et al., 1992; Udupa & Baum, 2003) combined with field screening (Flandez-Galvez et al., 2003a; Millán et al., 2003; Cho et al., 2004) would very much help to improve the reproducibility of the results, since severity and spread of the disease are highly dependent on environmental conditions and especially on humidity (which may change from year to year). Indeed, Cho et al. (2004) observed dramatic increases in severity of blight symptoms, if 100% relative humidity was maintained for more than 2 days after inoculation, as compared to normal greenhouse conditions. Further, different loci may contribute to resistance at different points of the life cycle of the plant (Collard et al., 2003). As the scale used for disease evaluation to blight (1–9 scale; Singh et al., 1981; Reddy & Singh, 1984) is subjective particularly for intermediate values, a bias may be introduced by the researcher that may also affect the accuracy of tagging blight-resistant genes with markers. In fact, some authors detected different QTLs measuring disease reaction in the same environment using two disease scoring systems (Flandez-Galvez et al., 2003a).

To improve the reliability of mapping QTLs for ascochyta blight resistance, recombinant inbred lines (RILs) were used as mapping populations and rated in subsequent years. For example, Tekeoglu et al. (2000a) employed three different RIL populations and tested them in the field for two growing seasons against the *A. rabiei* pathotype prevalent in the United States. This study defined two major recessive and complementing resistance genes together with several modifiers. A third gene could only be detected in 1 year. Absence of either one of the two major genes led to susceptibility, whereas the modifiers determined the degree of resistance (Tekeoglu et al., 2000a). Two QTLs (QTL-1 and QTL-2) were identified in one of these populations and together accounted for 50.3% and 45.0%, respectively, of the estimated phenotypic variation in two subsequent years. Interval mapping located the QTLs with high LOD scores on LGs 6 and 1 of the maps of Gaur and Slinkard (1990) and Kazan et al. (1993), respectively. Two RAPD markers (UBC733b and UBC181a) flanked QTL-1, whereas QTL-2 was located between an ISSR and an isozyme marker 5.9 cM apart (Santra et al., 2000). Rakshit et al. (2003) used the population and ascochyta blight data set from the Pullman group (Tekeoglu et al., 2000; Santra et al., 2000) to identify two DNA amplification fingerprinting (DAF, Caetano-Anollés et al., 1991) markers, OPS06-1 and OPS03-1, in close vicinity of markers UBC733b and UBC181a flanking QTL-1. Marker OPS06-1 was located between UBC733b and UBC181a, and is probably the most closely linked marker for QTL-1 available to date. Another, more loosely coupled marker, OPS03-1, could be transferred to the population on which the map of Winter et al. (2000) was based. There, it mapped to LG 4, thus identifying this LG as LG 6 of Gaur and Slinkard (1990) and Kazan et al. (1993).

STMS markers mapped on LG 4 were transferred to the population segregating for ascochyta resistance, locating QTL-1 to the interval STMS 11, GAA47, GA2 and TR20. Twelve out of 14 STMS markers could be used in both populations. The study by Rakshit et al. (2003) showed that dominant markers can be transferred to other populations and located on the reference map of chickpea via closely linked STMS markers. Another study by Tekeoglu et al. (2002) confirmed the QTL reported by Santra et al. (2000) and, through the use of STMS markers (Hüttel et al., 1999; Winter et al., 1999) located QTL-1 on their LG 8 close to STMS GAA47 and QTL 2 on LG 4 (indicative markers TA72 and GA2).

A QTL was also detected in a genomic region saturated with RAPD markers using ILC3279 as source of resistance in an intraspecific cross (Millán et al., 2003). Recently, SCAR markers tightly linked to this QTL have been developed (Iruela et al., 2006), and STMS analysis revealed that this QTL could be the same as QTL-2 of Santra et al. (2000), since it was linked to the same markers TA72 and TA146.

Rating for ascochyta resistance in all these studies was performed in the field using natural inoculum without control over the fungal pathotypes or the environment. Udupa and Baum (2003) were the first to employ defined *A. rabiei* pathotypes (I and II) in a controlled greenhouse environment for scoring of disease symptoms. They mapped a major QTL for resistance to pathotype I (QTL ar1, indicative marker GA16; Figure 2) to LG 2. Two QTLs against pathotype II, QTL ar2a and QTL ar2b, were identified as independent recessive major resistance loci with complementary gene action and were mapped to LGs 2 and 4, respectively (indicative markers TA130, TA72, TS72 on linkage group 4). QTL ar2a resided in close vicinity of the pathotype I specific resistance locus, indicating a clustering of resistance genes (Figure 2).

Also, Cho et al. (2004) employed both, controlled greenhouse and field conditions, to screen their intraspecific RIL mapping population with two defined isolates of pathotype I and one isolate of pathotype II. As already shown by Udupa and Baum (2003) the pattern of blight resistance in the RILs varied depending on the pathotype. The greenhouse screening with the pathotype I isolates (Ar19 and Ar21d) revealed two QTLs for resistance located on LG2A + 6B, with LOD scores of 3.08 and 2.66, respectively. Those two QTL were postulated to be joined in a single one (QTL ar1a) on the basis of the expected linkage between LG 2A and LG2B + 6B, in accordance with the Winter et al. (2000) map (indicative markers GA20 and GA16). A second QTL (called ar1b here), located on LG 2B (indicative markers TA37 and TA200) with a LOD score of 3.69, was specific for pathotype I isolate Ar21d, suggesting that races may co-exist within the pathotypes.

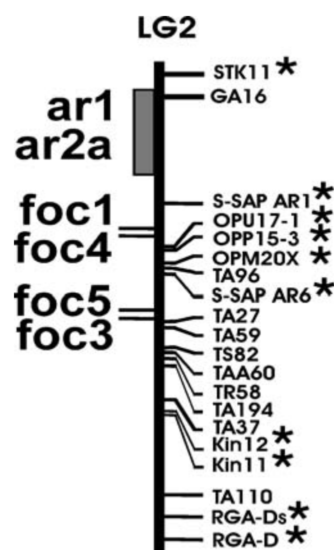

Figure 2. Detailed map of linkage group 2 in vicinity of the fusarium resistance gene clusters including fusarium resistance (foc) genes and QTL for ascochyta blight resistance (ar1, ar2a, indicated by the shaded box) on the left side of the vertical bar. Markers on the right are STMS and DAF markers from Benko-Iseppon et al. (2003) and RGA markers from Hüttel et al. (2002). Loci marked with an asterisk are potentially involved in pathogenesis, either encoding RGAs or pathogenesis-related proteins. See text for more detail.

