FINE MAPPING OF THE "*QTL-hotspot*" REGION FOR DROUGHT TOLERANCE IN CHICKPEA (*Cicer arietinum* L.)

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CERTIFICATE

This is to certify Ms. Deepa Jaganathan has carried out the research work embodied in the present thesis entitled "**Fine mapping of the "*QTL-hotspot*" region for drought tolerance in chickpea (*Cicer arietinum* L.)**" for the degree of Doctor of Philosophy under the joint-supervision of Dr. Rajeev K Varshney, Research Program Director, Grain Legumes, Director, Center for Excellence in Genomics (CEG), ICRISAT, Patancheru and Prof. PB Kavi Kishor, Department of Genetics, Osmania University, Hyderabad.

This is an original work carried out at ICRISAT and is satisfactory for the award of Doctor of Philosophy. Any part of this work has not been submitted for the award of any degree or diploma of any other university or institute.

Dr. Rajeev K Varshney _____

Supervisor

Prof. PB Kavi Kishor _____

Co-supervisor

DECLARATION

I hereby declare that the research work presented in this thesis entitled "**Fine mapping of the "*QTL-hotspot*" region for drought tolerance in chickpea (*Cicer arietinum* L.)**" has been carried out under the supervision of Dr. Rajeev K Varshney at International Crops Research Institute for the Semi-Arid tropics (ICRISAT), Patancheru and co-supervision of Prof. PB Kavi Kishor at Department of Genetics, Osmania University, Hyderabad.

This work is original and no part of the thesis has been submitted earlier for the award of any degree or diploma of any University.

Date: **(Deepa Jaganathan)**

Place: Hyderabad

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Dedicated to my dearest and respectful grandfather
Late Mr. B. A. Palaniappan, who was my inspiration
and my first teacher!

CONTENTS

ABBREVIATIONS

100-SDW	: 100-seed weight
°C	: degree Celsius
µl	: microliter
χ^2	: Chi square
ABA	: Abscisic acid
AFLP	: Amplified Fragment Length Polymorphism
BAC	: Bacterial Artificial Chromosome
BES	: BAC-end Sequences
BIBAC	: Binary Bacterial Artificial Chromosome
BLAST	: Basic Local Alignment Search Tool
BM	: Biomass
bp	: base pair
BP	: Biological Processes
CAPS	: Cleaved Amplified Polymorphic Sequences
CaM	: *Cicer arietinum* Microsatellite
CC	: Cellular Components
cDNA	: complementary DNA
CKAM	: Chickpea KASPar assay markers
CIM	: Composite Interval Mapping
cM	: centiMorgan
COS	: Conserved Orthologous Set
CTAB	: Cetyl-trimethyl-ammonium Bromide
DArT	: Diversity Array Technology

DAS : Days after Sowing

dCAPS : derived Cleaved Amplified Polymorphic Sequences

DC : Delta Carbon ratio

DF : Days to 50% Flowering

DM : Days to Maturity

DNA : Deoxyribonucleic Acid

DREB : Drought Responsive Element Binding protein

DSI : Drought Susceptibility Index

DTI : Drought Tolerance Index

DUG : Durgapura

EDTA : Ethylenediaminetetraacetic acid

EST : Expressed Sequence Tag

EST-SSR : Expressed Sequence Tag-Simple Sequence Repeat

E-QTL : Epistatic QTL

FAO : Food and Agriculture Organization of the United Nations

GBS : Genotyping-by-sequencing

Gb : gigabases (one billion bases)

GCP : Generation Challenge Programme

G × E : Genotype × Environment

GMM : Genic Molecular Marker

GO : Gene Ontology

GWAS : Genome Wide Association Studies

HI : Harvest Index

HIR : Hiriyur

Hrs : Hours

ICCM : ICRISAT Chickpea Microsatellite

ICRISAT : International Crops Research Institute for the Semi-Arid Tropics

ISSR : Inter Simple Sequence Repeat

KASPar : KBioscience Competitive Allele-Specific Polymerase chain reaction

Kbp : kilo base pairs

Kg : kilogram

LDW : Leaf Dry Weight

LG : Linkage Group

LOD : Logarithm of odds (base 10)

M : Molar

MAF : Minor Allele Frequency

MAS : Marker Assisted Selection

Mb : Million bases

MF : Molecular Function

Mg : Milligram

MJ : Megajoule

ml : millilitre

mM : millimolar

Mt : metric ton

M-QTL : Major effect QTL

NAN : Nandyal

NaCl : Sodium Chloride

NaOAc : Sodium Acetate

NCBI : National Center for Biotechnology Information

NGS : Next-Generation Sequencing

ng : nanograms

NIL : Near Isogenic Lines

PAT : Patancheru

PBS : Primary branches

PCR : Polymerase Chain Reaction

PHT : Plant height

pH : logarithmic measure of hydrogen ion concentration

POD : pods/plant

PVE : Phenotypic Variation Explained

Q score : Phred quality score

QTL : Quantitative Trait Locus

RAD : Restriction site Associated DNA

RAPD : Random Amplified Polymorphic DNA

RDp : Rooting Depth

RDW : Root Dry Weight

RECORD : REcombination Counting and ORDering

RFLP : Restriction Fragment Length Polymorphism

RGA : Resistance Gene Analogues

RIL : Recombinant Inbred Line

RL : Root Length

RLD : Root Length Density

rpm : revolutions per minute

RSA : Root Surface Area

RT : Root Dry Weight/Total Dry Weight ratio

RT-PCR : Real-Time PCR

RV : Root Volume

RWL : Leaf water loss

RWC : Relative Water Content

SAD : Sum of Adjacent Distances

SCAR : Sequence Characterized Amplified Region

SDW : Shoot Dry Weight

SHE : Sehore

SNP : Single Nucleotide Polymorphism

SOAP : Short Oligonucleotide Analysis Package

SPD : seeds/pod

SSR : Simple Sequence Repeat

StDW : Stem Dry Weight

STMS : Sequence Tagged Microsatellite Sites

TE : Tris (10 mm)-EDTA (1 mm)

TIC : TIME FOR COFFE

TOG : Tentative Orthologous Genes

WGRS : Whole Genome Re-Sequencing

w/w : weight/weight

w/v : weight/volume

YLD : Yield

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ABSTRACT

Chickpea (*Cicer arietinum* L.) is the third most important grain legume cultivated in the arid and semi-arid regions of the world. Drought is one of the major constraints leading up to 50% production losses in chickpea. In order to understand the basics of drought tolerance, two recombinant inbred line (RIL) mapping populations (ICC 4958 × ICC 1882 and ICC 283 × ICC 8261) segregating for root traits were developed and a promising "*QTL-hotspot*" region was reported on these populations. With an objective to fine map this region, two approaches were adopted, i) genotyping-by sequencing (GBS) and ii) skim sequencing. GBS approach enabled identification of 828 single nucleotide polymorphism (SNP) markers. A high-density genetic map was developed, comprising 1,007 marker loci including 49 SNP markers in the "*QTL-hotspot*" region and spanning a distance of 727.29 cM. QTL analysis using the extended genetic map along with precise phenotyping data generated earlier, re-estimated the "*QTL-hotspot*" from 29 cM to 14 cM. In addition, these 49 SNPs were converted into cleaved amplified polymorphic sequence (CAPS)/derived CAPS (dCAPS) markers which can be used in marker assisted breeding. An ultra-high-density bin map was developed using 53,223 SNPs obtained through skim sequencing approach and its analysis with the phenotyping data, split the "*QTL-hotspot*" region into two sub-regions namely "*QTL-hotspot_a*" of 139.22 kb with 15 genes and "*QTL-hotspot_b*" of 153.36 kb with 11 genes. To validate and find more recombination in these regions, a large mapping population was developed. Flanking SNP markers of the two regions were converted to KASPar assays and screened on 1,911 F_2 lines. Progeny testing on $F_{2:3}$ lines revealed the role of "*QTL-hotspot_a*" in controlling 100-SDW. A total of 15 candidate genes were reported in this region. In summary, the refined region will help in precise introgression of the "*QTL-hotspot*" in breeding program for yield improvement under drought conditions and the reported genes can be used for further cloning studies to dissect the molecular basis of drought tolerance in chickpea.

INTRODUCTION

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the world's third most important food legume crop after common bean (*Phaseolus vulgaris* L.) and soybean (*Glycine max* L.) and cultivated primarily on marginal lands in the arid and semi-arid regions of South Asia and Sub-Saharan Africa. It is a self-pollinated species with eight chromosomes (Arumuganathan and Earle 1991) and genome size of 738 Mb (Varshney et al. 2013a). Globally chickpea is cultivated on 13.5 Mha with an annual production of 14.23 Mt (FAO 2014). It is a rich source of protein (20-30%), carbohydrates (~40%) and minerals, and thus serves as an important sources of nutrients in vegetarian diets, especially in developing countries (Jukanti et al. 2012). It is a member of Leguminosae family, which includes 18,000 species, grouped into 650 genera (http://www.ildis.org/Leguminosae). Like other legumes, chickpea can fix atmospheric nitrogen through symbiosis with rhizobia. The two well-known types of cultivated chickpeas are, *desi*, which is a smaller, brown tough coated seed and *kabuli* which is a bold and cream soft coated seed. In India, the *desi* cultivar accounts for nearly 90% of total area under cultivation and the remaining area is occupied by the *kabuli* type (Pundir et al. 1985). Over 95% of chickpea production and consumption occur in developing countries, most of them are grown in rainfed conditions. India is the largest producer of chickpea (~43%), followed by Australia, Pakistan, Turkey and Myanmar (FAO 2014).

Legume crops production is affected extremely due to increasing water limitation and recent persisting fluctuations in rainfall conditions (Daryanto et al. 2015). Chickpea production is affected by various abiotic and biotic stresses. Among abiotic stresses, drought causes heavy yield losses to chickpea. As chickpea is predominantly cultivated on residual soil moisture, terminal drought is a serious problem and will become more prevalent due to climate changes and global warming (Tuberosa 2012). During terminal drought stress, the crop suffers at

grain filling stages due to less or no water availability, which directly affects the yield (Zaman-Allah et al. 2011). In the past three decades, there has been a shift in cultivation of chickpea from cooler to warmer regions both in Asia and Africa (Kimurto et al. 2014; Krishnamurthy et al. 2013) which has also increased the impact of drought on crop productivity. Therefore, the current focus in different breeding programs is to enhance drought tolerance in chickpea to stabilize yield and increase production.

Yield losses due to terminal drought can be overcome through combined approaches of genomics with breeding and physiological methods, known as 'genomics assisted breeding' (Varshney et al. 2005). It is essential to identify quantitative trait loci (QTLs) or genes that confer drought tolerance, which can be deployed in breeding programmes to generate superior cultivars. Marker assisted selection (MAS) methods have proven to accelerate the process of variety development (Ibitoye and Akin-Idowu 2010). However, breeding efforts towards developing drought tolerant chickpea varieties has remained slow, mainly because of precision issues in phenotyping for drought, low genetic base and limited availability of genomic resources. Nevertheless, in recent years, the availability of large-scale genomic resources and physiological methods with high throughput phenotyping and analyzing methods have facilitated progress towards understanding the genetic basis of drought tolerance in chickpea. Various studies have mapped QTLs for different drought component traits like flowering time, seed weight, plant height, root length etc. (Or et al. 1999; Cho et al. 2002; Rehman et al. 2011; Varshney et al. 2014; Bajaj et al. 2015).

Recent advances in sequencing technology provide a cost effective way to develop thousands of single nucleotide polymorphisms (SNPs) in a limited period of time on large mapping populations, by sequence-based genotyping methods (Cronn et al. 2008). One such technique, genotyping-by-sequencing (GBS) offers simultaneous SNP detection and genotyping, therefore is now being used in several crops for diversity assessment, trait mapping, genome-

wide association studies (GWAS) and genomic selection (Elshire et al. 2011; Deschamps et al. 2012; Poland et al. 2012). GBS approach, however, suffers from the limitation of missing regions of the genome and bias based on methylation and restriction site abundance (Chen et al. 2014). An alternate approach to overcome missing data challenges is whole genome re-sequencing (WGRS) wherein samples can be sequenced at greater depth thereby reducing the missing data issue. To save the cost of sequencing, WGRS can be done at a lower depth and in that scenario the approach is referred to as skim sequencing (Golicz et al. 2015). This approach is very useful to identify sequence variants/SNPs in species where the reference genome sequence is available. However, the SNPs identified using next-generation sequencing (NGS) technologies cannot be directly used for QTL studies as: i) NGS technologies are prone to small but unrecoverable sequencing errors and therefore an individual SNP site cannot be used as a reliable marker for genotyping, ii) it is very difficult to score all SNP sites in an entire recombinant population, and iii) limitations of QTL analysis software to handle such a huge dataset. To address these issues, a parent dependant sliding window approach was used to identify true recombination breakpoints and to construct a recombination bin map using SNP data of an entire recombinant population in rice (Huang et al. 2009).

Recently, Varshney et al. (2014) reported 45 robust main-effect QTLs (M-QTLs; QTLs which explain >10% phenotypic variation (PVE)) and 973 epistatic QTLs (E-QTLs) explaining 58.2% and 92.19% PVE respectively, using two intra-specific recombinant inbred line (RIL) mapping populations (ICC 4958 × ICC 1882, referred as ICCRIL03, where ICCRIL denotes ICRISAT Chickpea RIL, 03 denotes population number and ICC 283 × ICC 8261 referred as ICCRIL04). In addition, the study also revealed nine QTL clusters including a genomic region on linkage group 4 (CaLG04) referred to as "*QTL-hotspot*", harboring several QTLs for drought tolerance related traits. Generally QTL analysis on bi-parental

populations accounts for the QTL represented by the specific allelic segregation which may not be identified in other population (Holland 2007; Jamann et al. 2015). In this context, "*QTL-hotspot*" region was considered to be an important region for improving drought component traits in chickpea, as it was also identified in another intra-specific mapping population (ICC 283 × ICC 8261). However, the "*QTL-hotspot*" was large (~29 cM on the genetic map and 7.74 Mb on the physical map) (Varshney et al. 2014) and contained only few SSR markers, making it difficult to identify polymorphism between the recurrent and target genotypes in a backcrossing strategy and also to identify genes associated with drought tolerance in this region (Thudi et al. 2014). Enriching this region with additional markers will facilitate fine mapping and precision breeding for drought tolerance. Further, dissection of this region will enable identification of candidate genes for drought tolerance that can be deployed for chickpea crop improvement.

In order to fine map the "*QTL-hotspot*" region and identify candidate genes, this study was planned to propose with the following objectives:

1. SNP discovery and genotyping of the RIL population (ICC 4958 × ICC 1882)
2. Marker saturation in the "*QTL-hotspot*" region
3. Development of high resolution mapping population
4. Identification of the candidate gene(s) for drought component traits

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Chickpea

2.1.1 Origin and history

Chickpea (*Cicer arietinum* L.) is one of the oldest and most used legumes in the Middle and Far East. Its domestication started before 12,000-10,000 years ago in the Fertile Crescent. It was cultivated mainly in the semi-arid and temperate to warm regions of the Old World (Zohary and Hopf 1973; van der Maesen 1972). Chickpea was introduced by Spanish and Portuguese to the new world in 16th century and *kabuli* types moved to India through Mediterranean in 18th century. *Cicer reticulatum* is the wild relative of domestic chickpea and it was cultivated in South-East Turkey. Chickpea is preferably cultivated during summer in Middle East and North America whereas it is cultivated during dry winter in India while in Australia it is grown during winter (Redden and Berger 2007).

2.1.2 Taxonomy and types

Wild and cultivated chickpeas are in the genus *Cicer* which comprises of 44 species among which 9 are annual and 35 are perennial. *Cicer* belongs to the family, Fabaceae under *Cicerae* tribe. The only domesticated member of *Cicer* is *Cicer arietinum* which is a self-pollinated, diploid crop with 16 chromosomes. Pollination is completed within the bud before flowering and usually one seed is set per pod. Cultivated chickpea comprises of two genetically distinct sub-types, *desi* and *kabuli*. *Kabuli* types seem to be evolved from *desi* and the genetic diversity is comparatively high in *desi* than *kabuli* (Redden and Berger 2007).

2.1.3 Nutritional value

From the beginning of agriculture, grain legumes have been used extensively for human consumption and for animal feed. Chickpea plays a major role in vegetarian diets and it is a staple food in many parts of the world especially in Asian countries. Chickpea is a good source of energy (14 to 19 MJ/kg), protein (12.6 to 30.6%), carbohydrates (51 to 71%), minerals, vitamins, fiber and phytochemicals (Wood and Grusak 2007). Chickpea has been consumed in several forms; it has been consumed directly or by boiling, roasting, puffing, frying and also in the form of dhal, flour and sprout. Apart from its nutritional value, several reports have stated that in ancient times, chickpea has been used for medicinal purposes. For instance, Dodonaeus (16^{th} century) believed drinking of chickpea soaked water would cure kidney and bladder stones, the same water with rosemary would cure jaundice (van der Maesan 1987). Additionally, chickpea can meet around 80% of the nitrogen through symbiotic nitrogen fixation. Chickpea can fix up to 140 kg nitrogen/ha (Saraf et al. 1998).

2.2 Constraints for Chickpea Production

Though chickpea is grown in more than 50 countries worldwide, the major producers includes India, Australia, Pakistan, Turkey, Myanmar, Iran, Ethiopia, Canada and USA. The top five producers of chickpea in the world includes, India contributing 43.41% (6.18 Mt), followed by Turkey (0.58 Mt), Pakistan (0.58 Mt), Australia (0.33 Mt), and Iran (0.27 Mt) (FAO 2014). In spite of its high yield potential, chickpea production is majorly affected due to adverse biotic and abiotic stresses. Globally, abiotic stresses lead to major yield losses compared to biotic stresses. The important causative abiotic stresses include drought, heat, cold and salinity. The major biotic stresses include pod borer, ascochyta blight, fusarium wilt, botrytis grey mould, dry root rot, collar rot, foot rot and stem rot (Toker et al. 2007). Among the abiotic stresses, drought plays a major role in yield loss by making the crop loss up to

33% globally. In the case of biotic stresses, pod borer causes 21% of yield losses. Especially in India insect pest are estimated to cause 7-15% yield loss and fusarium wilt can cause up to 100% yield loss during favorable adverse conditions (Chattopadhyay and Mohapatra 2015).

2.2.1 Drought stress in chickpea

Drought is defined as a water stress due to inadequate rainfall or water supply in which the crop fails to meet its transpiration needs (Tuberosa 2012). It is not only determined by rainfall and also due to evaporation, soil water holding capacity and crop water requirement. It is the most important environmental stress in agriculture. Drought stress in chickpea is mainly due to the growing season and environment. Chickpea faces two types of drought stresses, (i) terminal drought - which occurs due to the depletion of water in soil especially during the end of the growing season and (ii) intermittent drought - which occurs due to insufficient rainfall during growing season (see Toker et al. 2007).

Chickpea is grown widely in the arid and semi-arid zones and ~90% of the world's chickpea is grown under rainfed conditions (Kumar and Abbo 2001). Especially in South Asia and Northern Australia, chickpea is predominantly grown in post rainy season where terminal drought is a major limiting factor of productivity. Terminal drought affects mainly during pod filling and seed development resulting in high yield losses up to 80% (Gaur et al. 2014). The yield loss can go up to 100% based on the genotype and type of drought experienced in the given environment. Drought accompanied with heat can cause severe yield losses due to shortening of growing season by affecting the yield component traits like biomass, pod number, seed number, seed weight and quality (Saxena 1993; Leport et al. 1999; see Toker et al. 2007).

Drought resistance mechanism includes drought escape, drought avoidance and drought tolerance. Drought escape can be achieved through earliness, early vigour and dehydration

postponement. Dehydration postponement is obtained by managing water uptake and thereby reducing water loss. Other water management technique includes osmotic adjustment, turgor potential, root length density, rooting depth and leaf types and movements (Turner et al. 2001).

Drought tolerance can be achieved by the ability of the cells to metabolize at low water status which can be obtained through membrane stability, lethal water potential, chlorophyll content, polyamine, brassinosteroids, jasmonates, phosphatidic and salicylic acids synthesis (Nayyar et al. 2005; Davies 1995).

2.3 Breeding for Drought Tolerance

Breeding for abiotic stresses is limited due to the quantitative nature of these stresses which is also affected by genotype-by-environment (G × E) interaction (Jha et al. 2014). Breeding for drought escape can be achieved by developing early maturing varieties where the plant flowers and completes the cycle before it faces the drought stress. Drought avoidance can be achieved by improved root traits whereas drought tolerance can be achieved by searching of germplasm for the donors with suitable drought tolerant traits. Though drought resistance is obtained through escape, avoidance and tolerance mechanisms, cases of selection for escape and avoidance are only reported (Saxena 1987; Kashiwagi et al. 2005). Wild species are the natural sources of stress resistance which are unnoticed and lost during crop domestication and selection, resulting in narrow genetic components (Zamir et al. 2001).

Several studies during the past three decades focused to determine the factors controlling drought tolerance in chickpea. For instance, Singh et al. (1997) screened around 4,000 chickpea lines to find the tolerant sources for drought in the spring chickpea in the Mediterranean. This study identified 19 drought tolerant lines among 4,165 lines screened during 1992 to 1995. These lines are being used for developing drought resistant cultivars. In

2002, Anbessa and Bejiga evaluated around 400 Ethiopian landraces for drought tolerance. This study reported two better performing resistance genotypes, ACC 41235 and ACC 209025 selected from 19 drought tolerant lines which were identified earlier by screening 482 genotypes. Smaller leaf area and root traits like root dry weight (RDW), root volume (RV) and rooting depth (RD) are the sources of resistance as these traits helps in reduced water loss and extensive soil moisture extraction respectively. Serraj et al. (2004) reported the evaluation of RIL population derived from a longer rooting genotype ICC 4958 and an agronomically important genotype Annigeri during 2001 and 2002. It was observed that high yielding lines were possessing longer roots, however general correlation was not observed between these two traits whereas, high shoot biomass and harvest index contributed to high yield.

At ICRISAT, screening of 216 genotypes including mini-core germplasm collection revealed two drought tolerant genotypes ICC 8261 and ICC 4958 possessing longer root length density (RLD). This study concluded deeper root system can be a trait of drought avoidance to improve yield under less soil moisture conditions and provides a baseline for breeding and selection for drought avoidance root traits in chickpea (Kashiwagi et al. 2005). Subsequent studies identified that wild species are more drought resistant than the cultivated lines including the highly tolerant ICC 4958 (Toker et al. 2007). Consideration of including such wild lines in breeding will contribute to developing best drought tolerant cultivars. Intra-specific mapping populations (ICC 4958 × ICC 1882 and ICC 8261 × ICC 283) segregating for root traits were also developed and used for drought tolerance studies (Gaur et al. 2008). Further, few studies reported some important physiological traits like excised leaf water loss (RWL), relative water content (RWC), membrane permeability, ascorbic acid, proline content to be considered for selection procedure (Gunes et al. 2008). In another study, two *desi* chickpeas, ACC 316 and ACC 317 for drought and heat tolerance were reported by screening

377 chickpea accessions (Canci and Toker 2009). This study also found seed weight as an early selection trait and reported phenological traits like days to flowering and maturity should be considered for drought avoidance. Root traits had been majorly focused in terminal drought tolerance of chickpea (see Kashiwagi et al. 2015).

2.4 Chickpea Genomics

2.4.1 Genomic resources for chickpea

Until late 1990s morphological and isozyme marker based maps were available for chickpea (Gaur and Slinkard 1990; Kazan et al. 1993). Weising et al. (1989, 1991, 1992) reported fingerprinting of *C. arietinum* and its wild relatives using oligonucleotide probes. In the following years, several studies reported the development of microsatellite markers in chickpea (Table 1). For instance, Sharma et al. (1995) studied the polymorphism of di-, tri- and tetra- nucleotide repeats and reported the use of these SSR (simple sequence repeat) markers in linkage mapping. Subsequently, development of STMS (sequence tagged microsatellite sites) and large set of SSR markers from bacterial artificial chromosome (BAC) and binary bacterial artificial chromosome (BIBAC) libraries were reported (Hüttel et al. 1999; Winter et al. 1999; Lichtenzveig et al. 2005; Nayak et al. 2010)

Along with microsatellite marker development, transcriptomic studies were also made available in the early 2000s. For instance, studies identified dehydration specific transcripts and few potential candidate genes for drought, heat and cold stress through transcriptional profiling of differentially expressed genes (Boominathan et al. 2004; Mantri et al. 2007). Consequently, development of EST-SSR markers from the EST libraries were also reported for drought and salinity stresses (Buhariwalla et al. 2005; Varshney et al. 2009). Further, sequencing and annotation of these libraries resulted in identification of stress specific genes and also resulted in the identification of SSR and SNP markers from the generated ESTs

(Table 1). Physical map of chickpea with 1,945 BAC/BIBAC contigs with average length of 559 kb was reported by Zhang et al. (2010). This map covered 1,088 Mb and this is the first genome wide physical map in chickpea. Further, the genome sequence of chickpea was made available in 2013 (Varshney et al 2013a; Jain et al. 2013).