Growth chamber experiments with a mixture of pathotype II isolates revealed one QTL (named QTL ar2a), which mapped to the same position on LG4A (indicative markers GA24 and GAA47, LOD score 4.17) as the QTL for blight resistance in the field (LOD score 2.83). Two QTLs for blight resistance in the field on LGIV and LGVIII of the interspecific linkage map of Tekeoglu et al. (2002) appeared to be the same as the QTL on LG4A of the map of Cho et al. (2004). Since both field experiments were conducted on the same experimental farm in Washington State, USA, ascochyta pathotype II seems to be prevalent there.

Flandez-Galvez et al. (2003a) also detected association to blight resistance in a linkage group equivalent to LG4 using a F2 population derived from an intraspecific cross. In this group, QTL 5 (indicative marker TA146) was the only one showing significant

Table 1. QTLs for resistance to ascochyta blight and diagnostic markers allowing their assignment to linkage groups (LG) of the map of Winter et al. (2000)

Pathotype	Name of QTL	Indicative marker[a]	LG	Reference
nd	1	GAA47	4	Tekeoglu et al. (2002)
nd	2	TA72, GA2	4	
nd	1	TS12b		Flandez-Galvez et al. (2003b)
	2/3	TA3a/TA3b		
	4/5/6	TA30/TA146/TR20		
nd	AR2	SC/OPK13$_{603}$	4	Millán et al. (2003)
		SC/OPM02$_{935}$ TA72, TA146		Iruela et al. (2006)
nd	I	STMS11, GA2, GAA47, TR20	4	Rakshit et al. (2003)
I	ar1	GA16	2	Udupa and Baum (2003)
II	ar2a	GA16	2	
II	ar2b	TA130, TA72, TS72	4	
I	ar1a	GA20, GA16	2B–6B	Cho et al. (2004)
I	ar1b	TA37, TA200	2B	
II	ar2a	GA24, GAA47	4A	
nd	1*	STMS 11, GA2, TR20	4	Collard et al. (2003)
nd	2*	XLRRb$_{280}$	4	

nd: not determined.
[a]XLRRb$_{280}$ is an RGA marker; SCAR markers are named with SC prefix; rest are STMS markers.
*Seedling resistance.

association in two different environments (controlled and field trial), five other QTLs were also detected (1, 2, 3, 4, 6) but in only in a particular environment (Table 1).

In all the studies undertaken so far, cultivated chickpea was the source of resistance to ascochyta blight. To exploit resistances available in the wild *Cicer* species, Collard et al. (2003) employed an interspecific F_2 population derived from a cross between a susceptible chickpea cultivar and a resistant *C. echinospermum* accession to generate a preliminary linkage map of low density. Six out of the eight detected LGs could be correlated to LGs from the integrated map (Winter et al., 2000) by STMS markers that generally function also in *C. echinospermum* (Choumane et al., 2000). The two remaining linkage groups contained no STMS and thus could not be related to known LGs. The F_2 population was evaluated for seedling and stem resistance in glasshouse trials. Interval mapping and single-point analysis identified at least two QTLs for seedling resistance, and both were located on LG 4 (QTL 1, interval STMS 11, GA2, TR20; QTL2, no significantly linked STMS, indicative marker RGA XLLRb$_{280}$). Out of five markers associated with stem resistance, four were also linked to seedling resistance. No significant association of stem resistance with STMS markers was detected. A summary of the QTLs for ascochyta blight resistance detected is provided in Table 1 together with markers indicating the respective linkage groups.

The application of STMS markers in all these studies now allows us to conclude that *A. rabiei* pathotype I resistance is governed by a major QTL on LG 2 close to marker GA16 (Figure 2). This, or an adjacent locus is also partly responsible for resistance to pathotype II. Another, (eventually race-specific) QTL for resistance to pathotype I may be located on the same LG in the vicinity of marker TA37. A QTL flanked by STMS 11 and TR20 on LG-4 confirmed by all workers is responsible for resistance to pathotype II and eventually also for resistance during the seedling stage (see summary map in Figures 1 and 2). Since apparently all major blight resistance QTLs are tagged with STMS markers, pyramiding of resistance genes via MAS should now be feasible and awaits its proof-of-principle. The genetic control of this disease bred into cold tolerant germplasm would be a major breakthrough for yield increases in Mediterranean-type environments in many parts of the world.

Infection of chickpea by Ascochyta rabiei: The transcriptome

Functional genomics of chickpea is still very much in its infancy, yet there is great scope for its application in the development of gene-based markers for molecular breeding of complex traits. Moreover, better knowledge of the activity of genes involved in pest and disease resistance, and tolerance to environmental stresses promises increases in productivity and yield stability. Until recently, only some 500 chickpea expressed sequence tags (ESTs) were deposited in public databases. However, more recently several groups initiated systematic gene expression analyses associated with specific stresses.

The group of Prof. W. Barz (Muenster, Germany) was the first to focus on transcriptomics of chickpea cell cultures upon infection with *A. rabiei* (Mackenbrock et al., 1993). On the physiological level, elicitation of cultured cells stimulated a signal transduction pathway leading to several rapid responses including an oxidative burst, extracellular alkalinisation followed by extracellular acidification, transient K+ efflux, and activation of defence-related genes, all within 2 h. Rapidly and transiently expressed genes encoded the first soluble enzyme in the pterocarpan biosynthesis part of the medicarpin and maackiain malonylglucoside phytoalexin pathway (Mackenbrock et al., 1993), a NADPH: isoflavone oxidoreductase (IFR; Tiemann et al., 1991), and at least eight members of the cytochrome P450 protein family also involved in isoflavone synthesis. Thus, it seems that isoflavone metabolism is of considerable importance for resistance to *A. rabiei* (Barz & Mackenbrock, 1994; Overkamp et al., 2000). Increased expression was also found for mRNAs of rab and rac type small GTP-binding proteins (Ichinose et al., 1999), and for genes encoding two glycine-rich proteins (GRPs), which displayed maximum expression 5 days post infection and are probably involved in fortification of cell walls by oxidative cross-linking of cell wall components (Cornels et al., 2000). In *planta*, a pathogenesis-related thaumatin-like protein (TLP) gene, PR-5a, and a second cDNA coding for a slightly larger TLP (PR-5b), presumably located in the vacuole, were elicited much faster in an *A. rabiei*-resistant cultivar than in a susceptible cultivar. However, PR-5a was not effective as an antifungal agent against *A. rabiei* (Hanselle et al., 2001). Generally, genes elicited by *A. rabiei* could be assigned to five groups according to their function in plant metabolism including primary metabolism, regulation of gene expression, defence-related, signal transduction and catabolic pathways (Ichinose et al., 2000). Full-length sequences for chalcone synthase and phenylalanine ammonia-lyase (PAL) cDNA, both important enzymes in defence response, were provided by the same group (Hanselle et al., 1999; Hein et al., 2000). At least a part of these elicitor-induced responses are inhibited by the serine/threonin protein kinase inhibitor staurosporine and the anion channel blocker anthracene-9-carboxylic acid, and stimulated by the ser/thr protein phosphatase 2A inhibitor cantharidin (Otte et al., 2001), indicating that the signal cascade leading to activation of pathogenesis-related genes requires the action of serine/threonin kinases. Interestingly, a major QTL for ascochyta resistance on LG 2 (indicative marker GA16; Figure 2) is closely linked to a serine/threonin kinase gene (STK11; Hüttel et al., 2002). To determine if this gene is the resistance gene requires more research.

However, the belief that ascochyta blight or fusarium wilt resistance in chickpea is initiated by, or correlated to, differential expression of conventional, defence-related genes may be misleading. This cautionary note refers to recent results of Cho and Muehlbauer (2004), who investigated expression patterns of genes related to defence in chickpea after pathogen inoculation and exogenous treatments with systemic signals such as salicylic acid (SA) and methyl-jasmonate (Me-JA). The authors demonstrated significant differential expression of the defence-related genes in two blight and SA- and Me-JA differential germplasm lines (one resistant and SA and Me-JA responsive, the other susceptible and SA and Me-JA non-responsive) after inoculation with *A. rabiei* and exogenous treatment with SA and Me-JA. However, resistance in the RILs from a cross of these varieties did not co-segregate with the expression of the genes induced either by the pathogen or by the signal chemicals. Also, resistance to fusarium wilt did not require induction of these genes. Moreover, these authors also did not observe a hypersensitive response (HR) in their resistant varieties, previously claimed to be part of chickpea's defence against *A. rabiei* (Hoehl et al., 1990). Thus, systemic regulation of defence-related transcripts associated with disease resistance in several pathosystems in e.g. *Arabidopsis* or other host species does not necessarily confer resistance against the two necrotrophic fungi in chickpea. Instead, either constitutively expressed or unknown defence systems independent of SA- and JA may be necessary for resistance against these pathogens (Cho & Muehlbauer, 2004).

New mechanisms of defence have to be searched for depending on the pathosystem, since SA- and Me-JA-independent mechanisms of resistance are also found in other species (Branding et al., 2000; Roetschi et al., 2001). These are eventually only detectable by "open-source" transcription profiling systems such as Super-SAGE (Matsumura et al., 2003) that are not based on "prejudices".

Targeting of Fusarium oxysporum resistance genes

To date, eight races of *Fusarium oxysporum* f. sp. *ciceris* have been reported from India, Spain and the United States (0, 1A, 1B/C, 2, 3, 4, 5 and 6; Haware & Nene, 1982; Jiménez-Díaz et al., 1993; Jiménez-Gasco et al., 2004). Each of these races forms a monophyletic lineage, which acquired its virulence on different chickpea lines in simple, stepwise patterns (Jimenez-Gasco et al., 2004). Over 150 sources for resistance have been identified, and some are resistant to more than one race. The genetics of resistance is, however, complex, since at least for resistance to race 1 a minimum of two out of three detected resistance genes are required (van Rheenen, 1992).

Several studies in inter- and intraspecific RIL populations demonstrated the organisation of resistance genes for fusarium wilt races 1, 3, 4 and 5 (*foc*1 and *foc*3, *foc*4 and *foc*5; Mayer et al., 1997; Ratnaparkhe et al., 1998; Tullu et al., 1998; Winter et al., 2000; Sharma et al., 2004) in two adjacent resistance gene clusters on LG 2 flanked by STMS markers GA16 and TA96 (*foc1–foc4* cluster) and TA96 and TA27 (*foc3–foc5* cluster) respectively (Figure 2). However, not only resistance genes *per se*, but also other sequences coding for proteins putatively involved in the reaction of chickpea to pathogen attack were localised between, or in close vicinity, to the fusarium resistance gene clusters. For example, the sequence of one of the markers most tightly linked to the *foc4* and *foc5* loci is highly similar to a PR-5 thaumatin-like protein gene and another one is homologous to the gene for anthranilate *N*-hydroxycinnamoyl-benzoyltransferase, a regulator of the phytoalexin pathway, both important components of the plant's defence against pathogens (Figure 2). In the *Arabidopsis* genome, homologues of these genes are located in close vicinity on short segments of chromosome 1 and 5, respectively, suggesting synteny to the fusarium resistance gene cluster(s) of chickpea (Benko-Iseppon et al., 2003). Besides the fusarium resistance genes, the resistance loci *ar1* and *ar2a* against two different pathotypes of *A. rabiei* were also localised on LG 2 close to each other and to the *foc* gene clusters (Udupa & Baum, 2003; Figure 2). Moreover, several potential resistance and pathogenesis-related genes were localised on this LG (Hüttel et al., 2002; Pfaff & Kahl, 2003). It may therefore well be justified to call LG 2 a hot spot for pathogen defence.

However, not all fusarium wilt resistance genes are located on LG 2 of the genetic map of Winter et al. (2000). For example, Rubio et al. (2003) not only showed that two different genes can confer resistance to race 0 ($foc0_1$ and $foc0_2$) but also demonstrated linkage of the $foc0_1$ to RAPD marker $OPJ20_{600}$ (resistance derived from line JG62). More recently, this locus has been mapped on LG5 tightly flanked by a RAPD (3 cM apart) and an STMS (2 cM apart) marker (Cobos et al., 2005).