Sequencing by synthesis methods greatly reduced the time and cost of SNP identification and genotyping. Various approaches were introduced in sequencing and library construction methods which were further improvised. For instance, the technique of reduced representation library in which the size reduction was carried out using restriction enzymes was introduced in 2007 and was further improved by adding barcode and multiplexing with Illumina sequencing technologies in the subsequent years (Miller et al. 2007; Baird et al. 2008). Genotyping-by sequencing (GBS) method was proposed by Elshire et al. (2011) which follows the same principle but includes high multiplexing and less complicated steps. GBS is widely accepted for various studies like genome wide analysis, QTL mapping, and diversity studies and also applied in different species like Lolium, barley, wheat etc. (Pfender et al. 2011; Chutimanitsakun et al. 2011; Poland et al. 2012). The notable advantage of GBS approach is that SNP discovery and genotyping can be done at the same time (Poland and Rife et al. 2012).

A large scale development of EST-SSR markers were reported from transcriptome assembly of chickpea using Roche ⁄ 454 technologies/ pyro sequencing for various tissues of drought stressed genotypes (Hiremath et al. 2011; Garg et al. 2011). Illumina ⁄ Solexa sequencing was carried out to identify drought responsive genes between drought sensitive line, ICC 1882 and drought tolerant line, ICC 4958 (Hiremath et al. 2011). Using these same genotypes further studies identified genes that were up and down regulated between contrast genotypes under terminal drought stress (Deokar et al. 2011) and a total of 3,062 unigenes were identified in this study and few selected genes were also validated by real-time polymerase

chain reaction (RT-PCR) and northern blotting. Thudi et al. (2011) reported the development of large number of SSR markers from bacterial artificial chromosome (BAC) end sequences (BES-SSR) and diversity array technology (DArT) markers. A large set of SNP markers (2,486) was reported by Hiremath et al. (2012) using Solexa/Illumina sequencing, amplicon sequencing of tentative orthologous genes, mining of expressed sequence tags and sequencing of candidate genes. Among which, 2,005 SNPs were successfully converted into KASPar assays. Golden gate assays for few of these reported SNPs (96) were made available for the effective use of these SNPs in future studies (Roorkiwal et al. 2013).

During last 5 years, the number of marker resources emerged in surplus amount due to sequencing approaches and advances in SNP identification methods (Azam et al. 2012; Roorkiwal et al. 2013; Agarwal et al. 2015; Kujur et al. 2015a). Whole genome re-sequencing (WGRS) and GBS approaches resulted in generating plenty of marker resources and the availability of genome sequences enhanced several re-sequencing and marker discovery studies. At ICRISAT, to catalogue such marker information, two databases CicArVarDB and CicArMiSatDB were recently developed which describes the SSR and SNP marker information identified in chickpea genome. CicArMiSatDB helps the users to mine and filter the SSR markers, whereas CicArVarDB helps to visualize the read and mapping quality, SNP marker locations, etc. through webBLAST and JBrowse (Doddamani et al. 2014, 2015).

2.4.2 Genetic linkage maps

Molecular markers based first linkage map was reported by Simon and Muehlbauer (1997). This map had ~90 markers on ten linkage groups representing eight chromosomes of chickpea with restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers along with isozyme and morphological markers. Followed by, ISSR (inter simple sequence repeat) marker based maps were reported on a RIL

population (ICC 4958 × PI 489777) developed for fusarium wilt resistance (Ratnaparkhe et al. 1998). Later, linkage maps of few hundred markers including ISSR, RGS, STMS, and SSR were reported (Winter et al. 1999; Flandez-Galvez et al. 2003) on inter and intra-specific mapping populations. Mapping of gene specific markers for plant defense response on an inter-specific map was reported using RIL population (ICC 4958 × PI 489777) by Pfaff and Kahl (2003).

Further, studies reported the construction of consensus map for two or more mapping populations to avail the marker information on different maps. For instance, Cobos et al. 2005 reported the construction of consensus map for two intra-specific RIL populations (CA2156 × JG 62 and CA2139 × JG 62) with 160 markers spanning 427.9 cM. A consensus map of 10 mapping populations including 5 wide crosses (*C. arietinum* × *C. reticulatum*) and 5 narrow crosses (*desi* × *kabuli*) were constructed with 555 markers spanning 652.67 cM on wide crosses and with 299 loci spanning 426.99 cM on narrow crosses (Milan et al. 2010).

An inter-specific map on a RIL population (ICC 4958 × PI 489777) with 521 loci spanning 2,602 cM was constructed by integration of newly developed SSR markers and SNP markers (Nayak et al. 2010). A high density genetic linkage map with 1,291 loci was constructed by Thudi et al. (2011) which spanned a distance of 845.56 cM with DArT and BES-SSR markers. Further, SNP markers were integrated into this map which had 1,328 loci including 625 CKAM (chickpea KASPar assay markers) and 317 TOG (tentative orthologous genes) SNP and 396 published loci spanning 788.6 cM (Hiremath et al. 2012). A consensus map on two intra-specific mapping populations was reported by Varshney et al. (2014). This map includes 352 loci spanning 771.39 cM including SSR, DArT and genic molecular markers (GMM). It was constructed using two individual maps, including a map on ICC 4958 × ICC 1882 with 241 loci spanning 621.51 cM and on ICC 283 × ICC 8261 with 168 loci spanning

533.06 cM. Availability of NGS technologies has resulted in identifying 1000s of SNP and developing ultra-high density maps (Kujur et al. 2015c; Table 2).

2.4.3 QTLs for drought tolerance

Drought is a complex trait, inherited quantitatively and can be improved through its component traits like days to flowering, days to maturity, seed weight, seed size, biomass, yield and root traits and other physiological traits like water use efficiency, stomatal conductance, transpiration efficiency, osmotic adjustment, relative water content, canopy temperature and drought sensitivity index. Here listed are few studies in which QTLs for these traits were mapped in chickpea to improve yield under drought conditions. For instance, Cho et al. (2002) mapped a major gene for pigmentation on linkage group (LG) 8, four QTLs for 100-seed weight (100-SDW) on LG4 and LG9, one QTL for seed number on LG4 and a QTL for days to 50% flowering on LG3. Further a consensus map on two RIL populations, JG 62 × Vijay and Vijay × ICC 4958 was reported and used for QTL studies (Radhika et al. 2007). QTL analysis using these maps identified 8 QTLs for seed weight on LG1, LG3 and LG4. In another study, QTL for seed weight was mapped on three linkage groups (LG1, LG6 and LG4B) and the most significant QTL was found on LG4B flanked by GA24 and STMS11 (Abbo et al. 2005). This study also mapped QTLs for beta carotene on LG1, LG2, LG3 and LG4 and for lutein concentration on LG1 using F_2 population (Hadas × Cr205). Seed size and seed weight, are the most studied traits in chickpea. For instance, Cobos et al. (2007) mapped two QTLs for seed size on LG4 and LG8, and one QTL each for yield and days to flowering on LG4. Two major QTLs for seed weight were reported by Cobos et al. (2009) on LG4 and LG2. QTL on LG4 was flanked by GAA47 and STMS 11 with high phenotypic variation of 32%. This QTL was reported to be the same QTL identified earlier by Cho et al. (2002); Abbo et al. (2005); Cobos et al. (2007). Additionally, QTLs for seed coat thickness and flowering time were mapped on LG4 and LG3A respectively.

Table 1: List of molecular markers available for chickpea

Marker	Number of markers	Reference
Microsatellite markers		
SSR[1]	38	Sharma et al. 1995
STMS[2]	28	Hüttel et al. 1999
SSR	174	Winter et al. 1999
SSR	233	Lichtenzveig et al. 2005
STMS	13	Choudhary et al. 2006
SSR	92	Sethy et al. 2006a
SSR	11	Sethy et al. 2006b
SSR	311	Nayak et al. 2010
SSR	6,845	Thudi et al. 2011
SSR	728	Hiremath et al. 2011
STMS	181	Gaur et al. 2011
Functional markers		
EST-SSRs		
EST-SSR[3]	106	Buhariwalla et al. 2005
EST-SSR	64	Qadir et al. 2007
EST-SSR	60	Choudhary et al. 2009
EST-SSR	177	Varshney et al. 2009
EST-SSR	51	Gujaria et al. 2011
EST-SSR	202	Agarwal et al. 2015
SNPs		
CAPS[4]	73	Varshney et al. 2007
SNP[5]	21,405	Varshney et al. 2009
SNP	71	Nayak et al. 2010
SNP	495	Hiremath et al. 2011
CAPS	143	Gujaria et al. 2011
DArT[6]	5,397	Thudi et al. 2011
SNP	2,005	Hiremath et al. 2012
SNP	4,543	Azam et al. 2012
SNP	96	Roorkiwal et al. 2013
SNP	44,844	Kujur et al. 2015a
SNP	43,358	Kujur et al. 2015c
Others		
CISR[7]	87	Gujaria et al. 2011
ISR[8]	2,088	Hiremath et al. 2011
COS[9]	387	Hiremath et al. 2011

SSR[1]- simple sequence repeats; STMS[2]- sequence tagged microsatellite sites; EST-SSR[3]- expressed sequence tag-SSR; CAPS[4]- cleaved amplified polymorphic sequences; SNP[5]- single nucleotide polymorphism; DArT[6]- diversity array technology; CISR[7]- chickpea intron spanning region; ISR[8]- intron spanning region; COS[9]- conserved ortholog set.

A subsequent study on drought tolerance related traits by Rehman et al. (2011) identified 15 genomic regions, controlling drought related traits like harvest index, days to flowering, days to maturity, stomatal conductance, canopy temperature, by developing a linkage map and QTL analysis using ILC 588 × ILC 3279 RIL population. On the same population, Hamwieh et al. (2013) studied 12 drought related traits including drought resistance score, 100-seed weight, plant height, days to flowering, days to maturity, seed number, biological yield, percentage of empty pods, number of empty pods, harvest index, pod number and grain yield. In this study, LG3 and LG4 were found to contain QTLs for many traits and were reported to have pleiotropic effects. In another study, 45 QTLs for 20 drought component traits on two intra-specific RIL mapping populations namely ICC 4958 × ICC 1882 (ICCRIL03) and ICC 283 × ICC 8261 (ICCRIL04) were reported (Varshney et al. 2014). This study also reported nine important QTL clusters scattered on chickpea genome for 12 out of 20 traits studied which included a promising "*QTL-hotspot*" region on LG4 which explains a highest PVE of 58.20%. This region was used in molecular breeding program and successfully introgressed into the chickpea elite line JG11 (See Varshney et al. 2013b). The availability of SNP based ultra-high density maps allow to detect QTLs for any trait within the shortest defined locations (<1 cM). A high density map with >4,000 markers was constructed on RIL population (ICC 12299 × ICC 8261) using GBS based SNPs identified by mapping reads on *desi* genome, similarly >2,500 SNPs were mapped on the same population using SNPs identified through mapping of *kabuli* genome (Kujur et al. 2015b, c) (Table 3).

Table 2: List of linkage maps available for chickpea

Mapping population	Population	Number of markers	Map length (cM)	Reference
Inter-specific cross				
ICC 4958 × PI 489777	RIL[#]	112	613.00	Winter et al. 1999
ICC 4958 × PI 489777	RIL	354	2077.90	Winter et al. 2000
FLIP 84-92C × PI 599072	RIL	167	1174.50	Tekeoglu et al. 2002
ICC 4958 × PI 489777	RIL	296	2483.30	Pfaff and Khal 2003
Hadas × Cr205	F_2	72	344.60	Abbo et al. 2005
ICCL 81001 × Cr5-9	RIL	121	848.10	Cobos et al. 2009
ICC 4958 × PI 489777, FLIP 84-92C × PI 599072, Hadas × Cr205, ILC 72 × Cr5-10 and ICCL 81001 × Cr5-9	RIL, F_2	555	652.67	Milan et al. 2010
ICC 4958 × PI 489777	RIL	521	2602.00	Nayak et al. 2010
ICC 4958 × PI 489777	RIL	1,291	845.56	Thudi et al. 2011
ICC 4958 × PI 489777	RIL	300	766.56	Gujaria et al. 2011
ICC 4958 × PI 489777	RIL	1,328	788.60	Hiremath et al. 2012
Intra-specific cross				
ICCV 2 × JG 62	RIL	111	297.00	Cho et al. 2002
ICC 12004 × Lasseter	RIL	66	534.50	Flandez-Galvez et al. 2003
CA2156 × JG 62 and CA 2139 × JG 62	RIL	160	427.90	Cobos et al. 2005
JG 62 × Vijay and Vijay × ICC 4958	RIL	230	739.60	Radhika et al. 2007
ICCV 96029 × CDC Frontier	F_2	144	1285.00	Anbessa et al. 2009
ICCV 96029 × CDC Luna	F_2	76	497.00	Anbessa et al. 2009
ICCV 96029 × CDC Corinne	F_2	61	391.00	Anbessa et al. 2009
ICCV 96029 × Amit	F_2	42	276.00	Anbessa et al. 2009
ICCV 2 × JG 62, ILC 3279 × WR 315, WR 315 × C104, CA 2139 × JG 62 and CA 2156 × JG 62	RIL	229	426.99	Milan et al. 2010
ICCV 2 × JG 62	RIL	138	630.90	Garg et al. 2011
C 214 × WR 315	F_2	57	347.90	Sabbavarapu et al. 2013

C 214 × ILC 3279	F_2	58	386.30	Sabbavarapu et al. 2013
ILC 588 × ILC 3279	RIL	99	157.60	Rehman et al. 2011
ILC 3279 × ICCV 2	F_3	57	379.47	Jamalabadi et al. 2013
ILC 588 × ILC 3279	RIL	77	520.00	Hamwieh et al. 2013
ICC 4958 × ICC 1882	RIL	241	621.51	Varshney et al. 2014
ICC 283 × ICC 8261	RIL	168	533.06	Varshney et al. 2014
ICC 4958 × ICC 1882, ICC 283 × ICC 8261	RIL	352	771.39	Varshney et al. 2014
ICC 6013 × ICC 7346	F_4	292	785.60	Kujur et al. 2015b
ICCV 2 × JG 11	RIL	56	329.60	Pushpavalli et al. 2015
ICC 12299 × ICC 8261	RIL	4,448	714.09	Kujur et al. 2015c
ICC 12299 × ICC 8261	RIL	2,689	798.47	Kujur et al. 2015c

RIL[#]- recombinant inbred lines

Table 3: List of mapped QTLs for drought tolerance related traits

Mapping population	Trait	Linkage group (LG)	Reference
Inter-specific cross			
Hadas × Cr 205	Seed weight	LG1, LG6, LG4B	Abbo et al. 2005
ICCL 81001 × Cr 5-9	Flower colour	LG3A	Cobos et al. 2009
	Seed size	LG4, LG2	
	Seed coat thickness	LG4	
Intra-specific cross			
ILC 588 × ILC 3279	Harvest index	LG1, LG3	Rehman et al. 2011
	Days to flowering	LG1, LG3, LG4, LG8	
	Stomatal conductance	LG3, LG7	
	Days to maturity	LG1, LG3, LG7	
	Cooler canopies	LG1, LG3, LG4, LG6	
	Grain yield	LG1, LG4	
	Drought tolerance score	LG1, LG3, LG7, LG8	
	Reproductive period	LG1, LG3, LG7	
	Plant height	LG1, LG4	
ILC 3279 × ICCV 2	Days to flowering	LG3	Jamalabadi et al. 2013
	Plant height	LG3	
	100-seed weight	LG4	
ILC 588 × ILC 3279	Drought resistance score	LG3, LG4	Hamwieh et al. 2013
	100-seed weight	LG3, LG4, LG5, LG7	
	Pod number	LG3, LG4, LG7	
	Grain yield	LG3, LG4	
	Days to flowering	LG3, LG4	
	Plant height	LG1, LG2, LG3, LG7	
	Days to maturity	LG1, LG2, LG3, LG4	
	Harvest Index	LG1, LG2, LG3, LG4, LG5	
	Percentage of empty pods	LG1, LG2	
	Empty pods	LG2, LG3	

	Seed number	LG3, LG4	
	Biological yield	LG3, LG7	
ICCV 2 × JG 62	Pigmentation	LG8	Cho et al. 2002
	100-seed weight	LG4, LG9	
	Seed number/plant	LG4	
	Days to 50% flowering	LG3	
JG 62 × Vijay and Vijay × ICC 4958	Seed weight	LG1, LG3, LG4	Radhika et al. 2007
ICC 4958 × ICC 1882	Root length density	LG4, LG6, LG7	Varshney et al. 2014
	Root dry weight	LG1, LG3, LG7	
	Root surface area	LG4, LG6	
	Root volume	LG6	
	R-T ratio	LG4	
	Shoot dry weight	LG4, LG6	
	Plant height	LG3, LG4, LG6, LG7, LG8	
	Plant width	LG5	
	Primary branches	LG6	
	Days to 50% flowering	LG3, LG4, LG6, LG7, LG8	
	Days to maturity	LG4, LG6, LG7, LG8	
	Pods/plant	LG4	
	Seeds/pod	LG3, LG4, LG7	
	100-seed weight	LG1, LG4	
	Biomass	LG1, LG3, LG4, LG7, LG8	
	Harvest index	LG1, LG3, LG4, LG6, LG8	
	Yield	LG1, LG3, LG4, LG6	
	Delta carbon ratio	LG4	
	Drought tolerance indices	LG1, LG4	

	Drought susceptibility indices	LG7, LG4	
ICC 283 × ICC 8261	Root dry weight	LG8	Varshney et al. 2014
	Rooting depth	LG3	
	R-T ratio	LG1, LG2, LG3, LG4, LG5	
	Shoot dry weight	LG4	
	Plant height	LG1, LG3,LG4, LG5, LG6, LG7, LG8	
	Plant width	LG8	
	Primary branches	LG6	
	Days to 50% flowering	LG1, LG2 LG3,LG4, LG5, LG6, LG7	
	Days to maturity	LG1, LG2 LG3,LG4, LG5, LG6, LG7	
	Pods/plant	LG4, LG6, LG8	
	100-seed weight	LG1, LG4, LG6, LG8	
	Biomass	LG6	
	Harvest index	LG6, LG8	
	Yield	LG1, LG2, LG3	
	Drought tolerance indices	LG1, LG5	
	Drought susceptibility indices	LG3	
	Pod number/plant	LG1, LG2, LG4, LG5,	
ICC 6013 × ICC 7346		LG6, LG7, LG8	Kujur et al. 2015b
	100-seed weight	LG3, LG5, LG6	
	Seed number/plant	LG2, LG3, LG4, LG5, LG7	
		LG1, LG4, LG5, LG6,	
ICC 12299 × ICC 8261	100-seed weight	LG7, LG8	Kujur et al. 2015c
	Pod number /plant	LG1, LG2, LG4, LG6, LG7, LG8	
	Seed number/plant	LG1, LG2, LG5, LG8	

2.5 Fine Mapping Studies

In order to develop resistant material for any biotic or abiotic stress, it is necessary to identify QTL or genes linked to the particular trait. This can be achieved through bi-parental linkage map based QTL analysis or linkage disequilibrium (LD) based association mapping. QTL analysis aims to identify the sequence or nucleotide which is responsible for the trait effect via fine mapping and QTL cloning (Salvi and Tuberosa 2005; Holland 2007). The following step after identifying a major QTL for the trait of interest is to refine the region into a short interval to identify the genes controlling the trait. In brief, the process of identifying the closest marker of the target gene is known as fine mapping. It is always a concern that which population should be used for fine mapping. Several studies suggested the development of large mapping population while few studies used backcross populations, and near isogenic lines for fine mapping (Fridman et al. 2000; Jander et al. 2002; Xu et al. 2006; Uga et al. 2013; Song et al. 2015). Usually, numerous individuals are screened with the flanking markers of the target QTL, followed by phenotyping of the selected recombinant plants and progeny testing. The shortest segment obtained through this process is cloned and candidate genes are identified, however if the genome sequence is available the candidate genes can be directly pinpointed through precisely locating the QTLs (Price et al. 2006). Recent advances in sequencing technologies avoid laborious time taking genotyping methods by generating large number of SNP markers for map construction. It allows QTL mapping to obtain the resolution of fine mapping (Jamann et al. 2015).

Though fine mapping studies had started in early 1990s, several advancements have been made in recent years. “pre-NGS” era is described as where fine mapping has been done without using any NGS-derived markers. In early 1990s, non-PCR based markers were extensively utilized for fine mapping. In general, for the development of new markers in a targeted QTL region, markers flanking the QTL regions are mapped to their physical

positions by which the respective clones on the physical map can be identified. The identified clones are used for developing molecular markers. However by using such markers, several studies could reach finer intervals but not into gene levels (Eshed and Zamir et al. 1995), though limited studies reported fine mapping using SNP markers obtained from BAC/YAC clones (Fridman et al. 2000). Such studies could reach gene level of refinement, showing the power of SNP markers in refining the larger intervals.

While comparing with non-NGS technology based cloning methods, the advent of NGS technologies has reduced the time span into nearly half to clone a gene. Before the rise of genome sequences, gene isolation was possible only by isolating the respective YAC/BAC clones. Rather, post-NGS era has an advantage in direct identification of candidate genes from the genome sequence as the genome sequence is available for most of the important crops. Additionally genotyping-by sequencing approach for the entire population allow identifying several millions of SNPs in less cost and time. The high density of marker data allows identification of markers near or within the gene of interest.

Before NGS era, it was considered that using conventional populations like F_2 and RIL is difficult to identify and refine QTLs (Eshed and Zamir 1995). Currently this scenario has changed with the abundance of sequencing technologies and the knowledge of sequence variation. Now it is possible to use these populations for accurate QTL mapping and fine mapping. Recently, several studies have reported fine mapping and identification of genes on RIL population (Xu et al. 2011; Qi et al. 2014). About a decade earlier, it was thought that identification of candidate genes in shorter time period was possible only for model crops. Whereas the current situation with the genomic advancement made sure even for complex crops it is possible to identify candidate genes in shorter period.

Fine mapping and QTL cloning are must in order to understand the functional mechanism of a trait and to precisely perform marker assisted selection (MAS). In fact, sequencing technologies have brought remarkable changes in genomics and breeding in the last 10 years. The amount of time taken for identifying candidate genes has reduced tremendously in recent years. The projects which were taking few years of time to fine map and identify a candidate gene are expected to take few months to a year with the availability of genome sequences and other developments in NGS technologies.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Generation of SNP Markers Using Genotyping-by Sequencing (GBS) Approach

3.1.1 Plant material

Based on screening of mini-core collection for drought tolerance-related root traits at ICRISAT, ICC 4958 (*C. arietinum*), a drought tolerant breeding line developed by Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur, Madhya Pradesh, India was found to possess larger root system, while ICC 1882 (*C. arietinum*) which is a landrace collected from India was found to possess shorter root system. These genotypes were assembled at ICRISAT's gene bank in 1973. These phenotypically and genetically distinct genotypes were used for developing an intra-specific RIL mapping population of 264 lines, namely ICCRIL03. This population which segregates for root traits was used in the current study.

3.1.2 High throughput DNA extraction

DNA isolation was carried out for RILs and the parental genotypes, using high throughput mini-DNA extraction method as described by Cuc et al. (2008). A total of 208 RILs with good quality DNA were used for further sequencing. The steps involved in DNA extraction protocol are explained below.

3.1.2.1 Sample preparation

- Leaves were harvested from 15 days old seedlings.
- 70-100 mg of leaf tissue was placed in 12 × 8-well strip tube with strip cap (Marsh Biomarket, USA) in a 96 deep-well plate together with two 4 mm stainless steel grinding balls (Spex CertiPrep, USA).

3.1.2.2 Cetyltrimethylammonium bromide (CTAB) extraction

- For each sample 450 µl of preheated (at 65 ºC for half an hour) extraction buffer (100 mM Tris-HCl (pH-8), 1.4 M NaCl, 20 mM EDTA, CTAB (2-3% w/v), β-mercaptoethanol) was added and secured with eight strip caps.
- Samples were homogenized in a GenoGrinder 2000 (Spex CertiPrep, USA), following the manufacturer's instructions, at 500 strokes/min for 5 times at 2 min interval.
- Plate was incubated at 65 ºC for 10 min with shaking at periodical intervals.

3.1.2.3 Solvent extraction

- For each sample, 450 µl of chloroform-isoamyl alcohol (24:1) was added and mixed thoroughly by inverting.
- Plate was centrifuged at 5,500 rpm for 10 min. The aqueous layer (300 µl) was collected.