Cloning resistance genes via the candidate gene approach

Most resistance proteins are receptor-like protein kinases of the nucleotide-binding site-leucin-rich-repeat (NBS-LRR) class and composed of different combinations of conserved elements. Therefore, resistance gene analogues (RGAs) or candidate resistance genes can be isolated by PCR amplification with degenerate oligonucleotide primers derived from conserved amino acid motifs in the NBS (Kanazin et al., 1996; Shen et al., 1998). This approach was used by Hüttel et al. (2002) in an effort to directly clone *R*-genes against *F. oxysporum* and *A. rabiei*. These authors isolated a series of RGAs from both *C. arietinum* and *C. reticulatum* using two degenerate primer pairs targeting sequences in the NBS domain. A total of 48 different RGAs fell into 9 different sequence classes, and were members of the Toll-interleukin receptor (TIR)-NBS-LRR and coiled-coil (CC)-NBS-LRR groups. Thirty of these RGAs were mapped on the reference genetic map of chickpea (Winter et al., 2000), where they could be located on principally five linkage groups, some of them as clusters on LGs 2 and 5, respectively (Figure 1). One such cluster with the prominent RGA CaRGA-D mapped in the region harbouring the fusarium resistance gene cluster, but not close enough to the resistance to be a real fusarium resistance gene (Figures 1 and 2). Besides this most comprehensive direct approach towards the isolation of resistance genes from chickpea, Flandez-Galvez et al. (2003b) mapped 12 RGA markers that clustered on three LGs, and Collard et al. (2003), in a preliminary investigation of QTLs from *C. echinospermum* associated with seedling resistance to ascochyta

blight, mapped another two. One of them, XLRRb, could be located within a QTL for seedling resistance. However, the low LOD score of 2.6 suggests that it is probably not the resistance gene itself, but may be part of a resistance gene cluster containing this gene. Also, Tekeoglu et al. (2002) mapped an anonymous RGA marker to LG 3. In summary, efforts to directly clone a fusarium or ascochyta resistance gene via the candidate gene approach are not yet successful. A possible reason for the failure of this approach may be the low level of polymorphism in the chickpea genome combined with the high conservation of the NBS-coding region used for designing the primers that prevented the mapping of many RGAs. Targeting at the more variable LRR-coding region of the genes in accord with new methods to detect polymorphisms such as EcoTILLING (Comai et al., 2004) may be more successful.

Breeding for tolerance to abiotic stresses

Chilling tolerance at flowering

The change from spring to winter sowing in chickpea, resulting in efficient use of rain water and increased yield in Mediterranean type environments, implies that tolerance to low temperature becomes important for further crop improvement. Both freezing (below −1.5 °C) and chilling (−1.5–15 °C) are known to affect chickpea at various stages of development from germination to maturation (Croser et al., 2003b). Abortion of flowers at temperatures of 15 °C and below are documented from field in Australia (Siddique & Sedgley, 1986), the Mediterranean (Singh, 1993) and India (Savithri et al., 1980; Srinivasan et al., 1998), and in growth room studies (Srinivasan et al., 1999; Clarke & Siddique, 2004; Nayyar et al., 2005). Studies at low temperatures in controlled environments demonstrated that pollen tubes derived from chilling-tolerant phenotypes grew faster down the style to the ovary for fertilization than pollen derived from chilling-sensitive plants (Clarke & Siddique, 2004). Such knowledge of location and timing of chilling sensitive stages during reproduction in chickpea enables precise screening of germplasm for this trait. The variation in pollen derived from different genotypes and the co-expression of the trait in the pollen and its mother plant (haplo-diplo expression) opens up the possibility of using pollen selection to improve chilling tolerance.

The potential of pollen selection as a breeding tool, whereby selection pressure is applied at the gametophytic stage in the life cycle, has been demonstrated in a wide range of species (review by Hormaza & Herrero, 1996). Recently, this novel approach has been applied to chickpea improvement for winter sowing in the cool dry-land environment of southern Australia, contributing to the release of the two chilling tolerant desi chickpea cultivars Sonali and Rupali (Clarke et al., 2004). During the early generations of breeding, selection pressure was applied to the pollen tubes, as they grew down the style to fertilise the ovary, by chilling the mother plants for 3 days immediately after hand pollination. The pollen selection cycle can be repeated by cross pollination and backcrossing of sensitive cultivars with heterozygous pollen from F_2 donor plants segregating for chilling tolerance (Figure 3). The new cultivars set pods when the average maximum temperature over a 24-h period is 12 °C, compared to the check cultivars, which abort flowers at 15 °C. In the field, this translates to successful pod set about 2 weeks earlier, resulting in significantly higher yields in low-rainfall environments. Previous success in breeding chilling- or freezing-tolerant chickpea lines has been reported at ICRISAT (Khanna-Chopra & Sinha, 1987; ICRISAT, 1994) and at ICARDA (Singh, 1987).

Early identification of chilling-tolerant types combined with pollen selection has great potential to accelerate breeding for cold tolerance. Molecular markers based on amplified fragment length polymorphisms (AFLPs) have been linked to the trait using bulked segregant analysis for F_2 progeny of a cross between the chilling-sensitive cultivar Amethyst and the chilling-tolerant ICCV 88516 (Clarke & Siddique, 2003). Putative markers linked to traits for both chilling tolerance and chilling sensitivity overcome the limitations of the dominant AFLP marker system. Six pairs of specific 18–24-mer primers were designed directly from the sequence of the AFLP-based markers. The primers were then used to amplify the defined DNA fragment from genomic DNA of individual F_4 progeny with known phenotypes in an attempt to develop SCAR markers (Paran & Michelmore, 1993). The most promising primers were based on a 560 bp fragment containing a simple sequence repeat (3 bp repeat microsatellite), with 9 repeats in the susceptible parent and 10 in the tolerant parent (Clarke, unpublished). The three-base difference was visualised on a vertical acrylamide gel and was very useful in the selection of chilling-tolerant progeny derived from crosses between ICCV 88516 and Amethyst. Unfortunately, there has been no success in applying these SCAR markers to other breeding materials.

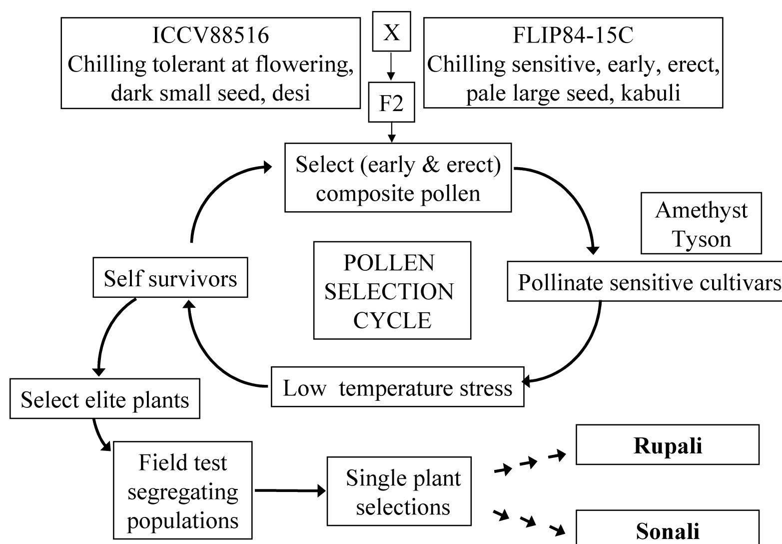

Figure 3. Schedule for chickpea improvement, including repeated cycles of pollen selection at low temperature stress, which was successfully used to develop new cultivars for Australia (source: Clarke et al., 2004).

A new method developed for marker-assisted breeding in lupins (Yang et al., 2001) could also be considered for chickpea in the future. Microsatellite-anchored fragment length polymorphism (MFLP) is highly efficient in producing DNA polymorphisms, and many MFLP markers can easily be converted into sequence-specific, simple PCR-based co-dominant markers. Several such markers are now being fully implemented in the Australian national lupin breeding program to select for resistance to fungal diseases (Yang et al., 2004).

Difficulties in screening and breeding for tolerance to low temperatures are further confounded by low genetic variability within cultivated chickpea (Abbo et al., 2003). Relatives of chickpea among the wild *Cicer* species offer a valuable genetic resource to overcome these limitations (Berger et al., 2003; Shan et al., 2005). Tolerance to cold has been reported in five annual and one perennial species (van der Maesen & Pundir, 1984; Singh et al., 1991, 1995, 1998; review by Croser et al., 2003a). The original collection and many selections of annual *Cicer* species held in world gene banks were analysed using DNA molecular markers, which are not affected by environmental influences, providing useful data for the selection of suitable parents for crosses (Iruela et al., 2002; Nguyen et al., 2004; Shan et al., 2005). To a certain extent, it will also be possible to use chickpea-derived STMS markers for the marker-based analysis of wide crosses because many STMS can be transferred between *Cicer* species (Choumane et al., 2000). Barriers in wide crosses are also being addressed through international collaboration with the aim to use embryo rescue to overcome incompatibility (Mallikarjuna, 1999; Clarke et al., 2004b).

Yield improvement in drought-prone environments: Altering phenology and root traits

Flowering time

Since about 90% of the chickpea crop is grown under rain-fed conditions, drought is the most important stress that limits chickpea production and yield stability. Therefore, matching growth duration of crop varieties to soil–moisture–availability is critical to realise high seed yield (Siddique et al., 2003). Time of flowering is a major trait of a crop's environmental adaptation, particularly when the growing season is restricted by terminal drought and high temperatures. Developing short-duration varieties has to date been the most effective strategy for minimizing losses from terminal drought, as early maturity helps the crop to avoid the period of greatest stress (Kumar et al., 1996; Kumar & Abbo, 2001). However, since yield is generally correlated with the length of crop duration under favourable

growing conditions, any reduction of crop duration below the optimum would have a penalty in yield (Saxena, 1987; Turner et al., 2001). Kumar and Rao (2001) developed the world's shortest-duration chickpea variety that was recently released as a catch cultivar for cultivation between rice and wheat in the Punjab province of India (Sandhu & ArasaKesary, 2003). The new variety led to substantial increases in chickpea area and productivity in short growing-season environments and expanded options to include chickpea in many prevailing and evolving new-production systems, such as rice fallows (Kumar & Abbo, 2001).

In Mediterranean environments, early flowering has a positive effect on seed yield (Siddique et al., 2003; Rubio et al., 2004). In a 4-year, multi-location trial in Southern Spain, early flowering was generally beneficial for productivity explaining 26% of the variation in yield in winter and 77% in spring-sown crops (Rubio et al., 2004). Chickpea generally is a long day plant (van der Maesen, 1972; Summerfield et al., 1981; Sethi et al., 1981). Day-length-insensitive lines have been obtained from crosses involving germplasm from low latitudes such as East Africa, the Indian Plateau and Mexico. Also, a day-length-insensitive line of chickpea from Iran has been reported (ICC 5810; Roberts et al., 1985). Early flowering lines derived from ICCV 96029 are also exploited in short-duration environments defined by low temperatures such as in Canada (Kumar & Abbo, 2000).

Limited information is available on the genetics of flowering in chickpea. A major gene (*efl-1*) for time of flowering was reported by Kumar and van Rheenen (2000), and another one (*ppd*) by Or et al. (1999). The latter gene controls time to flowering through photoperiod response (Hovav et al., 2003). It is unclear whether the *efl-1* and *ppd* genes differ from each other, as allelic tests have not been performed. The *efl-1* gene is extensively used to develop early maturing varieties (Kumar & Abbo, 2001; Musa et al., 2001). Anupama (personal communication) named two genes, *nff-1* and *nff-2*, that govern node number to first flowering, an indication of time of flowering. Significant correlation between time of flowering and number of nodes up to first flower was observed. Cho et al. (2002) mapped a QTL for days to 50% flowering to LG 3. Another QTL was also located on this linkage group in an interspecific RIL population and explained 28% of the total phenotypic variation (Cobos et al., 2004). Clearly, more research is required to understand this important phenological trait that can lead to major breakthroughs in increasing chickpea's productivity.

Drought tolerance/avoidance

Conventional breeding for drought tolerance is based on selection for yield and its components under a given water-limited environment. Because the large environmental variation necessitates evaluation of material at several locations and/or over years, trait-based selection could have an advantage. Efforts have been made to identify morphological traits that could contribute to drought tolerance/avoidance in chickpea (Turner et al., 2001). Two important drought avoidance traits have been suggested: a large root system is apparently more efficient for extraction of available soil moisture, and a smaller leaf area helps to reduce transpirational water losses (Saxena, 2003). More than 1500 chickpea germplasm and released varieties were screened for drought tolerance at ICRISAT. The most promising drought tolerant variety was ICC 4958 that had 30% more root volume than the popular variety Annigeri (Saxena et al., 1993). Promising drought-tolerant lines have been developed using ICC 4958 as one of the parents. Yield-based selections were effective in producing varieties with high yield, and trait-based selections resulted in lines with better drought tolerance obtained from a three-way cross involving ICC 4958, Annigeri and ICC 12237, a fusarium wilt resistant accession (Saxena, 2003). Efforts were also made to combine large roots trait of ICC 4958 and the few pinnules (smaller leaf area) trait of ICC 5680. Several lines combining these traits were more drought tolerant and yielded similar to the high yielding parent (Saxena, 2003).