3.1.2.4 Initial DNA precipitation

- 0.7 vol (210 µl) of isopropanol (stored at –20 ºC) was added to the collected aqueous layer and mixed thoroughly by inverting.
- Plate was centrifuged at 5,000 rpm for 15 min.
- Supernatant was discarded from each sample and pellet was air dried for 20 min.

3.1.2.5 RNase treatment

- 200 µl low salt TE (10 mM Tris EDTA (pH-8)) and 3 µl RNase (10 mg/ml) was added to each sample and incubated at 37 ºC for 30 min.

3.1.2.6 Solvent extraction

- 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added to each sample and inverted twice to ensure proper mixing.
- Plate was centrifuged at 5,000 rpm for 5 min.

- Aqueous layer from each sample was transferred to a fresh 96 deep-well plate.
- 200 µl of chloroform-isoamylalcohol (24:1) was added to the aqueous layer and inverted twice to ensure proper mixing.
- Plate was centrifuged at 5,000 rpm for 5 min. Aqueous layer was transferred to fresh 96 deep-well plate.
- A total of 315 µl ethanol-acetate solution (30 ml ethanol, 1.5 ml 3 M NaOAc (pH-5.2)) was then added to each sample and placed in 20 ºC for 5 min.
- Plate was again centrifuged at 5,000 rpm for 5 min.
- Supernatant was discarded from each sample and pellet was washed with 70% ethanol.
- Plate was centrifuged at 6,000 rpm for 10 min.
- Supernatant was again decanted from each sample. Samples were air dried for 1 hour.
- Pellet was re-suspended in 100 µl low-salt TE and stored at 4 ºC.

3.1.3 DNA quantification

Extracted DNA was quantified by loading the samples on 0.8% agarose gel containing 0.5 µl/10 ml ethidium bromide (10 mg/ml). DNA was normalized to 5 ng/µl concentration with visual comparison by loading DNA samples with the standard λ DNA molecular weight markers (2.5 ng/µl, 5 ng/µl, 10 ng/µl) on 0.8% agarose gel. DNA quantity was measured using spectrophotometer (Shimadzu UV160A, Japan).

3.1.4 Sequencing methods

The extracted DNA samples were subjected to genotyping-by sequencing (GBS) approach for SNP identification and genotyping as described by Elshire et al. (2011). In brief, GBS libraries from parental lines and RILs were prepared using *Ape*KI endonuclease (recognition site: G/CWCG) and sequenced using the Illumina HiSeq 2000 platform (Illumina Inc, San Diego, CA, USA). Genomic DNA of selected mapping population and parental lines was subjected to restriction digestion using endonuclease *ApeK*I for 2 hrs at 75 °C. Adapters with

unique multiplex sequence index (barcodes) were ligated to the sticky ends of the each sample using ligase buffer with ATP and T4 ligase. Reaction mix was incubated at 22 °C for 1 hr and heated to 65 °C for 30 min to inactivate the T4 ligase. Aliquots of each ligated sample (5 µl) were pooled (multiplexed) and purified to remove the excess adapters. DNA samples were eluted in a final volume of 50 µl.

PCR was performed to increase the restriction fragments from each library using primers complementary to corresponding adapters. The amplified pools constituting the "sequencing library" were cleaned up and evaluated for fragment sizes using a DNA analyzer. Libraries without adapter dimers were subjected to sequencing.

3.1.4.1 SNP calling

The reads obtained were first de-multiplexed according to the sample barcodes and adapter sequences were removed using custom perl script. The reads having more than 50% of low quality base pairs (Phred < 5%) were discarded and filtered data were used for calling SNPs after quality check (Q score > 20). The filtered, high quality data from each sample was aligned to the draft genome sequence (CaGAv1.0) of chickpea (Varshney et al. 2013a) using SOAP (Li et al. 2009). The nucleotide with highest probability at each position under a Bayesian model was identified for individual RILs and the consensus sequences were saved in FASTA format. Consensus sequences from all samples were compared to detect polymorphic loci. Polymorphic loci that were either heterozygous in any of the parents or present in < 50% individuals in the population were discarded and a high quality SNP dataset was generated.

3.1.5 Linkage mapping

Genotyping data for SNPs generated through GBS approach and genotyping data obtained from previous studies as mentioned in Varshney et al. 2014 were compiled for linkage analysis using JoinMap V4.0 (Van Ooijen and Voorrips 2006). Marker order was assigned

using regression mapping algorithm with maximum recombination frequency of 0.4 at minimum logarithm of odds (LOD) of 3 and jump threshold of 5. Ripple command was used after adding each marker locus to confirm marker order. The Kosambi mapping function was used to calculate the map distance (Kosambi 1943). To detect segregation distortion, chi square (χ^2) values were calculated using Joinmap V4.0. Highly distorted and unlinked markers were excluded from analysis. Mapchart 2.2 (Voorrips 2002) was used to visualize the constructed map for each linkage group. Linkage groups were named according to Varshney et al. (2014).

3.1.6 Phenotyping for root traits

Phenotyping data on the ICCRIL03 population was obtained from the earlier studies (Varshney et al. 2014). The procedure of phenotyping is described in the following sections.

Phenotyping data was collected for 20 drought tolerance traits including 6 root traits, 6 yield and yield related traits, 5 morphological traits, 2 phenological traits and one physiological trait in 1 to 7 seasons (2005, 2006, 2007, 2008, 2009, 2010 and 2011) at 1 to 5 locations in India namely Patancheru (PAT) located at 17.53°N 78.27°E, Nandyal (NAN) located at 15.48°N 78.48°E, Durgapura (DUG) located at 23.55°N 87.32°E, Hiriyur (HIR) located at 13.95°N 76.62°E and Sehore (SEH) located at 23.2°N 77.08°E. Details of the traits analyzed are provided in Table 4. Root traits namely, root dry weight (RDW), rooting depth (RD), ratio between RDW and total dry weight (R/T), root length density (RLD), root surface area (RSA) and root volume (RV) were measured in 2005 and 2007 with three replications by Physiology Division at ICRISAT, Patancheru.

For taking root related observations, chickpea plants were grown in PVC cylinders (18 cm diameter, 120 cm height) with three replications. The PVC cylinders, except the top 15 cm, were filled with an equi-mixture (w/w) of vertisol and sand, mixed with di-ammonium

phosphate at the rate of 0.07 g kg^{-1}. The soil water content of the mixture was equilibrated to 70% field capacity to create the conditions similar to those in the field at sowing time, where the soil is not fully saturated with water. A mixture of soil and sand was used to decrease the soil bulk density and facilitate root growth and extraction.

Plants were sampled at 35 days after sowing (DAS) avoiding any physical damage to plants, as previous studies showed that maximum variation in root dry weight and root length density among genotypes could be best noticed in Patancheru environment at 35 DAS, and that variation is reduced after 41 DAS (Krishnamurthy et al. 1996). The methods employed for recording the observations of the root traits are explained in brief in the following sections.

3.1.6.1 Stem dry weight (StDW)

The weight of dried stem (without leaves) was measured after drying the stem in hot air oven at 80 ºC for 72 hrs.

3.1.6.2 Leaf dry weight (LDW)

The weight of dried leaves (without stem) was measured after drying the leaves in hot air oven at 80 ºC for 72 hrs.

3.1.6.3 Shoot dry weight (SDW)

Total shoot which include stem and leaves of each plant were dried in a hot air oven at 80 ºC for 72 hrs. The weight of this dry matter was measured as SDW. SDW is the sum of StDW and LDW.

3.1.6.4 Root dry weight (RDW)

Root dry weight was recorded after drying the root in a hot air oven at 80 ºC for 72 hrs.

3.1.6.5 Rooting depth (RD)

Rooting depth was measured after removing the soil particles from the root system under running water. The roots were straightened and depth to which the roots had been penetrated was measured.

3.1.6.6 Ratio of RDW and total dry weight (R/T)

It is ratio between the RDW and the total dry weight. Total dry weight is the collective dry weights of shoot and root system of each plant.

3.1.6.7 Root length (RL)

The soil from the roots was removed by rinsing the roots in running water. The root length was measured by using a digital image analysis system (WinRhizo, Regent Instruments Inc., Quebec, Canada). The image of the root is taken and the total length is obtained by measuring the length of tap and branch roots of the chickpea root system.

3.1.6.8 Root length density (RLD)

The root length density (RLD) was calculated by dividing the total root length per cylinder by the cylinder volume at the maximum rooting depth.

3.1.6.9 Root surface area (RSA)

The root surface area was measured by using a digital image analysis system (WinRhizo, Regent Instruments Inc., Quebec, Canada).

3.1.6.10 Root volume (RV)

The root volume is the volume of the soil in the PVC cylinder, at the maximum rooting depth.

Table 4: Summary of traits evaluated at different locations and seasons

Trait	ICCRIL03 (ICC 4958 × ICC 1882)				
	2005*	2006*	2007*	2008*	2009**
Root traits#					
Root length density (RLD, cm cm^{-3})	PAT		PAT		
Root dry weight (RDW, g)	PAT		PAT		
Rooting depth (RDp, cm)	PAT		PAT		
Root surface area (RSA, cm^2)	PAT		PAT		
Root volume (RV, cm^3)	PAT		PAT		
Root dry weight / Total plant dry weight ratio (RTR, %)	PAT		PAT		
Morphological traits					
Shoot dry weight (SDW, g)	PAT		PAT		
Plant height (PHT, cm)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Plant width (PWD, cm)	PAT	PAT	PAT		
Primary branches (PBS)	PAT	PAT	PAT		
Secondary branches (SBS)	PAT	PAT	PAT		
Phenological traits					
Days to 50% flowering (DF)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Days to maturity (DM)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Yield and yield related traits					
Pods/plant (POD)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Seeds/pod (SPD)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
100-seed weight (100-SDW)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Biomass (BM, g)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Harvest index (HI, %)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Yield (YLD, g)	PAT	PAT	PAT	PAT,SEH,DUG,NAN	A
Transpiration efficiency related traits					
Delta carbon ratio ($\delta^{13}C$)				PAT,SEH,DUG	

#traits measured in PVC cylinders at rainout shelter. *measured in rainfed condition for the mentioned year. **measured in rainfed and irrigated condition.
A-Measured at all 5 locations (Patancheru, Sehore, Durgapura, Nandyal and Hiriyur).
PAT- Patancheru, SEH- Sehore, DUG- Durgapura, NAN- Nandyal.

3.1.7 QTL analysis

Genotyping data obtained from the current study along with the 318 markers generated earlier from Varshney et al. (2014) and the phenotyping data for 20 drought tolerance related traits obtained from Varshney et al. (2014) were used for QTL analysis using QTL Cartographer V.2.5 software (Wang et al. 2012). Composite interval mapping (CIM) was performed by selecting Model 6 with the default window size 10 cM, control marker number 5, and backward regression method. To obtain more precise results the default walk speed was reduced to 1 cM. LOD method (LOD >3) was used to determine the significance of each QTL interval with the threshold level performed at 1,000 permutations, significance level of $p \leq 0.05$.

3.1.8 Development of CAPS/dCAPS (cleaved amplified polymorphic sequences/derived cleaved amplified polymorphic sequences) markers

SNPs integrated in the "*QTL-hotspot*" region were converted to CAPS or dCAPS using dCAPS Finder 2.0 (Neff et al. 2002). The predicted CAPS and dCAPS candidates were amplified in a 20 µl PCR reaction using GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) on 5 parental genotypes of chickpea mapping populations (PI 489777, ICC 4958, ICC 1882, ICC 8261 and ICC 283). Amplicons for each CAPS and dCAPS were subjected to digestion using their respective restriction enzymes followed by separation on 2% agarose gel electrophoresis as described in Gujaria et al. (2011).

3.2 Marker Enrichment in the "*QTL-hotspot*" Region Using Skim Sequencing Approach

3.2.1 Library construction, skim sequencing and SNP calling

For RILs, a total of 50 ng DNA from each sample was used to prepare an Illumina Nextera library according to the manufacturer's instructions. In this procedure, the genomic DNA is

simultaneously fragmented and tagged with sequencing adaptors using an engineered transposome. The fragments were then amplified using a limited PCR cycle which also adds index sequences on both ends of the DNA, thus enabling dual-indexed sequencing of pooled libraries. Libraries for 96 individuals were pooled and sequenced on the Illumina HiSeq 2500 platform. Parental genotypes (ICC 4958 and ICC 1882) were sequenced separately at high sequencing depth (~8X) whereas ICCRIL03 population was sequenced at low coverage of ~0.70X (average of RILs).

The reads obtained were filtered to remove low quality bases and used for SNP identification. Initially, the reads from the parental genotypes (ICC 4958 and ICC 1882) were aligned to the draft chickpea genome (CaGAv1.0) using SOAP (Li et al. 2009). Uniquely mapped reads were considered for SNP calling using SGSAutoSNP software (Lorenc et al. 2012) with default parameters. Similarly, the low quality reads obtained from RILs were filtered out and SNPs were identified from the remaining reads. The identified SNPs were filtered with a minor allele frequency (MAF) cutoff of 0.2 and lines with ≥ 5% missing data were also excluded. SNPs were scored as "A" and "B" representing alleles from the two parents ICC 4958 and ICC 1882 respectively.

3.2.2 Identification of recombination break points

Sliding window approach was used to minimize the sequence errors during genotype calling and to determine the recombination break points using perl scripts as mentioned in Huang et al. 2009. Perl scripts from Huang et al. 2009 were used with necessary changes according to chickpea genome co-ordinates. In brief, a sliding window covering 15 SNPs moves from left to right one base at a time. For each window the ratio of the number of ICC 4958 to ICC 1882 SNPs (ICC 4958:ICC 1882) was calculated. Windows with nine or more SNPs (≥9:6 or ≥6:9) from either parent were considered as homozygous for an individual. For instance, a window with ≥9:6 (ICC 4958:ICC 1882) is considered homozygous for ICC 4958 and

genotype call for this window is denoted as “A”. While a window with ≤6:9 (ICC 4958:ICC 1882) is considered homozygous for ICC 1882 and genotype call for this window is denoted as “B”. While the window with 8:7≥ (ICC 4858:ICC 1882) ≥7:8 is considered as heterozygous genotype and denoted as “H”. As the window slides, genotypes are called and the recombination break points were determined. Recombination break point was defined at the transition from one genotype to another. The recombination breakpoints obtained from all the individual RILs were aligned and compared over 100 kb intervals. The successive intervals lacking recombination break points within the entire population were combined and considered as a single bin. These bins were then used as markers for linkage map construction.

3.2.3 Construction of bin map

The bins were used as genetic markers for the construction of a linkage map. Map construction was carried out using QTL IciMapping Version 3.3 software (Meng et al. 2015). The REcombination Counting and ORDering (RECORD) algorithm was used for marker ordering. Sum of adjacent criterion (SAD) ripple was performed to confirm the marker order. The marker order and their positions on genetic and physical map were visualized using Strudel V. 1.12.03.20 (Bayer et al. 2011).

3.2.4 QTL analysis using IciMapping

QTL IciMapping software was also used with the phenotype data for 20 drought related traits and bin map for QTL analysis. The ICIM-ADD mapping method, along with other default parameters was used. The LOD threshold was set by using permutation 1000 and p value ≤ 0.05. The results were compared with earlier studies (Varshney et al. 2014) and also with the results obtained from the GBS study (objective 1; chapter 3.1.4) to determine the quality and

accuracy of the bin map. Further, the QTL results were checked to refine the earlier identified "*QTL-hotspot*" region and also to identify candidate genes controlling respective traits.

3.3 Development of Large Scale Fine Mapping Population

3.3.1 Selection of parental lines and crossing

ICCX-110125-P18 (BC_5F_1) is a near isogenic line generated by Chickpea Breeding Division, ICRISAT, carrying "*QTL-hotspot*" segment on linkage group 4 from ICC 4958 in the genetic background of ICC 1882. This line was generated through continuous backcrossing of ICC 4958 with ICC 1882. It was termed as NIL^+ line as it carries "*QTL-hotspot*" from ICC 4958 in ICC 1882 genetic background. Background recovery of near isogenic lines was calculated using,

Background recovery = [(No. of recurrent allele + (No. of heterozygotes*1/2))/Total no. of markers] * 100

Selected progenies of ICCX-110125-P18 (BC_5F_1) NIL^+ lines were crossed with ICC 1882 during June 2013 at ICRISAT to develop F_1 population for generating large scale fine mapping population. F_1 plants were screened with 6 SSR markers from the "*QTL-hotspot*" region to identify a plant carrying heterozygous segment for the QTL region and homozygous for ICC 1882 in rest of the chromosomes. Using the selected F_1 lines, a high resolution segregating mapping population of F_2 lines were constructed during June 2014 at Indian Institute of Pulses Research-Regional Research Station cum Off-season Nursery, Dharwad, India located at 15°27′30″N 75°00′30″E.

3.3.2 Genotyping and progeny testing

DNA was extracted from parental lines (ICC 4958 and ICC 1882) and F_2 progenies using high throughput mini-DNA extraction method as described in Cuc et al. (2008). SNP markers

(no=6) flanking the "*QTL-hotspot*" regions obtained from high density map based QTL analysis (chapter 3.2.4) were used for genotyping the large scale F_2 fine mapping population. Based on the genotyping data, recombinant lines for the "*QTL-hotspot*" regions were selected and the selected plants were further advanced to obtain $F_{2:3}$ population. These F_3 lines were initially genotyped with the flanking SNP markers and the selected homozygous recombinant lines (a line carrying homozygous allele for both parent in a given segment) were further genotyped with all SNP markers (no=24) located in the "*QTL-hotspot*" regions. Phenotyping for yield related traits as mentioned in Table 4 was recorded. Progeny testing was carried out using the phenotyping data along with the genotyping data obtained on $F_{2:3}$ homozygous recombinant lines.

3.4 Candidate Gene Identification

The amino acid sequences of genes located in the "*QTL-hotspot*" region delimited through GBS approach were retrieved from draft genome sequence (CaGAv1.0) of chickpea (Varshney et al. 2013a) and searched against NCBI-nr protein database using blastp program implemented in Blast2GO software (Conesa et al. 2005) with an E-value threshold of $\leq e^{-20}$. Associated gene ontology (GO) terms were exported and searched for plant related GO terms using the GO slim viewer from the AgBase web server (http://www.agbase.msstate.edu), which also categorize terms into three different classes as biological processes (BP), molecular function (MF) and cellular components (CC). Similarly, co-ordinates of the delimited "*QTL-hotspot*" regions obtained from skim sequencing approach were mapped to the draft genome sequence (CaGAv1.0) of chickpea (Varshney et al. 2013a). The genes were extracted and looked for their functional role using Blast2GO. Corresponding amino acid sequences were mapped to uniprot database using BLASTp with an E-value threshold of $\leq e^{-20}$. Followed by the uniprot id's were extracted and mapping of these id's on retrieve/ID mapping resulted in finding out the annotation for these genes.

RESULTS

4. RESULTS

4.1 SNP Marker Development

4.1.1 Sequence data and SNP discovery using genotyping-by sequencing (GBS) approach

Parental genotypes of the mapping population (ICC 4958 and ICC 1882) were sequenced at higher depth (5X coverage) than RIL individuals (0.38X coverage on an average), and a total of 69.39 million reads amounting 6.24 Gb of ICC 4958 and 62.79 million reads amounting 5.65 Gb of ICC 1882 were generated. In addition, 701.05 million reads amounting 59.03 Gb were generated for 208 RILs (RIL001 to RIL208). The number of reads generated varied from 0.28 million (RIL078) to 19.23 million (RIL204) with an average of 3.37 million per line. The data obtained was filtered and used for SNP identification using SOAP software. The SNPs identified were again parsed to remove heterozygous SNPs in parents and a set of 828 SNPs were identified across 208 RILs. The flanking sequences of all the SNPs are made available through http://cmap.icrisat.ac.in/cmap/sm/cp/jaganathan/.

4.1.2 High density genetic map based on GBS derived SNP markers

Genotypic data for 828 polymorphic SNPs generated in this study along with 318 markers obtained from the earlier studies (Table 5), were used for genetic map construction. In total, 1,146 markers were used for genetic map construction, of which 1,007 (87.87%) were mapped on eight linkage groups (CaLG01 to CaLG08) covering 727.29 cM (Figure 1; http://cmap.icrisat.ac.in/cmap/sm/cp/jaganathan/). This map included 743 SNPs, 232 SSR, 21 DArT, 7 EST-SSR and 4 genic molecular markers GMM (Table 6). Highest number of markers was mapped on CaLG04 (386), while the lowest number of markers was mapped on CaLG05 (39). Distribution of marker loci on eight linkage groups has been shown in Figure 1.

The length of linkage groups varied from 59.41 cM (CaLG05) to 112.10 cM (CaLG04). Highest marker density was observed for CaLG04 with 3.44 markers per cM on an average whereas, lowest marker density was observed for CaLG07, which had an average of 0.62 markers per cM. Overall, the map had an average marker density of 1.30 per cM (Table 6 and Figure 1). Of 828 SNPs used for linkage map construction, 743 SNPs (89.73%) were mapped, among which 342 were mapped on CaLG04. Of 279 SSR markers used, 232 (83.15%) were mapped. Comparatively, SSR markers were mapped evenly on all the eight linkage groups. Highest number of SSR markers was mapped on CaLG03 (47), and the lowest number of SSR markers was mapped on CaLG02 (18). Out of 14 EST-SSRs, 7 markers were mapped, whereas all GMM and DArT markers used in the present study were mapped on different linkage groups. However, among 21 DArT markers mapped, 47.6% (10) were on CaLG01, 33.33% (7) on CaLG03, one each on CaLG02, CaLG05, CaLG06, and CaLG07. Out of four GMM, one each was mapped on CaLG02, CaLG04, CaLG07 and CaLG08.

4.2 Marker Enrichment in the "*QTL-hotspot*" Region

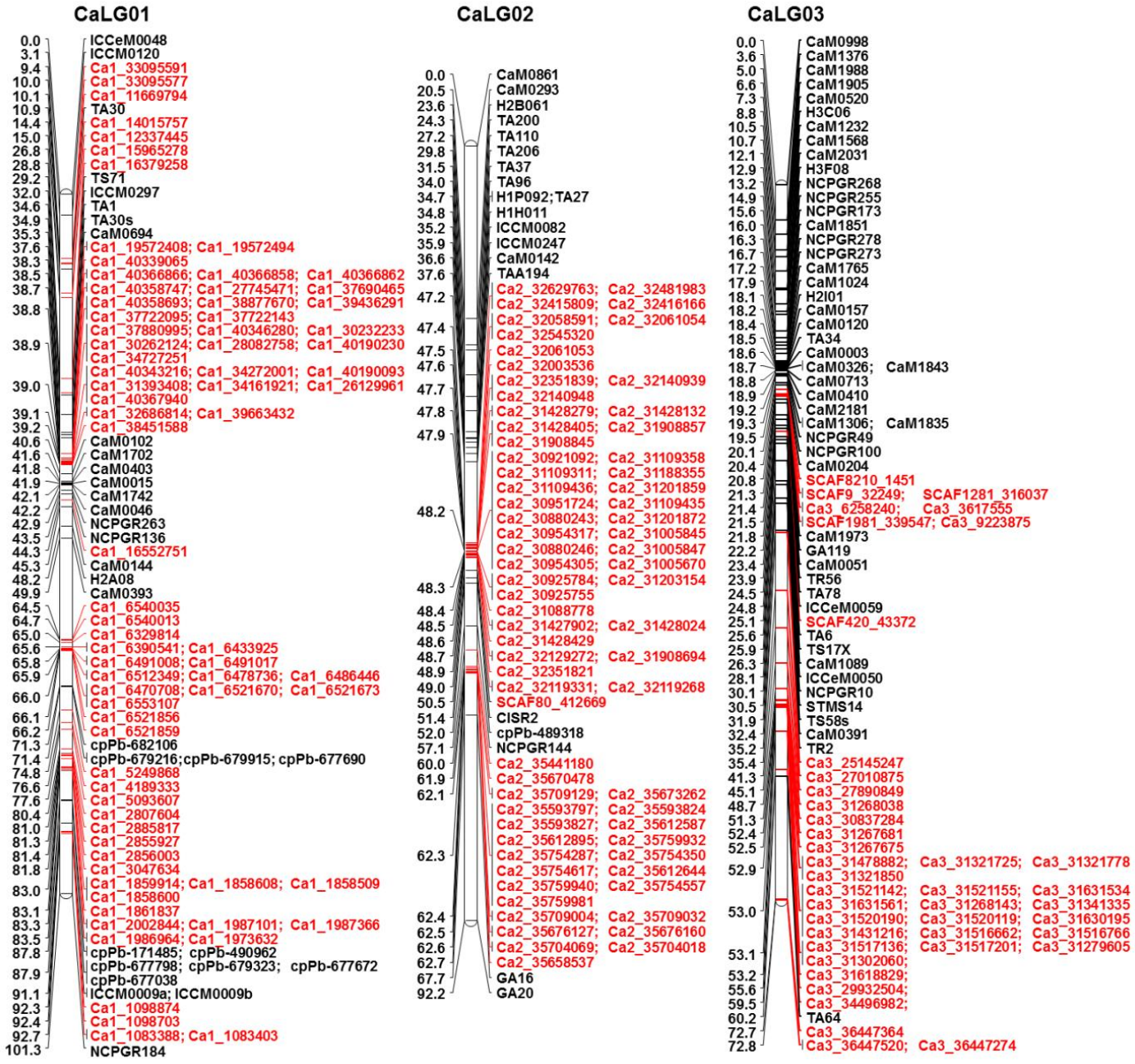
QTL analysis based on genotypic data for the mapped 1,007 markers and phenotypic data for 20 traits (as described in Varshney et al. 2014), identified a total of 164 robust M-QTLs by QTL Cartographer 2.5. More than 50% (91 M-QTLs) of these M-QTLs were located on CaLG04 and significantly, all 91 QTLs were detected in the "*QTL-hotspot*" region (Table 7). Earlier reported "*QTL-hotspot*" region (Varshney et al. 2014) had 7 SSR markers (ICCM0249, NCPGR127, TAA170, NCPGR21, TR11, GA24 and STMS11) and spanned 29 cM on linkage group 4. The current study integrates 49 new SNP markers into the "*QTL-hotspot*" region and the interval was re-estimated from 29 cM to 14 cM (Figure 2).