Selection for root traits is very difficult, since it involves laborious methods such as digging and measuring roots. Molecular tagging of major genes for root traits may enable MAS for these traits and could greatly improve the precision and efficiency of breeding. A set of 257 recombinant inbred lines (RILs) was developed from the cross Annigeri × ICC 4958 at ICRISAT and glasshouse-evaluated to identify molecular markers for root traits. Over 250 STMS and 100 EST markers were initially screened on parents of the RILs. Fifty-seven STMS markers detected polymorphisms and were mapped on the RIL population. A QTL flanked by STMS marker TAA 170 and TR 55 on LG 4A was identified that accounted for maximal phenotypic variation in root length ($Ra^2 = 33.1\%$), root weight ($Ra^2 = 33.1\%$) and shoot weight ($Ra^2 = 54.2\%$) (Chandra et al., 2004; Buhariwalla et al., personal communication). The locus also accounted for substantial variation (Ra^2 of 6.7–33.7%)

observed in these traits under simulated and actual field conditions.

Recently, the chickpea minicore collection of 211 accessions was evaluated for root traits along with 12 popular cultivars and 10 accessions of wild annual species (Krishnamurthy et al., 2003). The statistical differences of entries for both, root and shoot traits, were significant ($p < 0.001$). The root and shoot growth of the wild species was relatively poor compared to *C. arietinum* lines. However, several *C. arietinum* genotypes with higher maximum root depth and similar root mass than ICC 4958 were identified. Now, these newly identified genotypes serve as valuable alternative source for large root traits. Four accessions that contrasted extremely in rooting depth and total root biomass (ICC 8261, and ICC 4958 with large and ICC 283 and ICC 1882 with small roots) were selected for development of new mapping populations which will be useful in identification of additional markers for QTLs of root traits useful for drought avoidance.

Osmotic adjustment, the active accumulation of solutes within plant tissues in response to soil water potential (Morgan, 1984), is positively correlated with yield under drought environments in several cereals (Morgan, 1984; Morgan et al., 1986; Blum, 1989). Genotypic variation for osmotic adjustment also exists in chickpea (Morgan et al., 1991; Leport et al., 1999; Turner et al., 2001; Abbo et al., 2002; Moinuddin & Khanna-Chopra, 2004). However, the correlation of osmotic adjustment and yield under drought stress is not clear, since a positive association of osmotic adjustment with productivity (Morgan et al., 1991; Moinuddin & Khanna-Chopra, 2004) as well as no influence has been reported (Leport et al., 1999). The heritability of osmotic adjustment is low ($h^2 = 0.20$–0.33), indicating that gains from selection for increased osmotic adjustment are likely to be small (Abbo et al., 2002).

Genetic engineering is currently explored for enhancing the levels of drought tolerance in chickpea. ICRISAT has developed an efficient transformation and regeneration system (Jayanand et al., 2003), and developed transgenic plants with a dehydration responsive element (DRE) construct, where the expression of the DREB1A cDNA is driven by a drought-responsive rd29A promoter (ICRISAT, 2003). This construct is expected to enhance tolerance to several abiotic stresses, such as drought, chilling temperature and salinity, as it regulates a number of genes that act together in enhancing the tolerance to these stresses (Kasuga et al., 1999). The T1 generation transgenic plants are currently undergoing molecular characterisation. Plants have also been transformed with the gene P5CSF-129A that increases proline accumulation and improves tolerance to osmotic stress (Hong et al., 2000). Fifteen independently transformed lines tested positive in PCR, Southern blot hybridisation, and RT-PCR for integration and expression of the transgene. Some selected lines showed up to 15-fold overproduction of proline and a concomitant decline in free radicals. These lines are currently advanced to T3 generation for physiological characterization (ICRISAT, 2003).

Transcriptomics approaches to improve yield in drought-prone environments

To date, little attention has been paid to defining the molecular genetic determinants associated with drought avoidance and root traits. In order to generate markers for candidate gene mapping studies, functional genomics and allele mining of germplasm collections, only recently a targeted EST approach was employed at ICRISAT to identify genetic elements with putative involvement in chickpea drought avoidance and tolerance. Subtractive suppression hybridisation (SSH) was used for the isolation and characterisation of root-specific genes differentially expressed between two closely related chickpea genotypes (ICC 4958 and Annigeri) possessing different sources of drought avoidance and tolerance (Saxena et al., 1993; Saxena, 2003; Berger et al., 2003). The SSH process resulted in approximately 4000 clones including genes associated with root system development from the genotype ICC 4958 (tester), and possibly also some highly constitutively expressed genes from Annigeri. A total of 2858 EST sequences were analysed, of which 507 unique ESTs are available at GeneBank (CK148643–CK149150). All ESTs can also be accessed via a relational database at http://www.icrisat.org/gt1/Cpest/Home.asp (Jayashree et al., 2005). Tentative functional annotations with tBLASTx allowed the grouping of EST sequences into 12 general categories based on the biochemical functions of the predicted proteins as shown in Figure 4 (Buhariwalla et al., 2005). More than three-quarters of the 210 consensus sequences (77% equivalent to 164 contigs) were significantly similar (tBLASTx >100) to sequences in public databases. Over 379 sequences were similar at the nucleotide level to entries in the Medicago Gene Indices and 323 to the Soybean Gene Index (based on a cut-off