Table 5: List of marker genotyping data used from the earlier studies for linkage map construction

Marker	Number of markers	Reference
SSR[1]	103	Thudi et al. 2011
SSR	58	Winter et al. 1999
SSR	34	Gaur et al. 2011
SSR	32	Lichtenzveig et al. 2005
SSR	22	Nayak et al. 2010
SSR	22	Sethy et al. 2006
SSR	8	Huttel et al. 1999
EST-SSR[2]	14	Varshney et al. 2009
DArT[3]	21	Thudi et al. 2011
GMM[4]	4	Gujaria et al. 2011
Total	**318**	

SSR[1]- simple sequence repeats; EST-SSR[2]- expressed sequence tag-SSR; DArT[3]- diversity array technology; GMM[4]- genic molecular markers.

a.


CaLG01
0.0 ICCeM0048
3.1 ICCM0120
9.4 Ca1_33095591
10.0 Ca1_33095577
10.1 Ca1_11669794
10.9 TA30
14.4 Ca1_14015757
15.0 Ca1_12337445
26.8 Ca1_15965278
28.8 Ca1_16379258
29.2 TS71
32.0 ICCM0297
34.6 TA1
34.9 TA30s
35.3 CaM0694
37.6 Ca1_19572408; Ca1_19572494
38.3 Ca1_40339065
38.5 Ca1_40366866; Ca1_40366858; Ca1_40366862
38.7 Ca1_40358747; Ca1_27745471; Ca1_37690465
38.8 Ca1_40358693; Ca1_38877670; Ca1_39436291
Ca1_37722095; Ca1_37722143
38.9 Ca1_37880995; Ca1_40346280; Ca1_30232233
Ca1_30262124; Ca1_28082758; Ca1_40190230
Ca1_34727251
Ca1_40343216; Ca1_34272001; Ca1_40190093
39.0 Ca1_31393408; Ca1_34161921; Ca1_26129961
Ca1_40367940
39.1 Ca1_32686814; Ca1_39663432
39.2 Ca1_38451588
40.6 CaM0102
41.6 CaM1702
41.8 CaM0403
41.9 CaM0015
42.1 CaM1742
42.2 CaM0046
42.9 NCPGR263
43.5 NCPGR136
44.3 Ca1_16552751
45.3 CaM0144
48.2 H2A08
49.9 CaM0393
64.5 Ca1_6540035
64.7 Ca1_6540013
65.0 Ca1_6329814
65.6 Ca1_6390541; Ca1_6433925
65.8 Ca1_6491008; Ca1_6491017
65.9 Ca1_6512349; Ca1_6478736; Ca1_6486446
66.0 Ca1_6470708; Ca1_6521670; Ca1_6521673
Ca1_6553107
66.1 Ca1_6521856
66.2 Ca1_6521859
71.3 cpPb-682106
71.4 cpPb-679216;cpPb-679915; cpPb-677690
74.8 Ca1_5249868
76.6 Ca1_4189333
77.6 Ca1_5093607
80.4 Ca1_2807604
81.0 Ca1_2885817
81.3 Ca1_2855927
81.4 Ca1_2856003
81.8 Ca1_3047634
83.0 Ca1_1859914; Ca1_1858608; Ca1_1858509
Ca1_1858600
83.1 Ca1_1861837
83.3 Ca1_2002844; Ca1_1987101; Ca1_1987366
83.5 Ca1_1986964; Ca1_1973632
87.8 cpPb-171485; cpPb-490962
87.9 cpPb-677798; cpPb-679323; cpPb-677672
cpPb-677038
91.1 ICCM0009a; ICCM0009b
92.3 Ca1_1098874
92.4 Ca1_1098703
92.7 Ca1_1083388; Ca1_1083403
101.3 NCPGR184
CaLG02
0.0 CaM0861
20.5 CaM0293
23.6 H2B061
24.3 TA200
27.2 TA110
29.8 TA206
31.5 TA37
34.0 TA96
34.7 H1P092;TA27
34.8 H1H011
35.2 ICCM0082
35.9 ICCM0247
36.6 CaM0142
37.6 TAA194
47.2 Ca2_32629763; Ca2_32481983
Ca2_32415809; Ca2_32416166
Ca2_32058591; Ca2_32061054
47.4 Ca2_32545320
47.5 Ca2_32061053
47.6 Ca2_32003536
47.7 Ca2_32351839; Ca2_32140939
Ca2_32140948
47.8 Ca2_31428279; Ca2_31428132
47.9 Ca2_31428405; Ca2_31908857
Ca2_31908845
Ca2_30921092; Ca2_31109358
Ca2_31109311; Ca2_31188355
Ca2_31109436; Ca2_31201859
Ca2_30951724; Ca2_31109435
48.2 Ca2_30880243; Ca2_31201872
Ca2_30954317; Ca2_31005845
Ca2_30880246; Ca2_31005847
Ca2_30954305; Ca2_31005670
Ca2_30925784; Ca2_31203154
48.3 Ca2_30925755
48.4 Ca2_31088778
48.5 Ca2_31427902; Ca2_31428024
48.6 Ca2_31428429
48.7 Ca2_32129272; Ca2_31908694
48.9 Ca2_32351821
49.0 Ca2_32119331; Ca2_32119268
50.5 SCAF80_412669
51.4 CISR2
52.0 cpPb-489318
57.1 NCPGR144
60.0 Ca2_35441180
61.9 Ca2_35670478
62.1 Ca2_35709129; Ca2_35673262
Ca2_35593797; Ca2_35593824
Ca2_35593827; Ca2_35612587
Ca2_35612895; Ca2_35759932
62.3 Ca2_35754287; Ca2_35754350
Ca2_35754617; Ca2_35612644
Ca2_35759940; Ca2_35754557
Ca2_35759981
62.4 Ca2_35709004; Ca2_35709032
62.5 Ca2_35676127; Ca2_35676160
62.6 Ca2_35704069; Ca2_35704018
62.7 Ca2_35658537
67.7 GA16
92.2 GA20
CaLG03
0.0 CaM0998
3.6 CaM1376
5.0 CaM1988
6.6 CaM1905
7.3 CaM0520
8.8 H3C06
10.5 CaM1232
10.7 CaM1568
12.1 CaM2031
12.9 H3F08
13.2 NCPGR268
14.9 NCPGR255
15.6 NCPGR173
16.0 CaM1851
16.3 NCPGR278
16.7 NCPGR273
17.2 CaM1765
17.9 CaM1024
18.1 H2I01
18.2 CaM0157
18.4 CaM0120
18.5 TA34
18.6 CaM0003
18.7 CaM0326; CaM1843
18.8 CaM0713
18.9 CaM0410
19.2 CaM2181
19.3 CaM1306; CaM1835
19.5 NCPGR49
20.1 NCPGR100
20.4 CaM0204
20.8 SCAF8210_1451
21.3 SCAF9_32249; SCAF1281_316037
21.4 Ca3_6258240; Ca3_3617555
21.5 SCAF1981_339547; Ca3_9223875
21.8 CaM1973
22.2 GA119
23.4 CaM0051
23.9 TR56
24.5 TA78
24.8 ICCeM0059
25.1 SCAF420_43372
25.6 TA6
25.9 TS17X
26.3 CaM1089
28.1 ICCeM0050
30.1 NCPGR10
30.5 STMS14
31.9 TS58s
32.4 CaM0391
35.2 TR2
35.4 Ca3_25145247
41.3 Ca3_27010875
45.1 Ca3_27890849
48.7 Ca3_31268038
51.3 Ca3_30837284
52.4 Ca3_31267681
52.5 Ca3_31267675
52.9 Ca3_31478882; Ca3_31321725; Ca3_31321778
Ca3_31321850
53.0 Ca3_31521142; Ca3_31521155; Ca3_31631534
Ca3_31631561; Ca3_31268143; Ca3_31341335
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53.2 Ca3_31618829;
55.6 Ca3_29932504;
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60.2 TA64
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72.8 Ca3_36447520; Ca3_36447274

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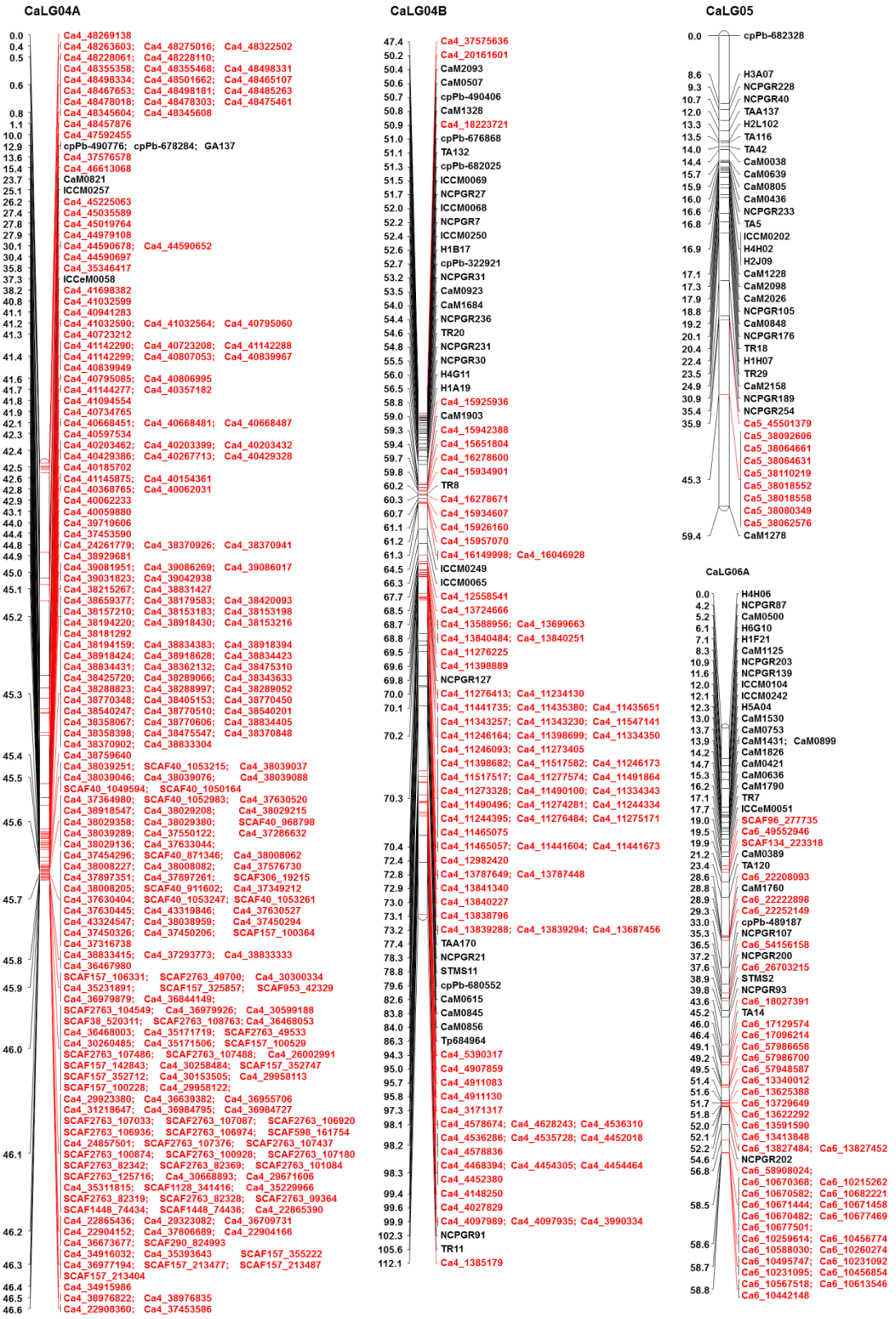
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13.5 TA116
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15.7 CaM0639
15.9 CaM0805
16.0 CaM0436
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16.8 TA5
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16.9 H4H02
H2J09
17.1 CaM1228
17.3 CaM2098
17.9 CaM2026
18.8 NCPGR105
19.2 CaM0848
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20.4 TR18
22.4 H1H07
23.5 TR29
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5.2 CaM0500
6.1 H6G10
7.1 H1F21
8.3 CaM1125
10.9 NCPGR203
11.6 NCPGR139
12.0 ICCM0104
12.1 ICCM0242
12.3 H5A04
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14.7 CaM0421
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16.2 CaM1790
17.1 TR7
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36.5 Ca6_54156158
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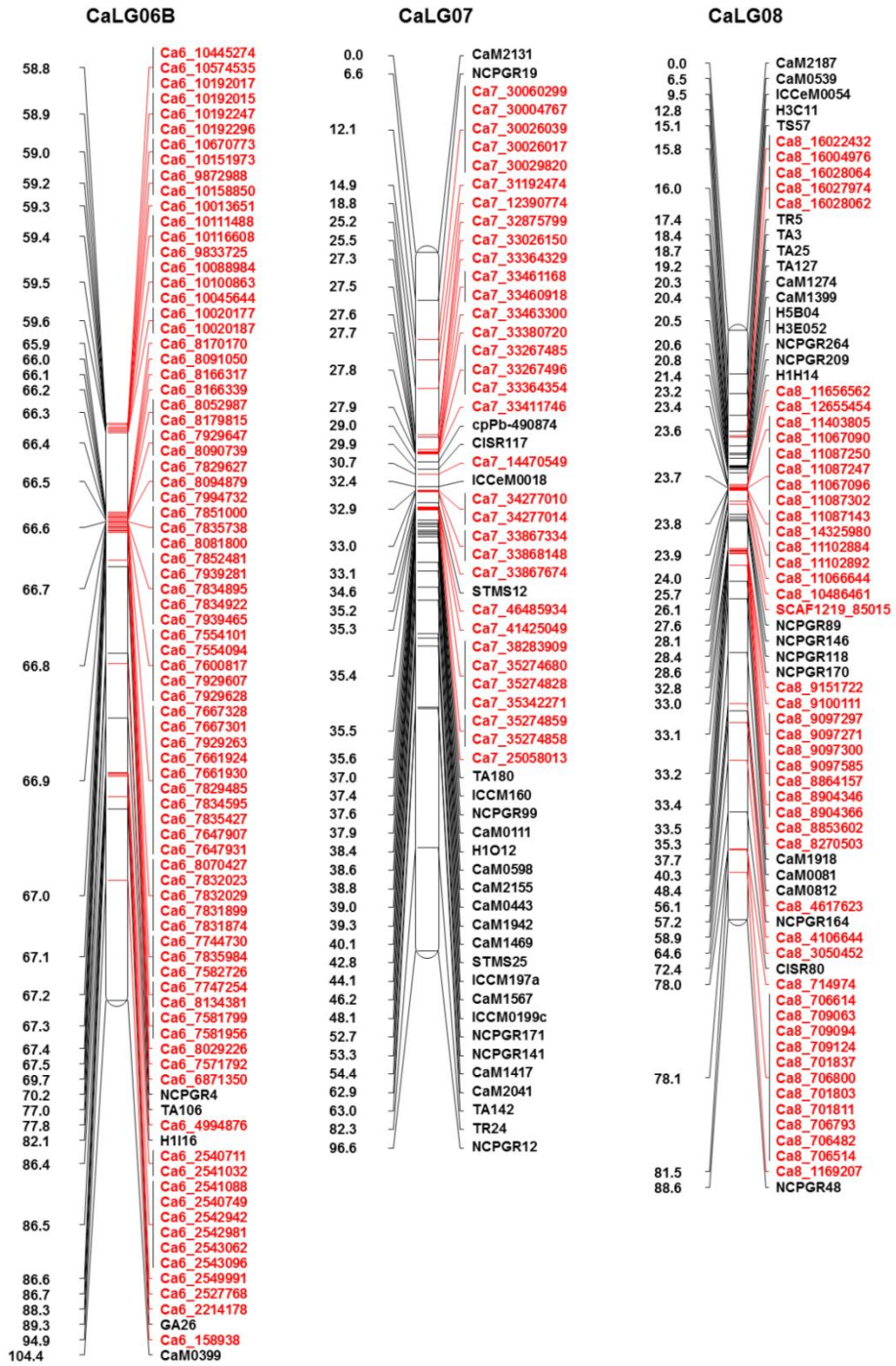

Figure 1: High density intra-specific genetic map of chickpea (ICC 4958 × ICC 1882) using GBS-SNP markers. a. Linkage groups 1-3; b. Linkage groups 4-6A; c. Linkage groups 6B-8. This map is comprised of 1,007 markers including 743 novel SNPs from GBS approach and spans 727.29 cM. Genetic distances (cM) were shown on the left side and the markers were shown on the right side of the bars. Map was constructed using JoinMap 4.0 and Kosambi mapping function. Markers in black colour font are from the framework map and markers in red colour font are newly generated SNP markers. For clear visualization, CaLG04 and CaLG06 were split into two parts and named as CaLG04A, CaLG04B, CaLG06A and CaLG06B.

Table 6: Distribution of different types of markers on the intra-specific genetic map of ICC 4958 × ICC 1882 RIL population using GBS-SNP markers

Marker series	**SNP**	**SSR**	**EST-SSR**	**GMM**	**DArT**	**Total markers**	**Map length (cM)**	**Density (Markers/cM)**
Total markers used	828	279	14	4	21	1146		
Total markers mapped	743	232	7	4	21	1007		
Percent mapped	89.73	83.15	50.00	100.00	100.00	87.87		
Markers unlinked	85	47	7	0	0	139		
Percent unlinked	10.3	16.85	50	0	0	12.13		
Markers mapped on different linkage groups								
CaLG01	77	21	1	-	10	109	101.27	1.08
CaLG02	70	18	-	1	1	90	92.16	0.98
CaLG03	41	47	2	-	-	90	72.78	1.24
CaLG04	342	35	1	1	7	386	112.10	3.44
CaLG05	9	29	-	-	1	39	59.41	0.66
CaLG06	124	34	1	-	1	160	104.36	1.53
CaLG07	33	24	1	1	1	60	96.59	0.62
CaLG08	47	24	1	1	-	73	88.62	0.82
Total	**743**	**232**	**7**	**4**	**21**	**1007**	**727.29**	
Average						**125.88**	**90.91**	**1.30**

4.3 QTL Analysis for Drought Tolerance Related Traits

Out of 20 traits analyzed, QTLs were identified for 16 traits, including root length density (RLD, cm cm^{-3}), root surface area (RSA, cm^2), root dry weight/total plant dry weight (RTR, %), shoot dry weight (SDW, g), plant height (PHT, cm), primary branches (PBS), days to 50% flowering (DF), days to maturity (DM), 100-seed weight (100-SDW, g), biomass (BM, g), harvest index (HI, %), pods/plant (POD), seeds/pod (SPD), yield (YLD, g), drought susceptibility index (DSI), and drought tolerance index (DTI). QTL nomenclature was adopted as per Varshney et al. (2014). In case, a QTL reported for a given trait in Varshney et al. (2014) has been further resolved into two or more QTLs, the QTLs are further named using a decimal followed by a roman numeral. For instance *QR3rld01* reported earlier was resolved into three QTLs, hence named as *QR3rld01.1*, *QR3rld01.2*, and *QR3rld01.3* (Table 7). Here Q denotes QTL, R3 denotes ICCRIL03, rld denotes root length density and 01 denotes the QTL number. QTLs were considered as 'stable' (if they appeared in more than one location for the specified trait) and 'consistent' (if they appear in more than one year/season for the specified trait) as described in Varshney et al. (2014). Detailed description of the QTLs identified in this study for drought tolerance related traits is summarized below.

4.3.1 Root related traits

Three QTLs were identified, one each for RLD, RSA and RTR with PVE ranging from 10.65-13.56% (Table 8). Among them, RLD and RTR were identified in the "*QTL-hotspot*" as reported earlier, whereas a QTL for RSA was identified on CaLG06 (Table 7). The QTL for RLD, *'QR3rld01'* was refined to 3.23 - 5.37 cM from 10.54 cM, whereas that for RTR, *'QR3rtr01'* was refined to 1.81 - 5.37 cM from 5 cM (Table 7 and Table 8). Both QTLs were consistent across years (2005 and 2007).

4.3.2. Morphology related traits

A total of 3 and 9 QTLs were identified for SDW and PHT respectively, out of which 2 and 5 were newly identified for the respective traits. Overall, PVE ranged from 10.05 - 34.57% (Table 8). The QTL size for SDW *'QR3sdw01'* was similar as reported earlier, whereas QTL for PHT, *'QR3pht03'* was refined to 1.81 cM from 5.37 cM (Table 7). Out of 9 QTLs identified for PHT, 3 were stable and 5 were consistent. Interestingly, two QTLs, *'QR3pht06', 'QR3pht08'* were found consistent and stable, which were previously reported to be unstable and inconsistent by Varshney et al. (2014). A QTL for primary branches (PBS) '*QR3pbs02*' was newly identified in the current study which explained the PVE of 12.92% (Table 7 and Table 8).

4.3.3 Phenology related traits

For phenological traits, 3 and 2 QTLs were identified for DF and DM, respectively. The maximum PVE explained by the QTL, *'QR3df04'* was much higher (67.71%) as compared to the earlier study (26.87%) for DF (Table 7). This QTL has been refined to 1.81 cM from 5.14 cM. Similarly, the QTL, '*QR3dm01'* explained 47.43% PVE for DM which was comparatively higher than reported earlier (19.71%) and was refined to 7.33 cM from 15.13 cM (Table 7 and Table 8).

4.3.4 Yield-related traits and drought indices

A total of 16 QTLs including 5 novel QTLs were identified for yield and yield related traits, including 2 each for 100-SDW, POD and 3 each for BM, HI, SPD and YLD. Overall, the QTLs spanned the same size as reported previously (Varshney et al. 2014), however QTLs for BM and POD has been refined to 15.13 cM and 5.37 cM respectively (Table 8). PVE explained by each QTL was comparatively high, especially for 100-SDW, '*QR3100sdw03*', this QTL explained phenotypic variation of 60.41% (Table 7).

Table 7: Robust main-effect QTLs (M-QTLs) identified for various drought tolerance related traits on ICC 4958 × ICC 1882

Trait	Year	Location[a]	Environment[b]	Linkage group (LG)	QTL name	Marker interval	Size of the QTL (cM)	PVE (%)[**]	Additive effect	LOD[#]
Root traits										
Root length density (RLD, cm cm^{-3})	2005	PAT	CC	CaLG04	***QR3rld01.1***	ICCM0065 - Ca4_11276225	3.23	12.09	0.02	4.59
	2005	PAT	CC	CaLG04	***QR3rld01.2***	Ca4_13687456 - NCPGR21	5.14	11.90	0.02	7.09
	2007	PAT	CC	CaLG04	***QR3rld01.3***	Ca4_13840227 - NCPGR21	5.37	10.66	0.02	6.20
Root surface area (RSA, cm^2)	2007	PAT	CC	CaLG06	*QR3rsa02*	NCPGR4 - Ca6_4994876	7.57	11.04	62.35	5.02
Root dry weight/total plant dry weight (RTR, %)	2005	PAT	CC	CaLG04	***QR3rtr01.1***	Ca4_12558541 - Ca4_11276225	1.81	11.94	-0.94	7.03
	2005	PAT	CC	CaLG04	***QR3rtr01.2***	Ca4_13840227 - TAA170	4.39	10.86	-0.90	6.64
	2007	PAT	CC	CaLG04	***QR3rtr01.3***	ICCM0065 - Ca4_13588956	2.39	12.22	-1.15	7.18
	2007	PAT	CC	CaLG04	***QR3rtr01.4***	Ca4_13840227 - NCPGR21	5.37	13.56	-1.20	8.96
Morphological traits										
Shoot dry weight (SDW, g)	2005	PAT	RF	CaLG04	***QR3sdw01.1***	Ca4_12558541 - Ca4_11276225	1.81	10.78	0.11	6.40
	2005	PAT	RF	CaLG04	***QR3sdw01.2***	Ca4_12982420 - NCPGR21	5.91	12.59	0.12	7.40
	2007	PAT	RF	CaLG04	***QR3sdw01.1***	Ca4_12558541 - Ca4_11276225	1.81	25.47	0.13	16.47
	2007	PAT	RF	CaLG04	***QR3sdw01.3***	Ca4_13840227 - TAA170	4.39	26.91	0.14	16.86
	2007	PAT	RF	CaLG04	*QR3sdw3**	Ca4_15651804 - TR8	0.73	16.99	0.11	10.01
	2007	PAT	RF	CaLG04	*QR3sdw4**	Ca4_16149998 - ICCM0249	3.15	18.94	0.12	10.08
Plant height (PHT, cm)	2006	PAT	RF	CaLG08	***QR3pht06.1***	NCPGR164 - CISR80	15.13	15.14	-1.70	9.28
	2007	PAT	RF	CaLG08	***QR3pht06.2***	Ca8_4106644 - CISR80	13.44	12.09	-1.19	7.42
	2009	HIR	IR	CaLG08	***QR3pht06.1***	NCPGR164 - CISR80	15.13	10.13	-1.24	7.14
	2007	PAT	RF	CaLG04	***QR3pht03.1***	Ca4_12558541 - Ca4_11276225	1.81	17.21	1.41	10.95

	2007	PAT	RF	CaLG04	***QR3pht03.2***	Ca4_13840227 - TAA170	4.39	13.99	1.27	9.65
	2008	NDL	RF	CaLG04	***QR3pht03.1***	Ca4_12558541 - Ca4_11276225	1.81	15.13	1.65	9.53
	2008	NDL	RF	CaLG04	***QR3pht03.2***	Ca4_13787649 - TAA170	4.56	18.36	1.82	10.57
	2008	PAT	RF	CaLG04	***QR3pht03.2***	Ca4_12982420 - TAA170	4.93	25.05	1.85	19.97
	2008	SEH	RF	CaLG04	***QR3pht03.2***	Ca4_13840227 - NCPGR21	5.37	20.37	1.91	11.75
	2009	HIR	RF	CaLG04	***QR3pht03.1***	Ca4_13724666 - Ca4_11276225	1.05	30.86	1.71	21.51
	2009	PAT	IR	CaLG04	***QR3pht03.1***	Ca4_12558541 - Ca4_11276225	1.81	17.92	1.21	12.83
	2009	PAT	RF	CaLG04	***QR3pht03.1***	Ca4_12558541 - Ca4_11276225	1.81	32.48	1.90	26.64
	2009	HIR	IR	CaLG04	***QR3pht03.2***	Ca4_13840227 - TAA170	4.39	21.59	1.88	13.49
	2009	NDL	RF	CaLG04	***QR3pht03.2***	Ca4_13840227 - NCPGR21	5.37	16.61	1.69	10.81
	2009	PAT	IR	CaLG04	***QR3pht03.2***	Ca4_13840227 - TAA170	4.39	22.40	1.35	15.26
	2009	PAT	RF	CaLG04	***QR3pht03.2***	Ca4_12982420 - TAA170	4.93	34.57	1.97	29.16
	2008	NDL	RF	CaLG04	***QR3pht08.1***	Ca4_16149998 - ICCM0065	4.99	11.80	1.47	6.45
	2008	PAT	RF	CaLG04	***QR3pht08.2***	ICCM0065 - Ca4_13588956	2.39	24.55	1.83	18.12
	2009	HIR	IR	CaLG04	***QR3pht08.1***	ICCM0065 - Ca4_11276225	3.23	21.77	1.89	14.83
	2008	DUG	RF	CaLG06	***QR3pht01***	Ca6_2540711 - CaM0399	17.94	10.46	2.00	3.97
	2008	DUG	RF	CaLG07	***QR3pht09****	Ca7_12390774 - Ca7_33364329	8.46	10.05	2.23	4.63
	2008	DUG	RF	CaLG07	***QR3pht10****	CISR117 - Ca7_34277010	3.02	10.16	2.50	5.67
	2008	PAT	RF	CaLG04	***QR3pht11****	STMS11 - CaM0615	3.80	13.62	1.36	9.02
	2009	PAT	RF	CaLG04	***QR3pht11****	STMS11 - CaM0615	3.80	13.07	1.20	8.06
	2008	PAT	RF	CaLG08	***QR3pht12****	Ca8_4106644 - CISR80	13.44	24.29	-1.82	18.75
	2009	PAT	RF	CaLG08	***QR3pht12****	Ca8_4106644 - CISR80	13.44	12.85	-1.18	11.37
	2009	NDL	IR	CaLG03	*QR3pht13**	CaM0520 - ICCeM0050	3.41	10.34	-1.36	3.75
Primary branches (PBS)	2006	PAT	RF	CaLG08	*QR3pbs02**	CaM0812 - NCPGR164	8.81	12.92	-0.18	5.27
Phenological traits										
Days to 50% flowering (DF)	2005	PAT	RF	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	31.99	-2.40	14.84
	2005	PAT	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	39.07	-2.81	17.63
	2005	PAT	RF	CaLG08	***QR3df01***	Ca8_3050452 - CISR80	7.79	40.92	-3.01	6.35

	2006	PAT	RF	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	42.47	-1.49	17.31
	2006	PAT	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	48.30	-1.56	27.94
	2007	PAT	RF	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	38.31	-4.05	21.77
	2007	PAT	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	47.23	-4.49	26.71
	2008	NDL	RF	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	16.71	-2.20	9.04
	2008	NDL	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	21.61	-2.50	10.37
	2008	PAT	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	61.86	-2.44	30.63
	2009	DUG	IR	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	23.79	-3.13	11.70
	2009	DUG	IR	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	29.45	-3.47	16.30
	2009	DUG	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	51.03	-5.63	32.82
	2009	HIR	IR	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	62.22	-2.82	31.91
	2009	HIR	IR	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	67.71	-2.95	46.03
	2009	HIR	RF	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	57.98	-2.86	28.60
	2009	HIR	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	66.08	-3.04	44.42
	2009	NDL	IR	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	16.63	-1.29	9.22
	2009	NDL	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	33.52	-2.02	19.09
	2009	PAT	IR	CaLG08	***QR3df01***	CaM0812 - NCPGR164	8.81	46.20	-3.24	25.72
	2009	PAT	IR	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	58.93	-3.64	37.55
	2009	PAT	RF	CaLG08	***QR3df01***	NCPGR164 - Ca8_3050452	7.33	27.70	-2.85	21.42
	2009	SEH	RF	CaLG08	***QR3df01***	NCPGR164 - CISR80	15.13	12.15	-1.46	7.22
	2008	SEH	RF	CaLG04	*QR3df04.1*	Ca4_12558541 - Ca4_11276225	1.81	13.74	-0.56	8.57
	2008	SEH	RF	CaLG04	*QR3df04.2*	Ca4_13687456 - NCPGR21	5.14	20.61	-0.66	11.77
	2008	PAT	RF	CaLG06	*QR3df06**	Ca6_158938 - CaM0399	9.48	10.86	1.02	5.33
Days to maturity (DM)	2005	PAT	RF	CaLG08	***QR3dm01***	CaM0812 - NCPGR164	8.81	10.31	-1.25	5.34
	2006	PAT	RF	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	21.79	-3.05	12.94
	2007	PAT	RF	CaLG08	***QR3dm01***	NCPGR164 - CISR80	15.13	12.98	-2.77	7.42
	2008	NDL	RF	CaLG08	***QR3dm01***	NCPGR164 - CISR80	15.13	18.47	-2.02	9.58
	2008	PAT	RF	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	36.58	-1.56	21.32
	2009	DUG	IR	CaLG08	***QR3dm01***	CaM0812 - NCPGR164	8.81	15.84	-2.55	7.66

	2009	DUG	IR	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	21.90	-2.99	11.36
	2009	DUG	RF	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	47.43	-5.24	30.01
	2009	HIR	IR	CaLG08	***QR3dm01***	CaM0812 - NCPGR164	8.81	25.93	-2.91	14.52
	2009	HIR	IR	CaLG08	***QR3dm01***	NCPGR164 - CISR80	15.13	26.47	-2.91	12.81
	2009	HIR	RF	CaLG08	***QR3dm01***	CaM0812 - NCPGR164	8.81	31.56	-3.80	18.16
	2009	HIR	RF	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	39.28	-4.22	23.20
	2009	NDL	RF	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	32.73	-3.65	22.67
	2009	PAT	IR	CaLG08	***QR3dm01***	CaM0812 - NCPGR164	8.81	15.62	-0.97	9.40
	2009	PAT	IR	CaLG08	***QR3dm01***	NCPGR164 - CISR80	15.13	28.49	-1.26	17.01
	2009	PAT	IR	CaLG08	***QR3dm01***	CISR80 - Ca8_714974	5.68	10.11	-0.73	5.62
	2009	PAT	RF	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	42.09	-2.80	33.34
	2009	SEH	RF	CaLG08	***QR3dm01***	NCPGR164 - Ca8_3050452	7.33	18.14	-0.86	11.01
	2008	SEH	RF	CaLG04	*QR3dm04*	Ca4_13687456 - NCPGR21	5.14	14.78	-0.43	8.67
Yield related traits										
100-seed weight (100-SDW, g)	2005	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	20.66	2.75	16.25
	2005	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_13687456 - NCPGR21	5.14	23.56	2.97	17.79
	2006	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	26.92	3.28	18.11
	2006	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_13687456 - NCPGR21	5.14	30.23	3.48	19.62
	2008	NDL	RF	CaLG04	***QR3100sdw03***	Ca4_13687456 - TAA170	4.16	35.61	3.00	21.51
	2008	NDL	RF	CaLG04	***QR3100sdw03***	STMS11 - CaM0615	3.80	20.45	2.29	11.33
	2008	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	56.15	3.21	45.59
	2008	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_13687456 - TAA170	4.16	60.41	3.33	50.13
	2008	SEH	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	21.68	2.82	16.28
	2008	SEH	RF	CaLG04	***QR3100sdw03***	Ca4_13687456 - TAA170	4.16	55.97	3.24	34.16
	2009	DUG	IR	CaLG04	***QR3100sdw03***	Ca4_16149998 - ICCM0249	3.15	22.70	1.68	12.39
	2009	DUG	IR	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	32.90	2.11	27.18
	2009	DUG	RF	CaLG04	***QR3100sdw03***	Ca4_16149998 - ICCM0249	3.15	18.82	2.10	11.75
	2009	DUG	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	42.35	2.86	41.58

	2009	DUG	RF	CaLG04	***QR3100sdw03***	Ca4_13687456 - TAA170	4.16	48.94	3.12	46.31
	2009	HIR	IR	CaLG04	***QR3100sdw03***	Ca4_16149998 - ICCM0249	3.15	12.79	1.92	8.56
	2009	HIR	IR	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	39.14	3.03	42.38
	2009	HIR	IR	CaLG04	***QR3100sdw03***	Ca4_13687456 - TAA170	4.16	42.74	3.20	44.68
	2009	HIR	RF	CaLG04	***QR3100sdw03***	Ca4_16149998 - ICCM0249	3.15	16.40	2.10	11.10
	2009	HIR	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	41.52	3.14	44.06
	2009	HIR	RF	CaLG04	***QR3100sdw03***	Ca4_13687456 - TAA170	4.16	48.76	3.35	51.43
	2009	NDL	IR	CaLG04	***QR3100sdw03***	Ca4_16149998 - ICCM0249	3.15	22.10	2.37	12.57
	2009	NDL	IR	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	47.92	3.55	38.22
	2009	NDL	IR	CaLG04	***QR3100sdw03***	Ca4_12982420 - STMS11	6.41	49.72	3.62	41.38
	2009	NDL	IR	CaLG04	***QR3100sdw03***	STMS11 - CaM0856	5.17	19.42	2.22	11.40
	2009	NDL	RF	CaLG04	***QR3100sdw03***	Ca4_16149998 - ICCM0249	3.15	21.93	2.32	12.49
	2009	NDL	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	46.45	3.45	36.96
	2009	NDL	RF	CaLG04	***QR3100sdw03***	Ca4_12982420 - STMS11	6.41	49.42	3.57	41.57
	2009	NDL	RF	CaLG04	***QR3100sdw03***	STMS11 - Ca4_5390317	15.41	20.28	2.22	12.20
	2009	PAT	IR	CaLG04	***QR3100sdw03***	Ca4_16149998 - ICCM0249	3.15	31.90	3.02	20.80
	2009	PAT	IR	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	37.32	3.53	39.69
	2009	PAT	IR	CaLG04	***QR3100sdw03***	Ca4_13687456 - TAA170	4.16	41.90	3.64	44.14
	2009	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_12558541 - Ca4_11276225	1.81	32.66	2.60	30.77
	2009	PAT	RF	CaLG04	***QR3100sdw03***	Ca4_12982420 - STMS11	6.41	33.57	2.60	33.86
	2009	PAT	RF	CaLG04	***QR3100sdw03***	STMS11 - CaM0856	5.17	12.29	1.54	9.06
	2009	SEH	RF	CaLG04	***QR3100sdw03***	Ca4_12982420 - STMS11	6.41	16.20	1.37	8.47
	2008	PAT	RF	CaLG01	***QR3100sdw06****	Ca1_1083403 - NCPGR184	8.62	10.12	1.38	10.38
	2009	HIR	IR	CaLG01	***QR3100sdw06****	Ca1_1098874 - NCPGR184	8.93	14.71	1.78	18.04
	2009	HIR	RF	CaLG01	***QR3100sdw06****	Ca1_1098874 - NCPGR184	8.93	12.87	1.62	15.73
	2009	PAT	IR	CaLG01	***QR3100sdw06****	Ca1_1083388 - NCPGR184	8.62	15.61	2.12	18.32
	2009	PAT	RF	CaLG01	***QR3100sdw06****	Ca1_1083388 - NCPGR184	8.62	16.23	1.75	14.88
Biomass (BM, g)	2008	PAT		CaLG08	*QR3bm08**	TS57 - TR5	2.21	10.11	-37.42	7.81
	2009	HIR	IR	CaLG04	*QR3bm03*	Ca4_12558541 - Ca4_11276225	1.81	13.07	8.12	7.52

	2009	HIR	RF	CaLG04	*QR3bm03*	Ca4_12558541 - Ca4_11276225	1.81	13.26	7.05	8.18
	2009	HIR	RF	CaLG04	*QR3bm03*	Ca4_13687456 - NCPGR21	5.14	16.63	7.82	9.85
	2009	NDL	RF	CaLG08	*QR3bm05*	CaM0812 - NCPGR164	8.81	11.42	5.26	6.33
	2009	NDL	RF	CaLG08	*QR3bm05*	NCPGR164 - CISR80	15.13	16.17	6.28	7.87
Harvest index (HI, %)	2005	PAT	RF	CaLG08	***QR3hi02***	NCPGR164 - Ca8_3050452	7.33	15.32	-0.85	8.96
	2005	PAT	RF	CaLG08	***QR3hi02***	Ca8_3050452 - CISR80	7.79	14.07	-0.81	5.70
	2008	PAT	RF	CaLG08	***QR3hi02***	NCPGR164 - CISR80	15.13	16.87	0.02	7.76
	2008	PAT	RF	CaLG08	***QR3hi02***	CISR80 - Ca8_714974	5.68	11.12	0.01	5.45
	2009	PAT	IR	CaLG08	***QR3hi02***	NCPGR164 - Ca8_3050452	7.33	25.94	0.03	14.52
	2009	PAT	RF	CaLG08	***QR3hi02***	NCPGR164 - Ca8_3050452	7.33	15.68	0.02	8.11
	2009	HIR	IR	CaLG01	*QR3hi06*	H2A08 - Ca1_6540035	16.29	10.14	0.03	3.30
	2009	PAT	RF	CaLG04	*QR3hi10*	Ca4_12558541 - Ca4_11276225	1.81	13.40	-0.02	8.69
	2009	PAT	RF	CaLG04	*QR3hi10*	Ca4_11441735 - NCPGR21	8.24	13.40	-0.02	7.83
Pods/plant (POD)	2005	PAT	RF	CaLG04	***QR3pod01***	Ca4_13687456 - NCPGR21	5.14	15.09	-13.59	9.49
	2006	PAT	RF	CaLG04	***QR3pod01***	Ca4_16149998 - ICCM0065	4.99	10.73	-5.16	5.34
	2006	PAT	RF	CaLG04	***QR3pod01***	Ca4_13724666 - Ca4_11276225	1.05	22.85	-7.54	13.78
	2007	PAT	RF	CaLG04	***QR3pod01***	Ca4_12982420 - TAA170	4.93	12.28	-6.86	7.13
	2009	PAT	IR	CaLG04	***QR3pod01***	ICCM0065 - Ca4_13588956	2.39	28.56	-9.43	15.85
	2009	PAT	IR	CaLG04	***QR3pod01***	Ca4_13687456 - TAA170	4.16	32.34	-10.00	18.79
	2009	PAT	RF	CaLG04	***QR3pod01***	Ca4_16278671 - Ca4_15926160	0.86	16.74	-5.06	9.44
	2009	PAT	RF	CaLG04	***QR3pod01***	ICCM0065 - Ca4_11276225	3.23	20.03	-5.80	14.28
	2009	PAT	RF	CaLG04	***QR3pod01***	Ca4_13840227 - NCPGR21	5.37	21.43	-6.04	14.22
	2006	PAT	RF	CaLG04	*QR3pod02**	STMS11 - CaM0615	3.80	12.81	-5.60	6.79
Seeds/pod (SPD)	2007	PAT	RF	CaLG04	*QR3spd04**	Ca4_16149998 - ICCM0249	3.15	25.98	2.58	14.71
	2007	PAT	RF	CaLG04	*QR3spd01*	Ca4_12558541 - Ca4_11276225	1.81	45.40	3.43	31.13
	2007	PAT	RF	CaLG04	*QR3spd01*	Ca4_13840227 - TAA170	4.39	30.80	3.09	25.26
	2007	PAT	RF	CaLG04	*QR3spd05**	STMS11 - CaM0845	4.96	11.09	1.78	7.27
Yield (YLD, g)	2009	HIR	IR	CaLG01	*QR3yld03*	CaM0046 - Ca1_16552751	2.08	14.19	5.22	9.11

	2009	HIR	RF	CaLG04	*QR3yld04*	Ca4_13687456 - NCPGR21	5.14	11.67	4.30	6.47
	2009	NDL	RF	CaLG08	*QR3yld10**	Ca8_4106644 - CISR80	13.44	18.64	3.39	10.94
Drought Indices										
Drought susceptibility index (DSI)	2009	DUG		CaLG07	*QR3dsi02**	ICCM0199c - CaM1417	6.28	13.00	-2.42	3.65
Drought tolerance index (DTI)	2009	HIR		CaLG04	*QR3dti02*	Ca4_12558541 - Ca4_11276225	1.81	10.10	0.13	6.54
	2009	NAN		CaLG01	*QR3dti05**	H2A08 - Ca1_6540035	16.29	10.61	0.08	3.17
	2009	NAN		CaLG08	*QR3dti06**	NCPGR164 - CISR80	15.13	10.76	0.08	6.00

[a]PAT - Patancheru; NDL - Nandyal; SEH - Sehore; DUG - Durgapura; HIR – Hiriyur.
[b]RF - Rainfed; IR - Irrigated; CC - Cylider culture.
* newly appearing QTL.
** phenotypic variation explained.
# logarithm of odds (base 10).
QTLs represented in red bold fonts are consistent; QTLs represented in blue bold fonts are stable and consistent.

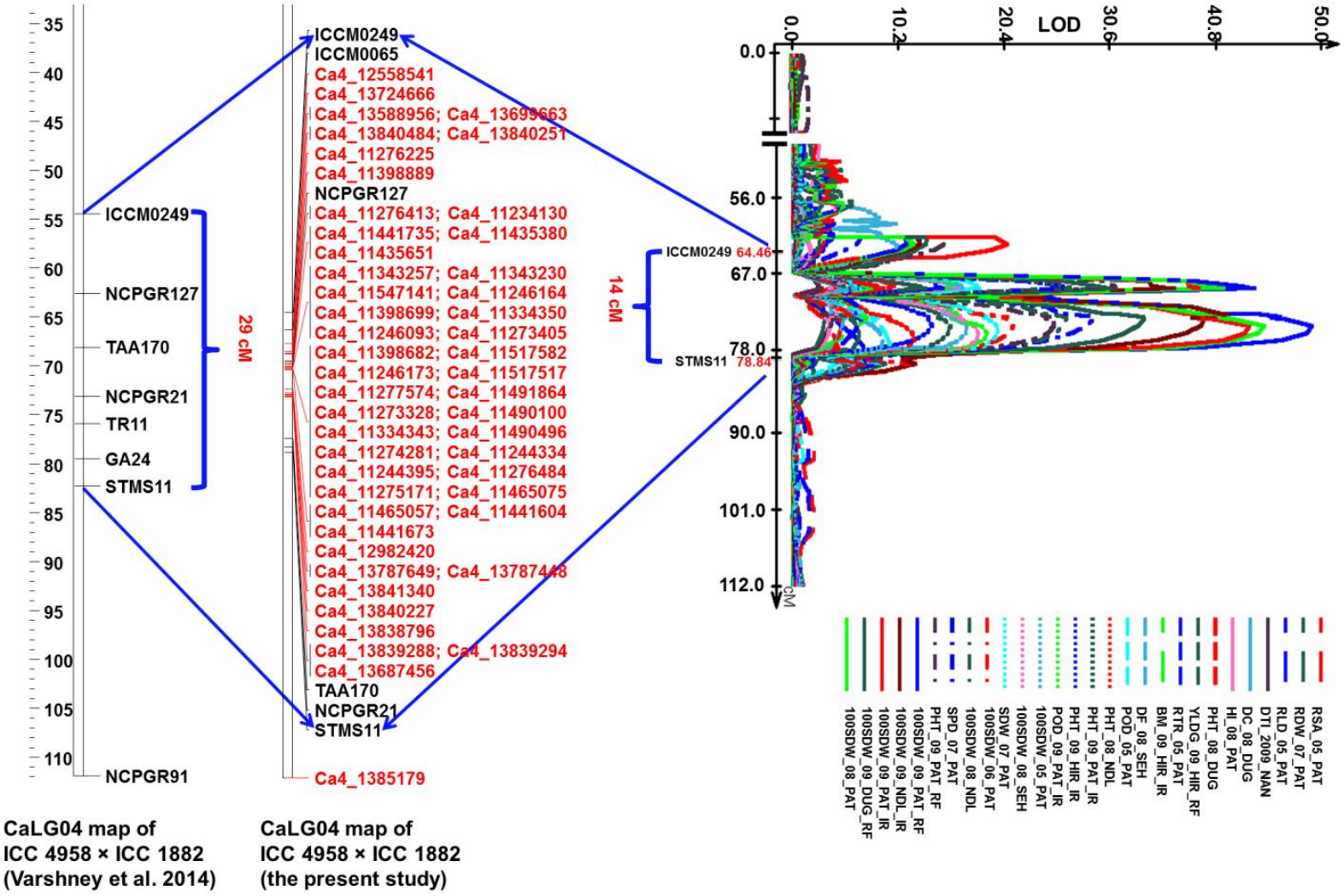

Figure 2: Saturated "*QTL-hotspot*" region with additional GBS-SNP markers. The figure shows comparison of the "*QTL-hotspot*" updated with 49 novel SNP markers in this study and with the one reported by Varshney et al. (2014).

In the case of drought indices, a novel QTL, '*QR3dsi02*' was identified explaining 13% phenotypic variation for DSI, whereas no QTL for DSI was reported in the earlier study (Varshney et al. 2014) (Table 8). Three QTLs were identified for DTI, of which 2 were novel. Interestingly, a QTL from the "*QTL-hotspot*", '*QR3dti02*' which was earlier reported to be a minor QTL, was ranked as robust QTL in this study (Table 7 and Table 8).

4.4 Conversion of SNP Markers into CAPS

As breeders are interested in an inexpensive and technically less demanding genotyping platform for marker assisted breeding, the SNPs integrated into the "*QTL-hotspot*" were converted to CAPS/dCAPS markers. A total of 16 CAPS and 33 dCAPS primer pairs were designed and verified for amplification (Table 9, Appendix 1). However only 20 out of 49 primer pairs showed single prominent amplicon and subsequently used for restriction digestion on a panel of 5 parental genotypes. As a result, 14 CAPS and 1 dCAPS were developed (Figure 3). In total, 8 CAPS markers were polymorphic in the parents of inter-specific mapping population ICC 4958 × PI 489777, while 14 (13 CAPS and 1 dCAPS) each in the parents of two intra-specific mapping populations ICC 4958 × ICC 1882 and ICC 283 × ICC 8261 were polymorphic (Table 9, Appendix 1).