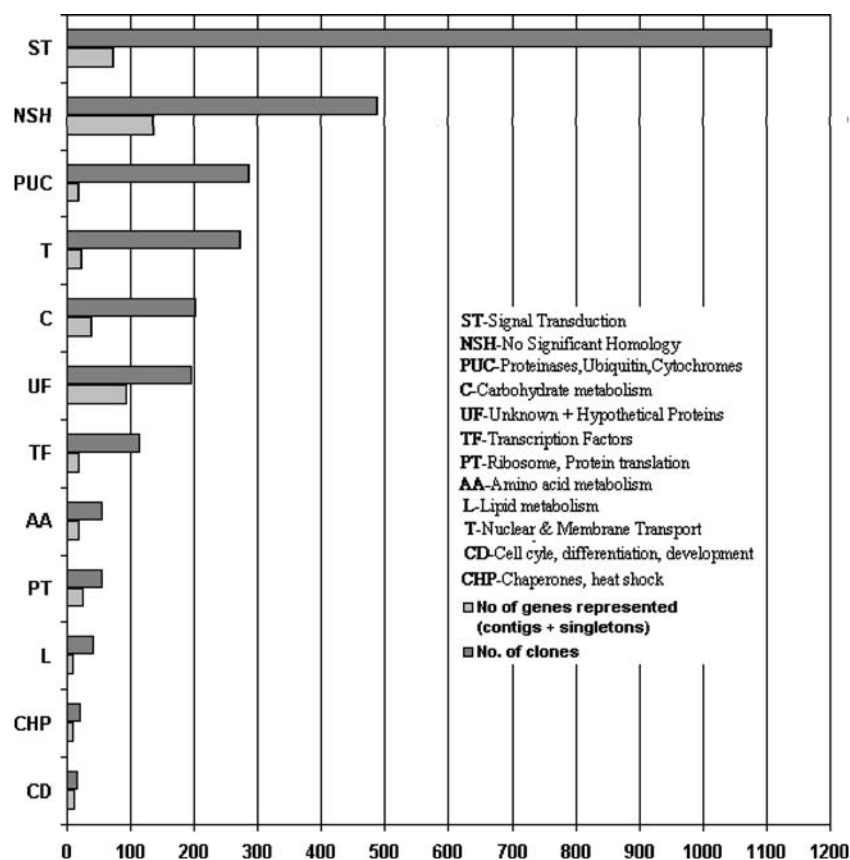

Figure 4. A summary of the number of clones and genes from chickpea roots in functional categories based on alignments with public databases in November 2004. Percentages indicate proportion of unigenes from the total number of unigenes identified in the whole dataset (source: Buhariwalla et al., 2005).

score of 200; $e < 10\text{-}5$). ESTs involved in signal transduction constituted the most abundant class of root ESTs (Figure 4), consisting largely of WD-repeat proteins (40%), protein kinases (25%) and arm-repeat containing proteins (13%). These sequences play a role in stress-response pathways in several organisms and thus provide an ideal resource for the development of candidate gene markers for studying root trait components and stress signalling response to drought. Other ESTs in chickpea root tissue that may be important for constitutive stress response and development include transcripts for proteases, T6P synthase, non-specific lipid transfer proteins, MRP-like ABC transporters, chaperones, HSP70, TCP-1-alpha, bZIP transcription factor, calcium ATPases, protein kinases, MRP4 glutathione-conjugate transporter, glutathione *S*-transferase, phosphoenol pyruvate carboxylase and *S*-adenosyl methionine synthetase. Primers designed from a selection of these EST are currently screened if they amplify polymorphic amplicons from parental genotypes of several recombinant inbred line populations designed specifically for mapping the root trait components of drought tolerance. Initial results suggest that around 20% of EST markers generated from this SSH library are polymorphic in these mapping populations, either directly or as cleaved amplified polymorphic sequence (CAPS) markers (Buhariwalla et al., 2005). Future developments will include the use of these EST markers for candidate gene mapping, allele mining of chickpea germplasm collections and the development of single nucleotide polymorphism (SNP) markers from the most promising EST markers.

Another study investigated changes in gene expression accompanying the adaptation to drought. Employing repetitive rounds of cDNA subtraction and differential DNA-array hybridisation followed by Northern blot analysis, Boominathan et al. (2004)

identified 101 dehydration-inducible transcripts in seedlings all of which were also induced by absisic acid (ABA). Steady-state expression levels of the dehydration-induced transcripts were monitored during the recovery period between two consecutive dehydration stresses. Seven of them maintained more than threefold expression after 24 h and more than twofold expression over the basic level even at 72 h after the end of stress. Noticeably, all of them were inducible by exogenous ABA treatment. When the seedlings were allowed to recover to a similar level after exposure to exogenous ABA, the steady-state abundance of six of them followed totally different kinetics returning to the basal expression level within 24 h. This observation suggests a correlation between the longer period of abundance of those transcripts in the recovery period and improved adaptation of the plants to subsequent dehydration stress and indicate that both ABA-dependent and -independent mechanisms are involved in the maintenance of the messages from the previous stress experience. Thus, the storage of stress-related messages could be one of the components necessary for increased tolerance of pre-exposed plants against subsequent exposures to stresses such as dehydration shocks.

The results presented by Boominathan et al. (2004) suggest that dehydration-stress-related gene expression is generally also regulated by ABA. However, Romo et al. (2001) demonstrated that at least the expression of two late embryo abundant (LEA) genes (CapLEA-1 and -2) in seedlings was not affected by ABA, but was up-regulated by dehydration stress imposed by NaCl and polyethylene glycol (PEG) treatment. PEG and, to an lesser extent, NaCL, but not ABA, also elicited the expression of a lipid transfer protein (LTP) gene (CapLTP) in almost all seedling tissues and especially in cotyledons to high levels. In this study, differential screening of a cDNA library from PEG-treated seedlings identified a total of seven up-regulated and six down-regulated cDNAs. In addition to those studied in more detail and mentioned earlier, up-regulated cDNAs coded for a proline-rich protein (PRP), a putative imbibition protein, a Glyoxylase I (GLX-I) gene and a protein of unknown function.

Salinity tolerance

Grain legumes, in general, are sensitive to salinity, and within legumes, chickpea, faba bean and field pea are more sensitive than other food legumes (Maas & Hoffman, 1977). The soil salinity affects germination resulting in poor plant stand. Chickpea plants show reduction in growth, high anthocynin pigmentation of foliage in desi type and yellowing of foliage in kabuli type, reduction in biomass, seed size and grain yield. Only salt-tolerant cultivars can be grown successfully in soils having electrical conductivity (ECe) higher than 4.0 dS/m^{-1}.