4.5 Refining the "*QTL-hotspot*" Region Using Ultra Density Bin Map

Coarse mapping for drought component traits resulted in identifying "*QTL-hotspot*" region with 7 SSR markers spanning 7.74 Mb on physical map and 29 cM on genetic map (Varshney et al. 2014). GBS based SNP marker analysis resulted in refining the region into 14 cM on genetic map and ca.3 Mb on physical map (chapter 4.2). However the refined region needs further fine mapping in order to delimit the "*QTL-hotspot*" into few genes.

4.5.1 SNP discovery through skim sequencing

ICCRIL03 population was genotyped using a skim sequencing approach. In the first instance, a total of 5.90 Gb and 5.94 Gb data were generated for ICC 4958 and ICC 1882 respectively, which represents an estimated coverage of 7.90X and 8.03X. SGSautoSNP identified 84,963 SNPs, out of which 64,581 were distributed over the 8 pseudomolecules and 20,382 were located on unplaced contigs. Subsequently, a total of 497.60 Gb of Illumina paired read sequence data were generated for 232 RIL samples. The data obtained ranged from 0.21X to 4.9X per RIL, with an average of 0.72X, however, RIL219 had exceptionally low (0.002X) data. A total of 2,836 SNPs predicted between the parents were found to be monomorphic in the population and were discarded, leaving 82,127 SNPs. However, only 62,370 SNPs that were located on pseudomolecules were considered for further analysis. These SNPs were further filtered, using the criteria of minimum allele frequency (MAF) of ≥ 0.20 and lines having $\geq 5\%$ missing data were discarded, which resulted in 53,169 SNPs across 222 RILs analyzed. Additionally 54 SNPs mapped within the "*QTL-hotspot*" (see chapter 4.2) were also included and thus in total 53,223 SNPs were used map construction. The distribution of SNPs on the eight chickpea pseudomolecules is represented in Figure 4 and Table 10. The highest number of SNPs were identified on Ca4 (18,989 SNPs), while only 954 SNPs were identified on Ca5.

Table 8: Comparison of robust main-effect QTLs (M-QTLs) identified for various drought tolerance related traits in the present study with that of Varshney et al. (2014)

	Varshney et al. (2014) (ICC 4958 × ICC 1882)						Current GBS study (ICC 4958 × ICC 1882)					
Trait	**Total QTLs**	**No. of QTLs in "*QTL-hotspot*"**	**Stable QTLs**	**Consistent QTLs**	**Position (cM)**	**PVE[a] (%)**	**Total QTLs**	**No. of QTLs in "*QTL-hotspot*"**	**Stable QTLs**	**Consistent QTLs**	**Position (cM)**	**PVE[a] (%)**
Root												
RLD	1	1	-	-	10.54	10.90	1	1	-	1	3.23 - 5.37	10.65 - 12.09
RSA	1	1	-	-	13.86	10.26	1	-	-	-	7.57	11.04
RTR	1	1	-	-	5.00	16.67	1	1	-	1	1.81 - 5.37	10.85 - 13.56
Morphological												
SDW	1	1	-	1	5.00	13.89 - 17.59	3*	3	-	1	0.73 - 5.91	10.78 - 26.91
PHT	4	1	1	2	0.96 - 42.39	10.00 - 30.20	9*	3	3	5	1.05 - 17.94	10.05 - 34.57
PBS	-	-	-	-	-	-	1*	-	-	-	8.81	12.92
Phenological												
DF	2	1	1	1	5.53 - 22.86	10.51 - 26.87	3*	1	1	1	1.81 - 15.13	10.86 - 67.71
DM	3	1	1	1	5.53 - 31.09	12.13 - 19.71	2	1	1	1	5.14 - 15.13	10.11 - 47.43
Yield related												
100-SDW	2	1	1	1	10.53 - 16.65	10.31 - 58.20	2	1	2	2	1.81 - 15.41	10.12 - 60.41
BM	2	1	-	-	10.54 -	10.95	3*	1	-	-	1.81 -	10.11 -

					22.86	- 21.32					15.13	16.63
HI	3	1	-	-	5.00 - 20.23	10.67 - 14.36	3	1	-	1	1.81 - 16.29	10.14 - 25.94
POD	1	1	-	1	10.54	10.19 - 23.18	2*	2	-	1	0.86 - 5.37	10.73 - 32.34
SPD	1	1	-	-	5.00	42.07	3*	3	-	-	1.81 - 4.96	11.09 - 45.40
YLD	2	1	-	-	1.92 - 5.00	13.98 - 15.71	3*	1	-	-	2.08 - 13.44	11.67 - 18.64
Drought indices												
DSI	-	-	-	-	-	-	1*	-	-	-	6.28	13.00
DTI	1	-	-	-	28.90	11.23	3*	1	-	-	1.81 - 16.29	10.10 - 10.76
Total	**25**	**13**	**4**	**7**			**41**	**20**	**7**	**14**		

*Newly identified M-QTLs; [a]PVE - phenotypic variation explained.

Table 9: Summary of validated CAPS/dCAPS markers on 5 parental lines of RIL populations

SNP ID	Marker*	Marker type	Enzyme	*Polymorphism status of the parental lines of different mapping populations		
				ICC 4958 × PI 489777	ICC 4958 × ICC 1882	ICC 283 × ICC 8261
Ca4_12558541	CGMM139	CAPS	*Mnl*I	M	P	P
Ca4_13840251	CGMM140	CAPS	*Mbo*II	P	P	P
Ca4_11276225	CGMM141	CAPS	*Aci*I	M	P	P
Ca4_11435380	CGMM142	CAPS	*Rsa*I	P	P	P
Ca4_11435651	CGMM143	dCAPS	*Hpa*II	M	P	P
Ca4_11547141	CGMM144	CAPS	*Hph*I	P	P	P
Ca4_11334350	CGMM145	CAPS	*Mbo*II	P	P	P
Ca4_11246093	CGMM146	CAPS	*Ava*I	P	P	P
Ca4_11517582	CGMM147	CAPS	*Hae*III	M	P	P
Ca4_11244334	CGMM148	CAPS	*Mse*I	M	P	P
Ca4_11465075	CGMM149	CAPS	*Bsa*BI	P	P	P
Ca4_11441673	CGMM150	CAPS	*Eco*RI	M	P	P
Ca4_13838796	CGMM151	CAPS	*Mnl*I	M	M	M
Ca4_13839288	CGMM152	CAPS	*Mnl*I	P	P	P
Ca4_13839294	CGMM153	CAPS	*Mse*I	M	P	P

*Validated CAPS/dCAPS nomenclature is as per Gujaria et al. (2011); *M - monomorphic, P - polymorphic.

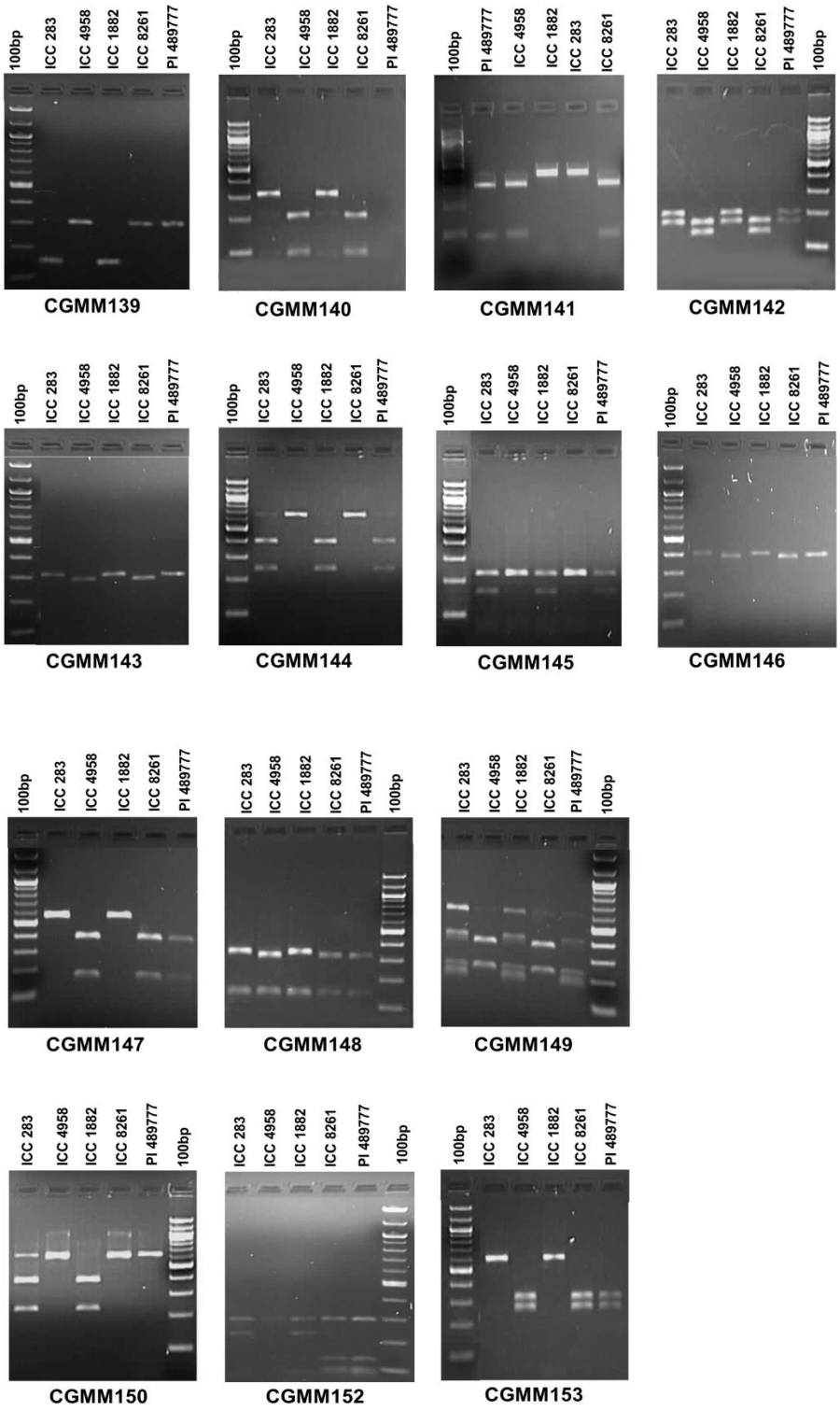

Figure 3: The electrophoretic profile of CAPS/dCAPS candidates after PCR and restriction digestion with their respective enzymes. Restricted digested products were shown on 1.2% agarose gel. Polymorphic bands were clearly observed between ICC 4958 and ICC 1882.

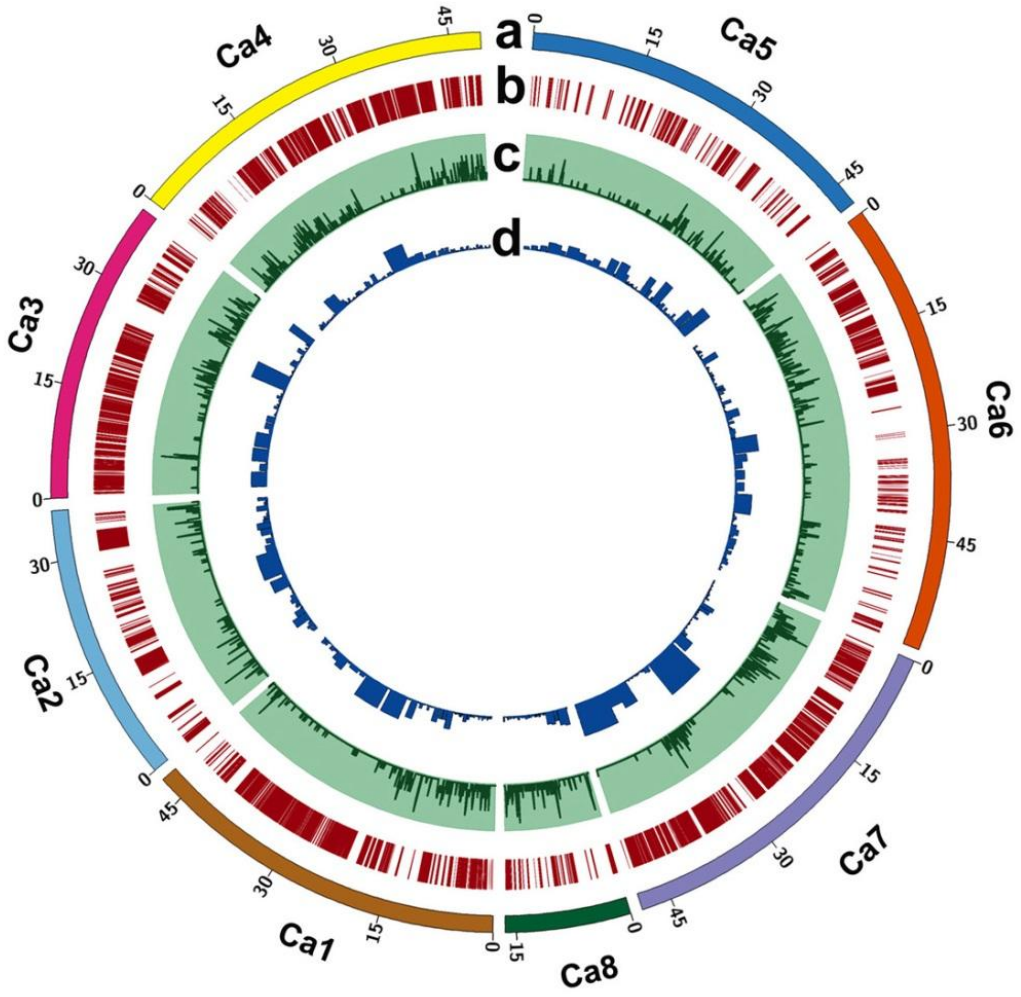

Figure 4: Genome-wide distribution of SNPs and recombination bins in chickpea using SNP markers generated from skim sequencing. (**a**) Chickpea pseudomolecules, labelled as Ca1 to Ca8 and each pseudomolecule is shown in a different colour. The numbers on arches represent the scale for the size of pseudomolecules in Mb; (**b**) Distribution of 53,223 SNPs on eight chickpea pseudomolecules. Each SNP is represented as a single vertical line. Aggregation of vertical lines indicates SNP dense regions, while SNP sparse regions are depicted by blank spaces; (**c**) Distribution of 1,610 recombination bins on chickpea pseudomolecules. The number of recombination bins within 100 kb intervals were calculated and plotted as a smooth line curve. The height of the line indicates the number of bins within the respective 100 kb interval. A flat line corresponds to no or limited recombination regions; (**d**) Distribution of genes among recombination bins. The number of genes located within each recombination bin was identified by comparing the coordinates of the respective bin with chickpea gene models. The width of the column is proportional to the recombination bin interval while column height is proportional to the number of genes within that interval.

4.5.2 Recombination breakpoints and bins

By using the sliding window approach on 53,223 SNPs segregating in 222 RILs, a total of 1,610 bins were identified (Figure 5 and Table 10). The average number of bins identified in an individual RIL was 35.71, while the number of bins per pseudomolecule ranged from 2.75 to 6.12. The minimum number of bins (112) was identified on Ca2, whereas the highest number of bins (292) was identified on Ca6 (Figure 4 and Figure 6a). The bin sizes ranged from 41 bp to 8.05 Mb, with an average of 210.60 kb and median of 54.77 kb. In total, 91% bins were of size ≤5 Mb while ~9% bins (144) had a size >5 Mb (Figure 6b). By considering 28,269 genes in the chickpea genome (Varshney et al. 2013a), the defined bin map covered 23,277 genes. In total, 8.68% (139) bins contained no gene, 14.29% (229) had only one gene, while 45.82% (734) had two to ten genes. Thus, 68.79% of bins had ≤10 genes (Figure 6c).

4.5.3 Bin markers based genetic linkage map

All 1,610 bins, as mentioned above, were used as molecular markers for developing a genetic map (http://cegresources.icrisat.org/cmap/Chickpea_bin_map). Out of which, 1,557 (96.71%) bins could be integrated into a genetic map that spans 973.94 cM (Table 10) and corresponds to 757.75 kb/cM by considering the chickpea genome size of 738 Mb (http://cegresources.icrisat.org/cmap/ Chickpea_bin_map/). The average inter-bin interval per linkage group ranged from 0.45 cM (CaLG04) to 0.93 cM (CaLG02) with an overall average value of 0.66. The size of the linkage groups varied from 70.57 cM (CaLG08) to 196.27 cM (CaLG06) (Table 10). The highest number of bins (286) was mapped on CaLG06, followed by CaLG04 (277), while the minimum number of bins was mapped on CaLG02 (103) (Table 10).

Table 10: Distribution of SNPs, recombination bins and markers mapped on chickpea pseudomolecules/linkage groups

Pseudomolecules	Number of SNPs identified	Number of bins identified	Number of bins mapped	Map length (cM)	Density (no.of bins/cM)
Ca1	12,225	223	223	179.85	0.81
Ca2	4,422	112	103	95.9	0.93
Ca3	2,365	188	174	80.44	0.46
Ca4	18,989	281	277	123.6	0.45
Ca5	954	151	143	119.06	0.83
Ca6	5,759	292	286	196.27	0.69
Ca7	7,158	248	242	108.25	0.45
Ca8	1,351	115	109	70.57	0.65
Total	**53223**	**1610**	**1557**	**973.94**	
Average					**0.66**

The order of bins on the genetic map was compared with their physical position on the chickpea genome sequence (CaGAv1.0). While excellent concordance was observed for linkage groups 4 and 8 and minor differences were observed on other linkage groups (Figure 7).

4.5.4 Bin map based QTL analysis

Genotyping data for 1,557 bins were analyzed together with phenotyping data collected for 20 traits at 1 - 5 locations in 1 - 5 seasons, mentioned in Table 4. QTLs with >10% PVE were considered as major QTLs while others were considered as minor QTLs. In summary, a total of 134 QTLs for 17 traits and two drought indices were identified, out of which 71 were major QTLs and 63 were minor QTLs (Table 11 and Appendix 2). PVE for major QTLs ranged from 10.14 to 59.83% at 3.12 to 43.56 LOD value while for minor QTLs, PVE ranged from 4.75 to 9.85% at 3.00 to 4.91 LOD value.

Major QTLs were identified for 11 traits (RLD, RTR, SDW, PHT, PBS, DF, DM, POD, 100-SDW, HI and DC) and minor QTLs were identified for all the analyzed traits (Table 11 and Appendix 2). The distribution of all the major QTLs in the chickpea genome has been shown in Figure 8 and summarized in Appendix 2. A total of 29 major QTLs for 9 traits (RLD, RTR, SDW, PHT, DM, POD, 100-SDW, HI and DC) were observed on CaLG04 and interestingly all were identified within the "*QTL-hotspot*" region. On the other hand, 30 major QTLs for five traits (PHT, PBS, DF, DM and HI) were observed on CaLG08.

4.5.5 Refined regions of "*QTL-hotspot*"

Although 1 - 17 major QTLs per trait were identified (Table 12), we targeted the topmost QTL (QTL which explained the highest phenotypic variation) for a given trait. As a result, 11 topmost

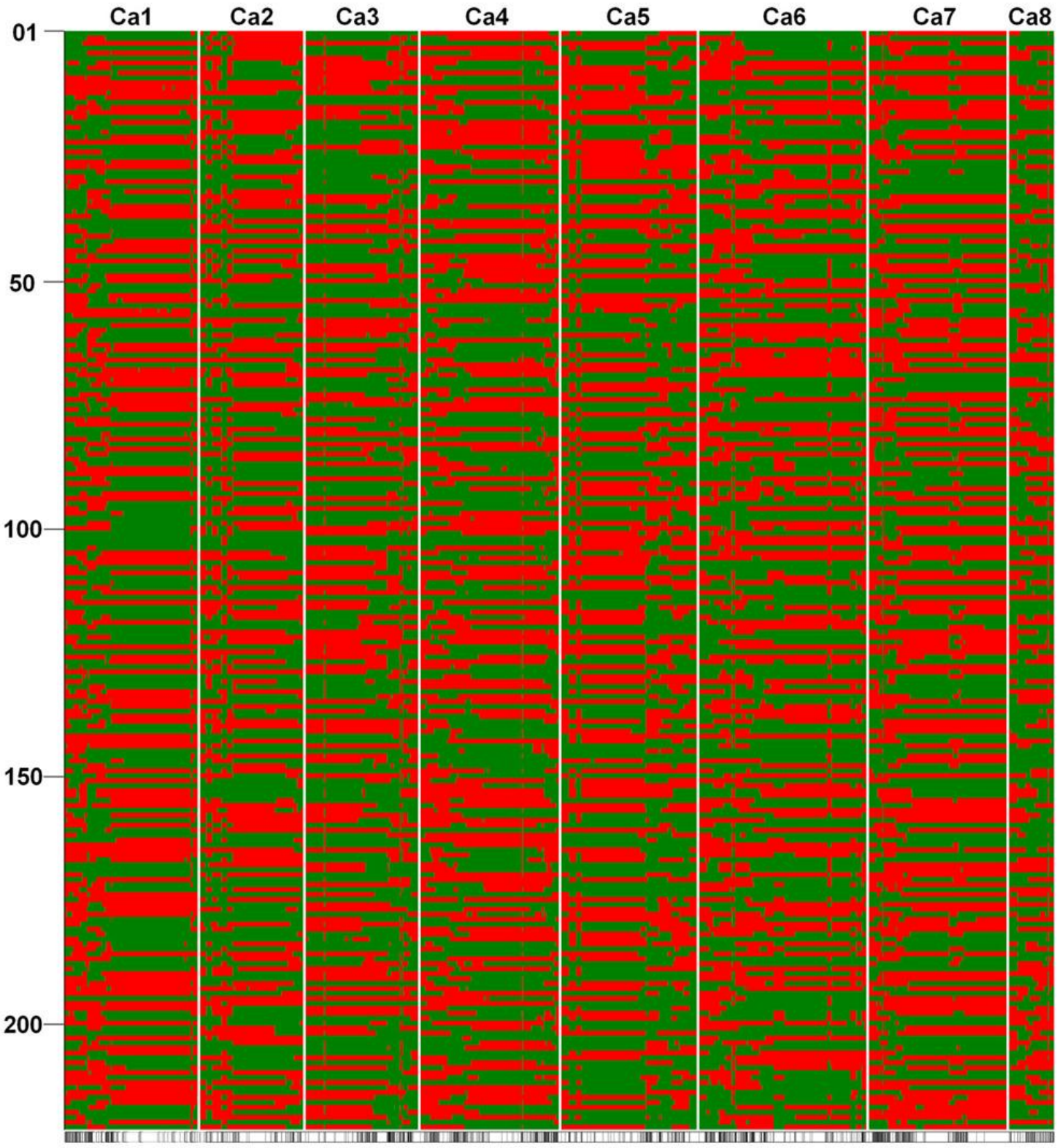
Ca1
Ca2
Ca3
Ca4
Ca5
Ca6
Ca7
Ca8
01
50
100
150
200

Figure 5: The recombination breakpoints identified in 222 recombinant inbred lines (RILs). A parent dependent 15 bp sliding window approach was used to identify true recombination breakpoints. A total of 53,223 SNPs identified were scored as "A" and "B" representing alleles from the two parents ICC 4958 and ICC 1882 respectively, and for each individual, the ratio of A and B alleles within the window was calculated using a perl script (Huang et al. 2009). Windows with nine or more alleles from either parent were considered as homozygous for the respective region. The recombination break point was defined at the transition from one genotype to another. The chromosomes are labelled as Ca1 to Ca8 and are separated by vertical lines while each horizontal line represents a single RIL. Green and red bars represent segments from ICC 4958 and ICC 1882 genotypes, respectively. The number of bins per pseudomolecule ranged from 2.75 to 6.12 while an average of 35.71 bins was identified in an individual RIL. The black and white panel at the bottom indicates the consensus 1,610 bins identified in the entire RIL population.

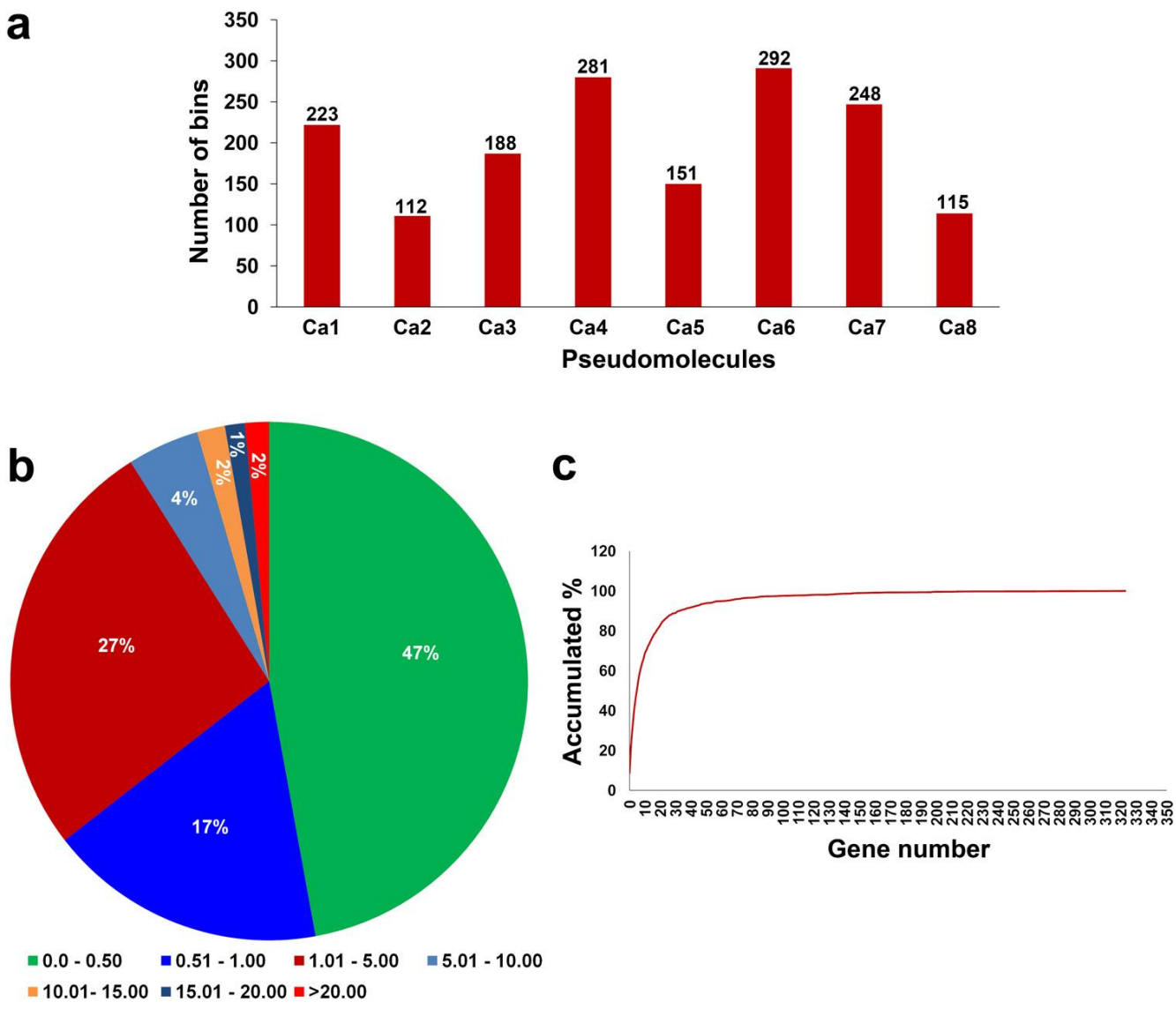

Figure 6: Features of recombination bin mapping in chickpea using skim sequencing based SNP markers. (**a**) Distribution of the recombination bins on eight chickpea pseudomolecules. The number of recombination bins identified in each psudomolecule was depicted on top of each column. A minimum of 112 bins were identified on Ca2 while maximum 292 bins were identified on Ca6; (**b**) Distribution of bin sizes identified in ICC 4958 × ICC 1882 population. Approximately, 50% of the bins were of ≤ 0.50 Mb size indicating majority of the recombination has been captured; (**c**) A plot of gene resolution. Number of genes in each recombination bin represented on the X-axis and the accumulated percentage of bins are on the Y-axis. In total, 8.68% (139) bins contained no gene, 14.29% (229) had only one gene, while 45.82% (734) had two to ten genes. Thus, 68.79% of bins had ≤ 10 genes.

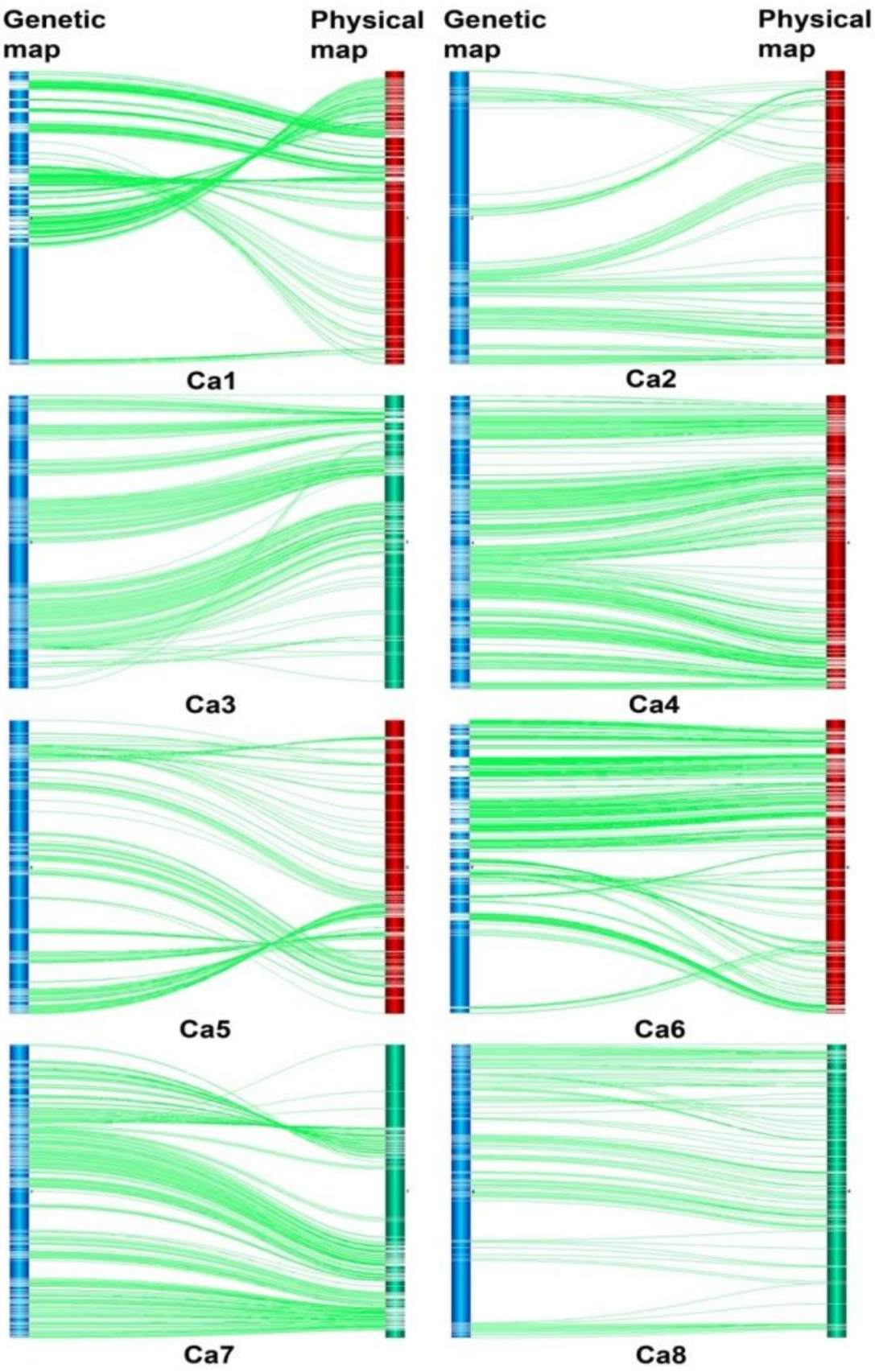
Genetic map
Physical map
Genetic map
Physical map
Ca1
Ca2
Ca3
Ca4
Ca5
Ca6
Ca7
Ca8

Figure 7: Concordance between the genetic map and physical map of chickpea. The order of each bin on linkage and physical map was visualized using Strudel V. 1.12.03.20 software. Each green line represents the position of the bin marker on respective genetic and physical map. Straight lines indicate markers on genetic and physical maps are in same order while crossing of lines indicate interchange of those markers on genetic and physical maps. Excellent consistency in marker order was observed between genetic and physical map in case of Ca3, Ca4, Ca6 and Ca8 pseudomolecules whereas slight deviation was observed for other pseudomolecules (Ca1, Ca2, Ca5 and Ca7). This may be due to the misassembly in some regions in the draft genome of chickpea.

QTLs with 10.14 - 59.83% PVE were selected for 11 traits (Table 12). While analyzing flanking markers for these topmost QTLs, we identified two genomic regions, namely bin_4_13239546 - bin_4_13378761 of 139.22 kb and bin_4_13393647 - bin_4_13547009 of 153.36 kb sizes on CaLG04 containing the topmost QTLs for RLD, PHT, POD, 100-SDW and DC; and for RTR and SDW, respectively (Table 12). Similarly, one genomic region (bin_8_6034209 - bin_8_5984553) of 49.66 kb size was identified on CaLG08 containing topmost QTLs for PBS, DF and HI (Table 12). However the topmost QTL for DM was present solitary on CaLG07. As expected, the two genomic regions on CaLG04 mentioned above were present within the "*QTL-hotspot*" region. In brief, this study split the "*QTL-hotspot*" region of ~3 Mb size into two smaller regions and we refer them as "*QTL-hotspot_a*" (bin_4_13239546 - bin_4_13378761, equivalent to 139.22 kb) and "*QTL-hotspot_b*" (bin_4_13393647 - bin_4_13547009, equivalent to 153.36 kb) (Figure 9).

4.6 Candidate Genes from Various Approaches for Drought Tolerance Related Traits

4.6.1 Genes identified from GBS-SNP based QTL analysis

A detailed analysis of QTLs from the "*QTL-hotspot*" region showed that, QTLs of 9 traits (RTR, SDW, PHT, DF, 100-SDW, DM, HI, SPD and DTI) were flanked by Ca4_12558541 - Ca4_11276225 marker. Further, the traits RTR, RLD, PHT, DF, DM, 100-SDW, BM, POD and YLD were flanked by Ca4_13687456 - NCPGR21. As the QTLs for 13 out of 16 traits fall between markers Ca4_11276225 and NCPGR 21 (whose physical position on genome is 14146315 bp), ~3 Mb regions between these markers were selected for candidate gene identification (Table 7). This 3 Mb region contained 286 genes. Amino acid sequences for these 286 genes were searched against the NCBI-nr protein database.

Table 11: Distribution of number of QTLs identified on chickpea genome from the RIL population of ICC 4958 × ICC 1882 cross using high density bin map

Trait	CaLG01	CaLG02	CaLG03	CaLG04	CaLG05	CaLG06	CaLG07	CaLG08	Total
Root length density (RLD, cm cm^{-3})				1 (1)					1 (1)
Root surface area (RSA, cm^2)								1	1
Root volume (RV, cm^3)								1	1
R-T ratio (RTR, %)				2 (2)					2 (2)
Shoot dry weight (SDW, g)				1 (1)					1 (1)
Plant height (PHT, cm)				14 (8)		7 (3)		5 (2)	26 (13)
Primary branches (PBS)	1						1	2 (1)	4 (1)
Secondry branches (SBS)	1			1				1	3
Days to 50% flowering (DF)	1 (1)				7	1		14 (13)	23 (14)
Days to maturity (DM)		1		1 (1)	4 (1)	3	3 (1)	14 (13)	25 (16)
Pods/plant (POD)				2 (2)					2 (2)
Seeds/pod (SPD)					1			1	2
100-seed weight (100-SDW, g)	9 (5)			12 (12)					21 (17)
Biomass (BM, g)	1			1			1		3
Harvest index (HI, %)				1 (1)		2 (1)	1	4 (1)	8 (3)
Yield (YLD, g)			1	1			1		3
Delta carbon ratio ($\delta^{13}C$)				3 (1)					3 (1)
Drought tolerance indices (DTI)	1						2	1	4
Drought susceptibility indices (DSI)								1	1
Total	**14 (6)**	**1**	**1**	**40 (29)**	**12 (1)**	**13 (4)**	**9 (1)**	**44 (30)**	**134 (71)**

Numbers in parenthesis represent major QTLs.

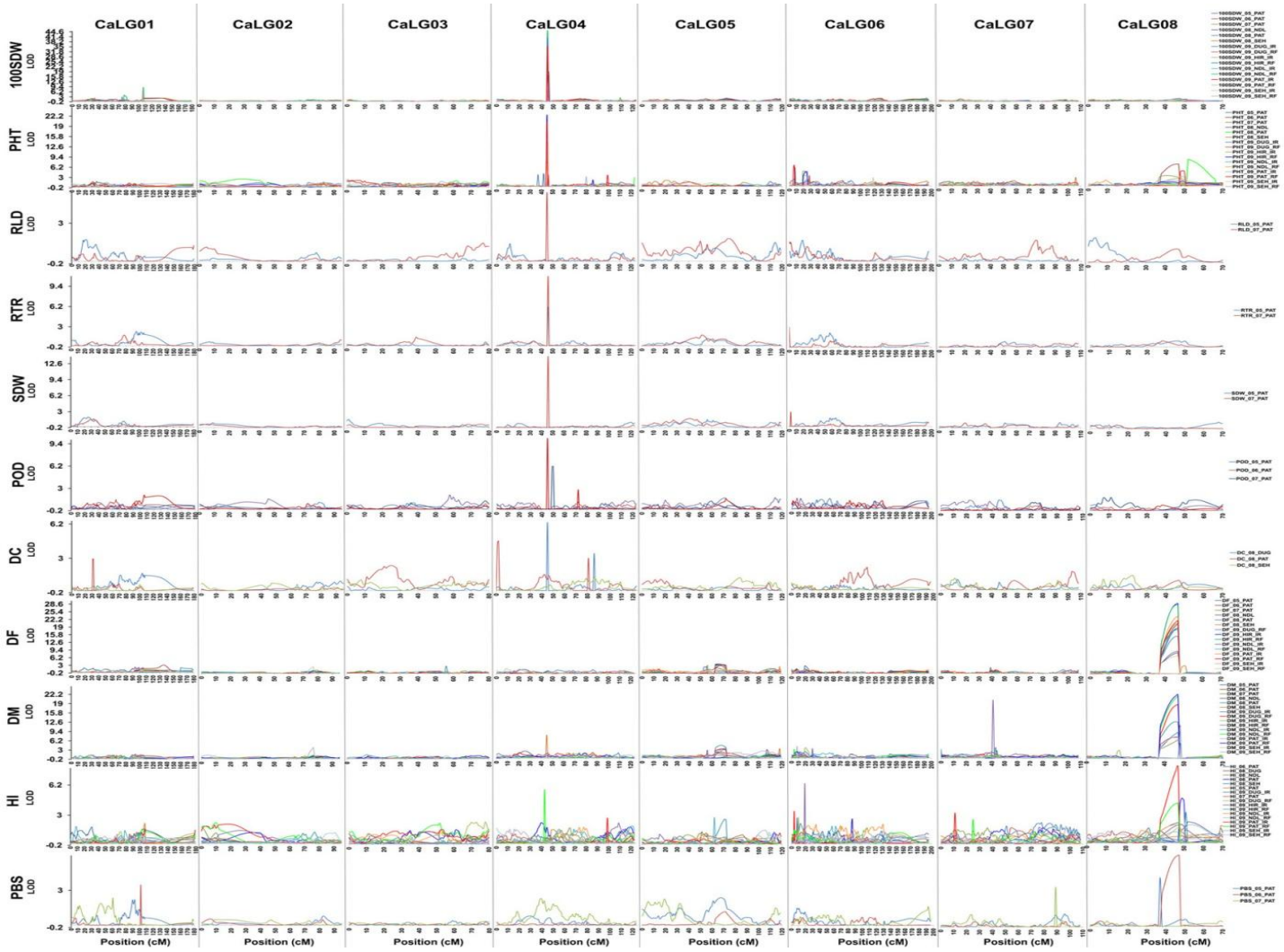

Figure 8: Genome-wide distribution of major QTLs identified for drought component traits using skim sequencing data. The linkage groups are separated by vertical lines, genetic distance is represented on the X-axis and LOD values are represented on Y-axis. Different coloured lines for each trait represent the phenotypic data collected over 1-5 seasons, 1-5 locations.

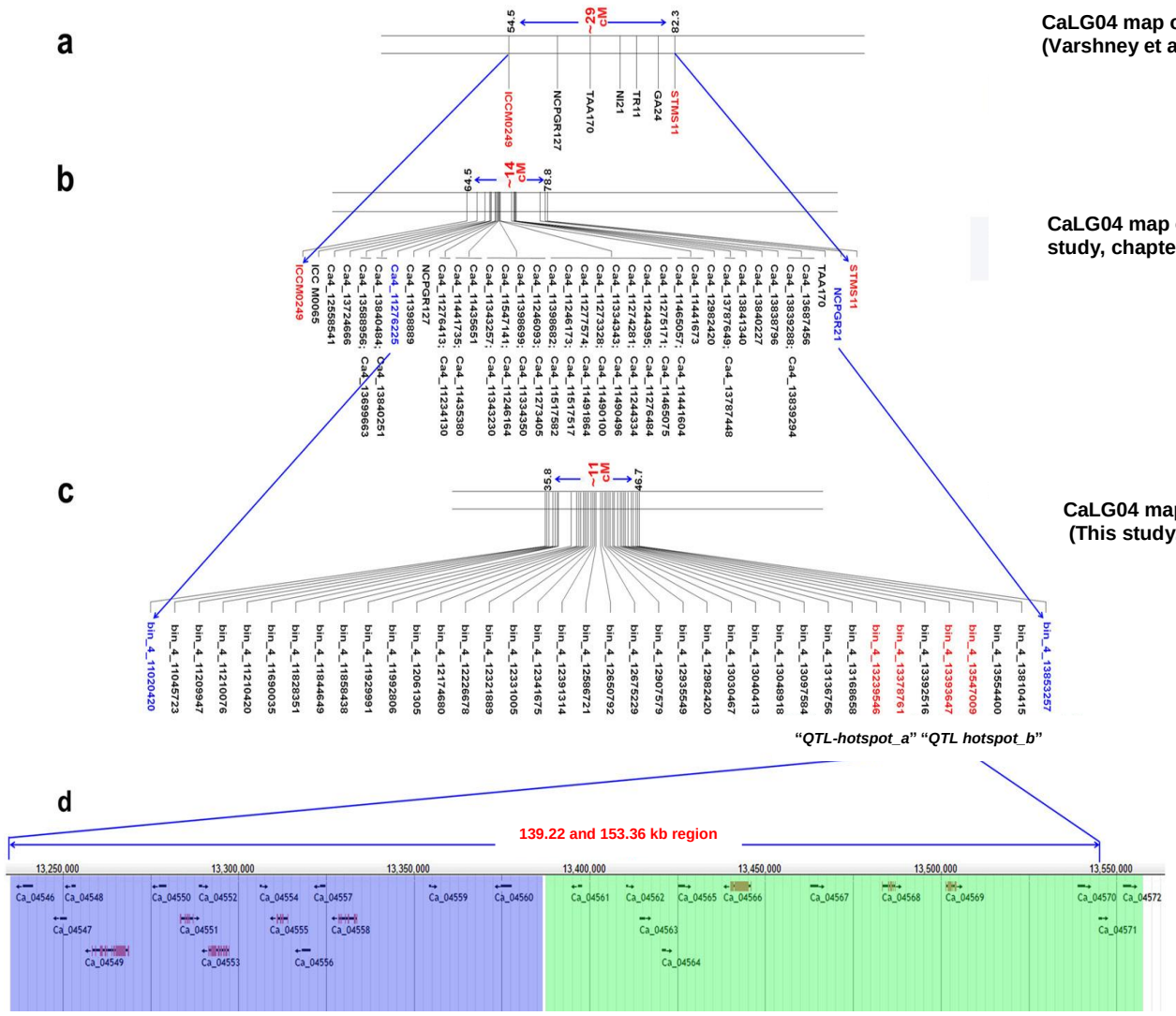
a
b
c
d
CaLG04 map of ICC 4958 × ICC 1882 (Varshney et al. (2014))
CaLG04 map of ICC 4958 × ICC 1882 (This study, chapter 4.2)
CaLG04 map of ICC 4958 × ICC 1882 (This study, chapter 4.5.5)
"QTL-hotspot_a" "QTL hotspot_b"
139.22 and 153.36 kb region

Figure 9: Refinement of "*QTL-hotspot*" region into "*QTL-hotspot_a*" and "*QTL-hotspot_b*" and identification of candidate genes.

a) The "*QTL-hotspot*" region, reported by Varshney et al. 2014, spanning 29 cM (corresponds 7.74 Mb on physical map) harbouring QTLs for several drought tolerance related traits on CaLG04; b) Refined "*QTL-hotspot*" region (~14 cM corresponding to ~3 Mb on physical map) reported from the current study consisted of 49 SNPs and six SSRs (chapter 4.2); c) Refined "*QTL-hotspot*" region on CaLG04 with newly integrated markers (recombination bins) from skim sequencing. The markers, viz. bin_4_11020420 and bin_4_13853257 correspond to the refined 3 Mb "*QTL-hotspot*" region reported in the current study (chapter 4.2). Integration of 1,421 SNPs to the "*QTL-hotspot*" region resulted in the identification of 38 recombination breakpoints and thereby split the "*QTL-hotspot*" region into "*QTL-hotspot_a*" (139.22 kb; 0.23 cM) and "*QTL-hotspot_b*" (153.36 kb; 0.22 cM). "*QTL-hotspot_a*" was flanked by bin_4_13239546 and bin_4_13378761 while "*QTL-hotspot_b*" was flanked by bin_4_13393647 and bin_4_13547009. These four flanking markers were shown in red colour font; d) A ~300 kb (13239546 - 13547009) snapshot of chickpea genome from JBrowse showing twenty six candidate genes identified in the "*QTL-hotspot_a*" and "*QTL-hotspot_b*" regions. A total of 15 genes (highlighted in blue colour area) were identified from "*QTL-hotspot_a*" while 11 candidate genes (highlighted in green colour area) were identified from "*QTL-hotspot_b*"region.

Table 12: Comparative analysis of topmost QTLs identified for drought related traits in chickpea from the RIL population of ICC 4958 × ICC 1882

	Bin mapping (current study)							Coarse mapping (Varshney et al. 2014)				GBS (current study)			
Trait Name	**Linkage group**	**Markers flanked**	**Cluster**	**Physical size (kb)**	**Genetic size (cM)**	**LOD#**	**PVE* (%)**	**Linkage group**	**Markers flanked**	**LOD#**	**PVE* (%)**	**Linkage group**	**Markers flanked**	**LOD#**	**PVE* (%)**
RLD	CaLG04	bin_4_13239546 - bin_4_13378761	1	139.22	0.23	5.23	10.36	CaLG04	NCPGR127 - NCPGR21	4.78	10.90	CaLG04	ICCM0065 - Ca4_11276225	4.59	12.09
PHT	CaLG04	bin_4_13239546 - bin_4_13378761	1	139.22	0.23	24.34	41.76	CaLG04	NCPGR127 - NCPGR21	16.55	30.20	CaLG04	Ca4_12982420 - TAA170	29.16	34.57
POD	CaLG04	bin_4_13239546 - bin_4_13378761	1	139.22	0.23	9.82	16.66	CaLG04	NCPGR127 - NCPGR21	13.14	23.18	CaLG04	Ca4_13687456 - TAA170	18.79	32.34
100-SDW	CaLG04	bin_4_13239546 - bin_4_13378761	1	139.22	0.23	43.56	59.83	CaLG04	NCPGR127 - NCPGR21	32.38	58.20	CaLG04	Ca4_13687456 - TAA170	50.13	60.41
DC	CaLG04	bin_4_13239546 - bin_4_13378761	1	139.22	0.23	6.11	11.90	CaLG04	NCPGR127 - NCPGR21	3.54	9.38	-	-	-	-
RTR	CaLG04	bin_4_13393647 - bin_4_13547009	2	153.36	0.22	10.57	20.09	CaLG04	TAA170 - NCPGR21	7.59	16.67	CaLG04	Ca4_13840227 - NCPGR21	8.96	13.56
SDW	CaLG04	bin_4_13393647 - bin_4_13547009	2	153.36	0.22	13.78	25.22	CaLG04	TAA170 - NCPGR21	7.97	17.59	CaLG04	Ca4_13840227 - TAA170	16.86	26.91
PBS	CaLG08	bin_8_6034209 - bin_8_5984553	3	49.66	9.47	5.70	11.27	CaLG06	TA106 - H1I16	3.01	8.73	CaLG08	CaM0812 - NCPGR164	5.27	12.92
DF	CaLG08	bin_8_6034209 - bin_8_5984553	3	49.66	9.47	28.19	44.76	CaLG08	NCPGR164 - CaM1918	9.63	26.87	CaLG08	NCPGR164 - Ca8_3050452	46.03	67.71
HI	CaLG08	bin_8_6034209 - bin_8_5984553	3	49.66	9.47	8.01	15.42	CaLG01	cpPb-679915 - CaM0393	4.96	14.36	CaLG08	NCPGR164 - Ca8_3050452	14.52	25.94
DM	CaLG07	bin_7_12870961 - bin_7_12856579	-	14.38	0.23	19.53	45.38	CaLG04	NCPGR127 - TAA170	8.75	19.71	CaLG08	NCPGR164 - Ca8_3050452	30.01	47.43

LOD# - logarithm of odds (base 10); PVE* - phenotypic variation explained.