The levels of salinity tolerance identified in chickpea are low to moderate. Several tolerant sources have been identified in India (Dua & Sharma, 1995; Kathiria et al., 1997), Pakistan (Asharf & Waheed, 1992) and Australia (Maliro et al., 2004). The salt-tolerant lines CSG 88101 and CSG 8927 identified by Dua and Sharma (1995) had lower Na^+ in roots than the sensitive genotypes. A salinity-tolerant desi variety, Karnal Chana 1 (CSG 8963), which can be grown in saline soils with ECe between 4 and 6 dS/m, has been released in India. Recently, 252 germplasm accessions (including 211 accessions of mini-core collection) and breeding lines were screened for salinity tolerance at ICRISAT. The majority of the highly tolerant genotypes were of kabuli type, whereas the majority of the highly sensitive accessions were of desi type (Serraj et al., 2004). None of the 19 accessions of five annual wild species (*C. reticulatum*, *C. echinospermum*, *C. bijugum*, *C. judaicum*, *C. pinnatifidum*) studied by Maliro et al. (2004) in Australia were found tolerant to salinity.

Conclusions

Increasing and stabilising seed yield while minimising inputs is the major aim of chickpea breeding. This goal can be achieved by cultivars better adapted to the various stresses in local environments. Besides this major challenge, preferences of consumers and industry as well as health aspects need to be considered.

Drought and cold are currently the main abiotic constraints to improve chickpea productivity. There is no simple way to breed for drought or cold resistance. However, in particular environments some metabolic processes can be modified through breeding, either as single traits (e.g. pollen fertility under cold stress) or as a combination of traits (e.g. large root trait in combination with small leaf area for drought). Matching plant phenology to the environment also contributes to breeding successes. Whether trait-based breeding is successful also for tackling biotic stresses, the second most important cause for unstable and low seed yields, needs to be determined for each pathogen and

insect pest. For example, resistance to fusarium wilt seems to be caused by clearly defined and tractable race-specific genes that may be combined with other desired traits with relative ease. Ascochyta resistance, however, becomes more and more obscure despite the fact that QTLs for resistance have been localised at a few loci in the chickpea genome. The possible existence of races within the pathotype classes, the possible association of resistance with unknown defence pathways, and most importantly, the failure of conventional breeding to efficiently control the pathogen despite all efforts, call for a thorough, in-depth investigation of the interaction of host and pathogen. It is likely that here gene-based breeding strategies need to be complemented by trait-based approaches. A much better understanding of the genetic basis of virulence and its expression as well as the population structure of the pathogen is required.

Trait-based breeding, however, requires trait dissection into components, defined genetic sources for these components and conditions to test for them. Moreover, well-tailored segregating populations are needed for pyramiding of resistance genes or QTLs controlling diseases. This is where molecular breeding enters the stage. STMS markers remain the marker of choice for many breeding programs and have been particularly useful for the comparison of chickpea maps among laboratories. Clearly, more markers are needed to cover the whole genome with a dense network to dissect adjacent QTLs into single factors, a prerequisite for pyramiding of genes positively influencing one or more characters. However, this network of markers should not be based solely on anonymous dominant or even STMS markers, but on gene-based markers, wich could allow us to compare maps from chickpea and related legumes. Thus, chickpea scientists will certainly benefit from the progress achieved in the model legumes *Medicago truncatula* and *Lotus japonicus*, or better investigated crops like soybean or pea.

Transcriptomics applied to chickpea has revealed an insight into mechanisms of drought tolerance/avoidance and pathogenesis-related and developmental processes. The future will certainly see much more impact of transcriptomics in chickpea breeding including application of microarrays. Thus, it will soon be possible to identify genes controlling complex traits by simply hybridising cDNAs to specialised chips. If combined with maps generated from markers detecting SNPs in differentially expressed genes such as single nucleotide amplification polymorphism markers (SNAP; Drenkard et al., 2000; Hayashi et al., 2004), it may be possible to land directly in the gene of interest as demonstrated for mouse (Schadt et al., 2003). The determination of genetic variability in these genes in the chickpea germplasm will then be the next step towards targeted molecular breeding and more efficient germplasm management.

Efforts to employ MAS have been initiated. However, the following aspects require more research: (i) the saturation of genomic areas of interest with markers, and their polymorphism in different genetic backgrounds (especially within *C. arietinum*); (ii) the mapping of resistance genes for fusarium wilt still not sited in current genomic maps which require the development of new RIL populations; (iii) the integration of genes or QTL controlling ascochyta blight resistance already located in different populations while simultaneously considering the importance of pathotype differentiation for this pathogen; and (iv) mapping and integration into the current genomic map of genes or QTL controlling tolerance to abiotic stresses such as chilling and freezing, drought, earliness and salinity, in addition to molecular markers tagging these traits. Insufficient efforts have been undertaken to target resistances to minor pests and diseases. Current mapping populations, available through the International Chickpea Genomics Consortium (http://www.icgc.wsu.edu/), should be evaluated for these traits in the first instance. Alternatively, new mapping populations segregating for these traits could be developed. Infestation of developing chickpea seeds by *Helicoverpa* larvae is a problem of pressing demand for increasing chickpea yield. However, this problem may be difficult to address by conventional breeding, since neither trypsin nor gut proteinase inhibitors that could prevent the larvae from feeding are available from chickpea or from wild *Cicer* species. Although there is now some evidence of antibiosis in several species of *Cicer* (Sharma et al., 2005), transgenic approaches may be required to develop new cultivars resistant to this ferocious pest (Patankar et al., 1999).

The development of molecular markers and of even more efficient tools in plant breeding will continue to be a very dynamic process in the coming years. Chickpea breeders look forward to a time when the presence of a gene is identified directly from a sample of a plant or its seed without resorting to the lengthy process of screening for physical and chemical characteristics. It is hoped that chickpea breeders, molecular biologists, geneticists and physiologists coordinate their effort to integrate and use molecular techniques along with classical breeding methods for chickpea improvement.

Acknowledgements

Univ. of Córdoba research was supported by CICYT (Contract No. AGL2001-2018-C02-01) and EU (Ascorab, Contract No. ICA4-CT-2000-30003). CLIMA research on chickpea is supported by GRDC, ACIAR and COGGO. Research of PW and GK was supported by the Bundesminister für wirtschaftliche Zusammenarbeit (BMZ, Project No. 2001.7860.8-001.00), EU (IP Grainlegumes, Contract No. Food-CT-2004-506223) and IAEA (Research Contract 10974/R2).

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