Of these, 211 sequences were annotated and 1,050 GO terms were obtained. Categorization of these terms into biological processes (BP), molecular function (MF) and cellular components (CC) showed predominance of stress related GO terms in BP class while in MF class GO terms for binding, catalytic, transferase, hydrolyses and kinase activity were predominantly present. Genes having a direct role in stress such as dehydration responsive element binding protein (DREB), heat stress transcription protein, thiamine thiazole synthase and few uncharacterized proteins were also identified in the region (http://cmap.icrisat.ac.in/cmap/sm/cp/jaganathan/).

4.6.2 Genes identified from skim sequencing-SNP based QTL analysis

The number of genes obtained from GBS-SNP based QTL analysis was high (286 genes in ~3 Mb), which needs further refinement to identify candidate genes for drought component traits under study. Therefore QTL analysis based on bin mapping from skim sequencing approach was looked for candidate gene identification. It is evident from the research presented above that QTL analysis using high density bin mapping refined the "*QTL-hotspot*" region from 3 Mb (chapter 4.2) to two sub-regions of 139.22 kb ("*QTL-hotspot_a*") and 153.36 kb ("*QTL-hotspot_b*"). Comparison of these sub-regions with the chickpea genome assembly (Varsheny et al. 2013a) and genome annotations identified a total of 26 genes in these two regions. The "*QTL-hotspot_a*" sub-region contained 15 genes and the "*QTL-hotspot_b*" contained 11 genes. Further, a detailed analysis of recombination at "*QTL-hotspot_a*" and "*QTL-hotspot_b*" along with the phenotypic data for 100-SDW for 10 RILs (4 high and 6 low) clearly indicated the possible role of "*QTL-hotspot_a*" and "*QTL-hotspot_b*" to govern 100-SDW trait in chickpea (Figure 10).

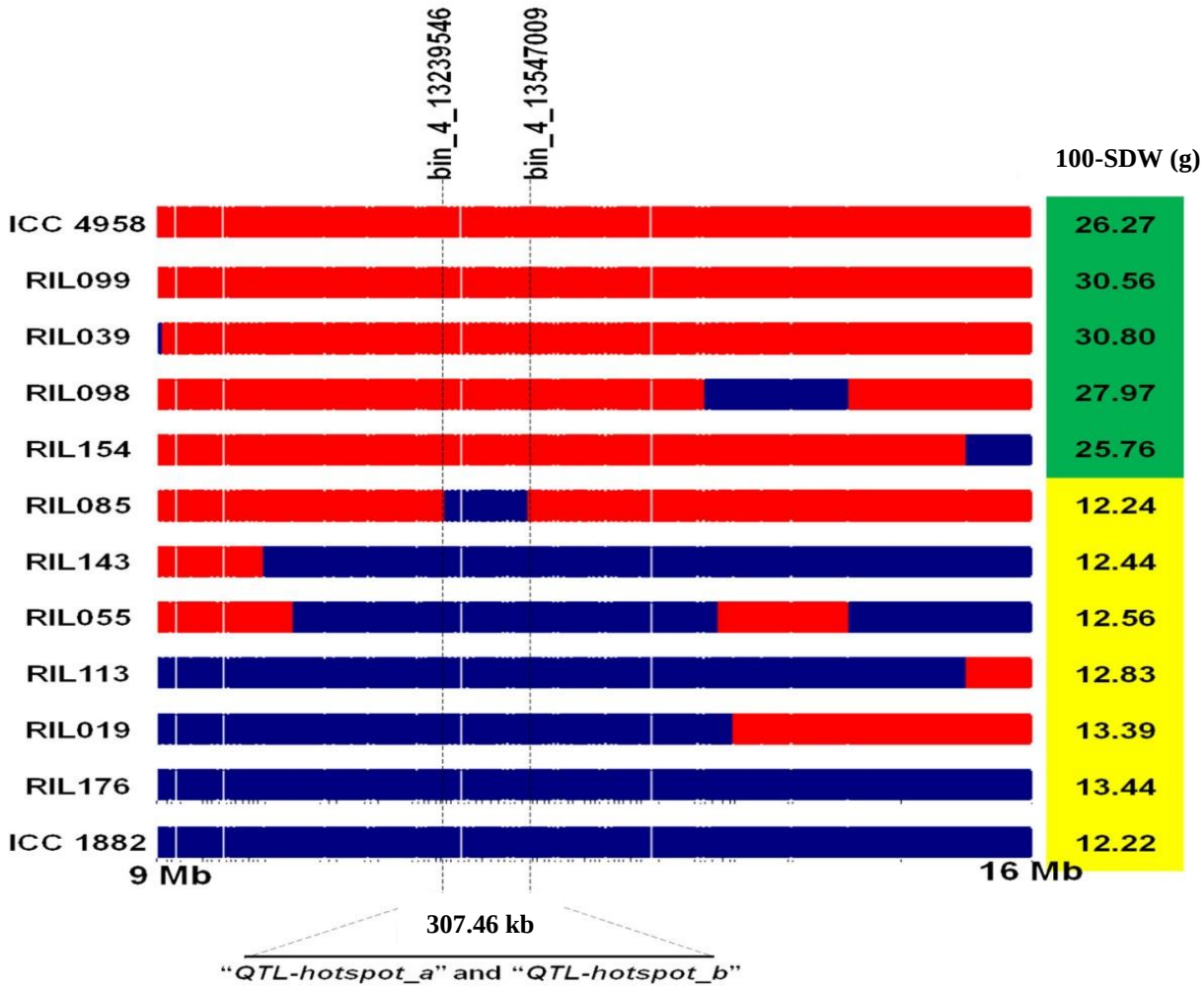

Figure 10: Fine mapping of "*QTL-hotspot*" for 100-seed weight (100-SDW) using RIL population. The recombination breakpoints in "*QTL-hotspot*" region spanning 7 Mb size (9-16 Mb on CaLG04 of physical map) among 4 recombinant inbred lines (RIL099, RIL038, RIL098, and RIL154) with high 100-SDW, 6 RILs (RIL085, RIL143, RIL055, RIL113, RIL019, and RIL176) with low 100-SDW and parental genotypes (ICC 4958 shown in red colour bars-high 100-SDW, ICC 1882 shown in blue colour bars-low 100-SDW) are shown. No recombination was observed within ~300 kb refined region ("*QTL-hotspot_a*" and "*QTL-hotspot_b*") in the case of RIL085 and other low and high RILs. This clearly indicates that the refined "*QTL-hotspot_a*" and "*QTL-hotspot_b*" are the candidate regions for 100-SDW in chickpea.

4.7 High Resolution Fine Mapping Cross

To confirm and further narrow down the two regions identified from skim sequencing based SNP analysis, a high resolution mapping population was developed which allows more recombination events leading to determine the possible shortest region responsible for controlling the traits of interest.

4.7.1 Marker screening and parent selection

In order to develop a large fine mapping population, a near isogenic line, BC_5F_1-ICCX-110125-P18 derived from continuous back crossing of ICC 4958 (male parent) with ICC 1882 (female parent) was selected. This line is termed as NIL^+ line as it carries "*QTL-hotspot*" from ICC 4958 in ICC 1882 genetic background. This NIL^+ line was selected by screening a total of 75 BC_5F_1 plants (coming from TL1 project) with 6 SSR markers (ICCM0249, TAA170, NCPGR127, GA24, NCPGR21 and STMS11) flanking the "*QTL-hotspot*". The plants showing heterozygous allele for all the flanking markers (no=22 lines) were selected and background recovery was calculated for the selected 22 lines using 51 SSR markers from overall genome. Based on the analysis, BC_5F_1-ICCX-110125-P18 showed highest genome (98.95%) recovery and was selected as NIL^+ line to be used further as male parent for the development of a large fine mapping population (Table 13). A total of 86 seedlings (BC_6F_1) which were obtained by the selfing of selected BC_5F_1-ICCX-110125-P18 were screened with flanking markers (Appendix 3). Among which, 8 lines showed homozygous allele for all the screened markers and these lines were crossed with the sensitive line (ICC 1882) and F_1s were obtained.

Table 13: Background recovery of ICCX-110125-P18 using genome wide SSR markers

Marker	Position (cM)	LG	ICC 4958	ICC 1882	ICCX-110125-P18
STMS21	23.18	CaLG01	A	B	B
H2A08	63.659	CaLG01	A	B	B
CAM0015	70.494	CaLG01	A	B	B
CaM0403	70.633	CaLG01	A	B	B
TA30	99.271	CaLG01	A	B	B
CAM0293	17.342	CaLG02	A	B	H
TA37	30.829	CaLG02	A	B	B
H1H011	34.198	CaLG02	A	B	B
TA194	46.494	CaLG02	A	B	B
TA103	50.158	CaLG02	A	B	B
GA16	60.325	CaLG02	A	B	B
GA20	78.502	CaLG02	A	B	B
H3C06	2.32	CaLG03	A	B	B
GA119	11.038	CaLG03	A	B	B
TR56	16.369	CaLG03	A	B	B
ICCeM50	21.807	CaLG03	A	B	B
CAM0520	26.371	CaLG03	A	B	B
CaM0615	6.675	CaLG04	A	B	B
CaM0845	7.863	CaLG04	A	B	B
CaM0856	8.106	CaLG04	A	B	B
ICCM0257	19.604	CaLG04	A	B	B
H1A19	35.73	CaLG04	A	B	B
TR20	37.031	CaLG04	A	B	B
NCPGR7	38.929	CaLG04	A	B	B
NCPGR27	39.088	CaLG04	A	B	B
CaM0507	39.671	CaLG04	A	B	B
CaM2093	40.063	CaLG04	A	B	B
CaM0923	40.487	CaLG04	A	B	B
TA132	44.88	CaLG04	A	B	B
CaM1328	46.787	CaLG04	A	B	B
ICCeM058	49.493	CaLG04	A	B	B
CaM1903	53.246	CaLG04	A	B	B
TR18	10.246	CaLG05	A	B	B
TA5	16.389	CaLG05	A	B	B
H2J09	16.518	CaLG05	A	B	B
H3A07	20.626	CaLG05	A	B	B
CAM2158	24.271	CaLG05	A	B	B
CaM0500	20.244	CaLG06	A	B	B
TA120	20.955	CaLG06	A	B	B

CAM1431	30.634	CaLG06	A	B	B
H1F21	37.493	CaLG06	A	B	B
H6G10	38.473	CaLG06	A	B	B
CAM1760	80.685	CaLG06	A	B	B
STMS25	32.149	CaLG07	A	B	B
CAM2155	36.962	CaLG07	A	B	B
H1O12	38.638	CaLG07	A	B	B
TR24	81.555	CaLG07	A	B	B
TA3	39.233	CaLG08	A	B	B
Recovery					98.95%

4.7.2 F_1 and F_2 populations

A total of 73 F_1s were generated by crossing of the selected 8 NIL^+ line (male parent) with ICC 1882 (female parent). These plants were tested with 2 SSR markers (TAA170 and NCPGR21) for selecting true hybrids. The hybridity test resulted in identifying 58 true F_1s (Table 14). Further self-pollination of these F_1s resulted in generating large F_2 mapping population comprised of 1,911 seedlings.

4.7.3 Recombinant lines from F_2 population

In order to identify recombination events within "*QTL-hotspot_a*" and "*QTL-hotspot_b*" regions, 6 KASPar markers namely CKAM2210, CKAM2177, CKAM2178, CKAM2179, CKAM2181 and CKAM2182 were employed for genotyping. The primer sequences and their physical locations can be found in Table 15. Based on the refined QTL analysis from section 4.6.2, CKAM2210 (13221840 bp on pseudomolecule 4) and CKAM2178 (13365751 bp on pseudomolecule 4) were selected as flanking markers for "*QTL-hotspot_a*". Similarly CKAM2179 (13391772 bp on pseudomolecule 4) and CKAM2182 (13550069 bp on pseudomolecule 4) were selected as flanking markers for "*QTL-hotspot_b*". A total of 1,911 seedlings were screened with these markers along with one additional marker from each region which identified 42 heterozygous recombinants. Out of 42 recombinants, 15 recombinants were identified for "*QTL-hotspot_a*" having recombination events between CKAM2210 and CKAM2178 and 11 recombinants were found to have recombination event between CKAM2179 and CKAM2182 for "*QTL-hotspot_b*". However, 16 plants were found to have recombination events for both the regions (Table 16). Detailed analysis of the recombination events between CAKM2210 and CKAM2182 revealed 22 types of genotype classes (Figure 11).

Table 14: Hybridity confirmation of F_1 plants using SSR markers

Line	TAA170	NCPGR21	Line	TAA170	NCPGR21
ICC 4958	A	A	ICCX-130026-P37	H	H
ICC 1882	B	B	ICCX-130026-P38	B	B
ICCX-130026-P1	B	B	ICCX-130026-P39	B	B
ICCX-130026-P2	H	H	ICCX-130026-P40	B	B
ICCX-130026-P3	H	H	ICCX-130026-P41	H	H
ICCX-130026-P4	B	B	ICCX-130026-P42	B	B
ICCX-130026-P5	H	H	ICCX-130026-P43	H	H
ICCX-130026-P6	H	H	ICCX-130026-P44	H	H
ICCX-130026-P7	H	H	ICCX-130026-P45	H	H
ICCX-130026-P8	H	H	ICCX-130026-P46	H	H
ICCX-130026-P9	H	H	ICCX-130026-P47	B	B
ICCX-130026-P10	B	B	ICCX-130026-P48	B	B
ICCX-130026-P11	H	H	ICCX-130026-P49	H	B
ICCX-130026-P12	H	H	ICCX-130026-P50	B	B
ICCX-130026-P13	H	H	ICCX-130026-P51	H	H
ICCX-130026-P14	H	B	ICCX-130026-P52	H	H
ICCX-130026-P15	H	H	ICCX-130026-P53	H	H
ICCX-130026-P16	H	H	ICCX-130026-P54	B	B
ICCX-130026-P17	H	H	ICCX-130026-P55	H	H
ICCX-130026-P18	H	H	ICCX-130026-P56	H	H
ICCX-130026-P19	H	H	ICCX-130026-P57	B	B
ICCX-130026-P20	H	H	ICCX-130026-P58	H	H
ICCX-130026-P21	H	H	ICCX-130026-P59	H	H
ICCX-130026-P22	H	H	ICCX-130026-P60	H	H
ICCX-130026-P23	H	H	ICCX-130026-P61	H	H
ICCX-130026-P24	H	H	ICCX-130026-P62	H	H
ICCX-130026-P25	H	H	ICCX-130026-P63	H	H
ICCX-130026-P26	H	H	ICCX-130026-P64	H	H
ICCX-130026-P27	H	H	ICCX-130026-P65	H	H
ICCX-130026-P28	B	B	ICCX-130026-P66	H	H
ICCX-130026-P29	H	H	ICCX-130026-P67	H	H
ICCX-130026-P30	H	H	ICCX-130026-P68	H	H
ICCX-130026-P31	H	H	ICCX-130026-P69	H	H
ICCX-130026-P32	H	H	ICCX-130026-P70	H	H
ICCX-130026-P33	H	H	ICCX-130026-P71	H	H
ICCX-130026-P34	H	H	ICCX-130026-P72	H	H
ICCX-130026-P35	B	B	ICCX-130026-P73	H	H
ICCX-130026-P36	B	B			

Table 15: List of KASPar markers used for screening F_2 plants

Primer ID	SNP position	Allele in ICC 4958	Allele in ICC 1882	Flanking sequences used for primer designing
CKAM2210	13221840	G	T	TTAAACTCACTTACCCTCTTTCCTTTCCATTTCCTTTCTTTCAAAATTCTCCTATATCCT[G/T]C TAATACAGATACTTTGCAACCCATTTTTTTTGTCAACAAAGTGTTATTGGGTGAGTTCA
CKAM2177	13323947	C	T	TATCAAATTTGAAAGATGACAATGACGTTCCCCTTCGATTAGTTCAAAAATCTCAAAGAA[C/ T]AAAGTTCAATCTAAATGGTTAACAAGTCAAAAATTAATCGTGGAATCGCCATATCTCTAC
CKAM2178	13365751	A	G	AATGATTGAGTTTTTCCTTATAATTGAGTTTCTCCTATGGCAATTATTATACTATAATTA[A/G] TTTTAACCTATTTATTTTGTCTAACAATTTTTTTTTCCTAATGTACAATATTATTAATAT
CKAM2179	13391772	G	T	ATGTCTTCGGCTTCCAGATTTGTGTTTGGTGACATGACCGAAGAAAGCTTGAAATGAGCT[G/ T]ATAGTGAAGAGCTCACTGCCTTTGATTCACACATATTGAATCTATTTAGAACCTTTCCAA
CKAM2181	13471138	C	T	TTGTCTGCCACGCCAAATTACGATAATCATCATCTCTCTTCCAGTTCTGTTCGTCAAGCC[C/T] CATTTGATATTGTTGGACATCGCAAATCTCAACGACACTGTAAACCACCTGCCCGTTTCC
CKAM2182	13550069	C	T	AACCACATGAAGAAAATAAATTATGTAAAATGTGTTGTTTCTTCGAATCAACTATGGTAT[C/ T]GAGGCTATTCTGGATATCGAAGGGACATAATGAAAGAGAGAGTAGTGGCTTCGAAATGCG

Table 16: Selected heterozygous F_2 recombinant lines for raising homozygous F_3 recombinants

F_2 recombinants	CKAM2210	CKAM2177	CKAM2178	CKAM2179	CKAM2181	CKAM2182
ICCX-130026-P34-P12	H	H	H	H	H	A
ICCX-130026-P65-P75	H	H	H	H	H	A
ICCX-130026-P20-P47	H	H	H	H	H	A
ICCX-130026-P20-P9	H	H	H	H	H	A
ICCX-130026-P6-P16	H	H	H	H	H	A
ICCX-130026-P31-P57	B	B	B	B	B	H
ICCX-130026-P9-P104	H	H	H	H	A	A
ICCX-130026-P27-P54	B	B	B	B	H	H
ICCX-130026-P34-P132	B	B	B	B	H	H
ICCX-130026-P37-P178	H	H	H	A	A	A
ICCX-130026-P17-P21	H	H	H	A	A	A
ICCX-130026-P6-P17	H	H	H	A	A	A
ICCX-130026-P18-P54	B	B	B	H	H	H
ICCX-130026-P29-P86	B	B	B	H	H	H
ICCX-130026-P9-P33	B	B	B	H	H	H
ICCX-130026-P24-P41	H	H	A	A	A	A
ICCX-130026-P25-P42	H	H	A	A	A	A
ICCX-130026-P44-P28	H	H	A	A	A	A
ICCX-130026-P24-P57	B	B	H	H	H	H
ICCX-130026-P43-P42	B	B	H	H	H	H
ICCX-130026-P73-P221	B	B	H	H	H	H
ICCX-130026-P20-P12	B	B	H	H	H	H
ICCX-130026-P37-P193	B	H	H	H	H	H
ICCX-130026-P27-P76	A	A	A	A	A	H
ICCX-130026-P29-P64	A	A	A	A	A	H
ICCX-130026-P56-P56	A	A	A	A	A	H
ICCX-130026-P18-P5	A	A	A	A	A	H
ICCX-130026-P9-P137	H	H	H	H	H	B
ICCX-130026-P46-P44	H	H	H	H	H	B
ICCX-130026-P13-P3	H	H	H	H	H	B
ICCX-130026-P41-P7	A	A	A	A	H	H
ICCX-130026-P67-P31	A	A	A	A	H	H
ICCX-130026-P29-P4	H	H	H	H	B	B
ICCX-130026-P20-P80	H	H	H	B	B	B
ICCX-130026-P17-P24	H	H	H	B	B	B
ICCX-130026-P21-P16	H	H	B	B	B	B
ICCX-130026-P33-P84	H	H	B	B	B	B
ICCX-130026-P73-P1	H	H	B	B	B	B
ICCX-130026-P73-P64	H	H	B	B	B	B

ICCX-130026-P20-P40	H	H	B	B	B	B
ICCX-130026-P30-P56	A	H	H	H	H	H
ICCX-130026-P43-P64	A	B	B	B	B	B

A - homozygous allele for ICC 4958; B - homozygous allele for ICC 1882; H - heterozygous alleles for both parents.

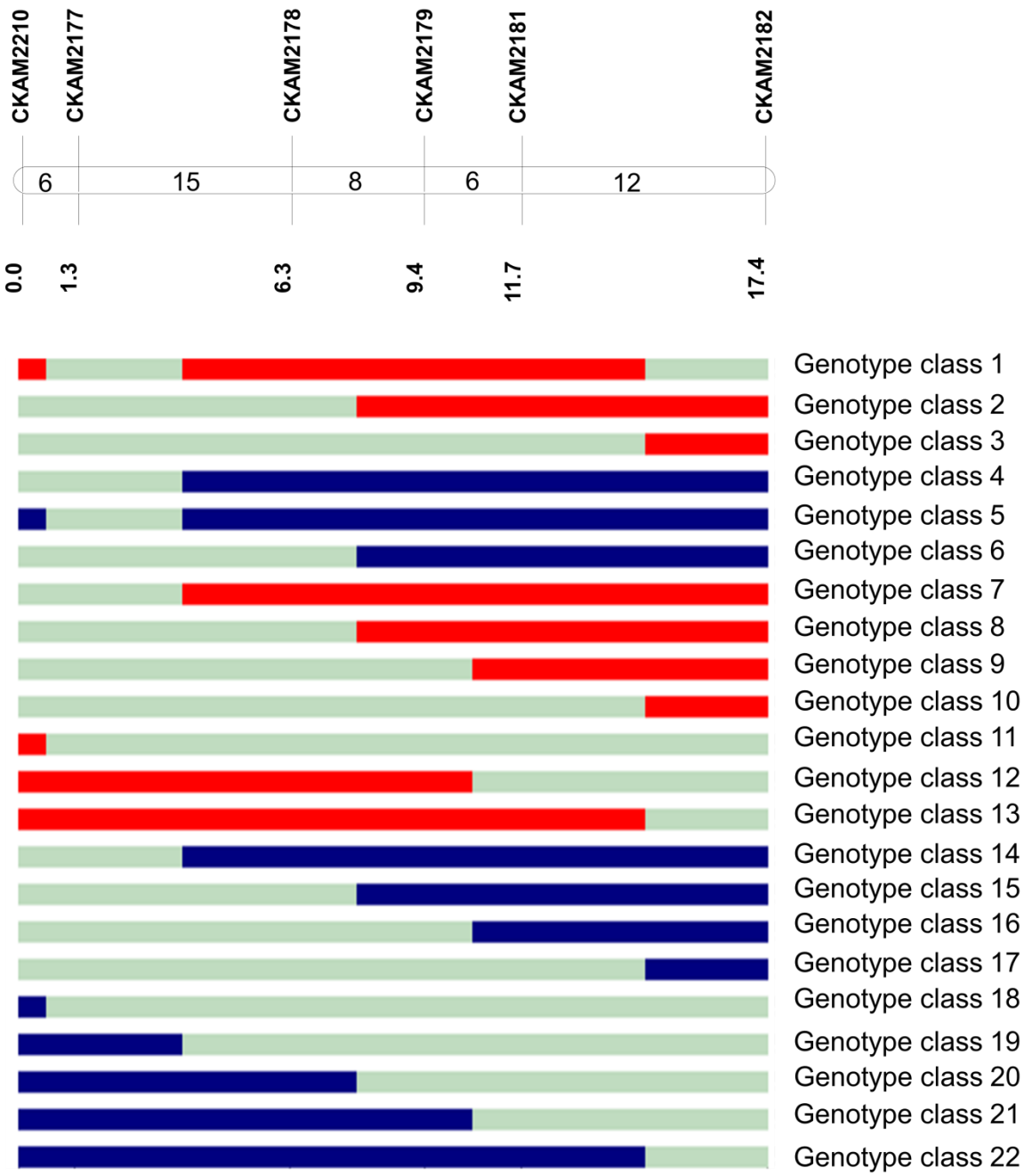

Figure 11: Genotype classes of F_2 fine mapping lines using KASPar markers. A total of 1,911 plants were genotyped with 6 KASPar markers and 22 types of genotypic classes were observed based on the recombination events occurred within this region.

4.7.4 High resolution map of selected recombinants

Further, to precisely locate and delimit "*QTL-hotspot_a*" and "*QTL-hotspot_b*" into few candidate genes, 18 SNP markers located within these regions were designed for KASPar assay (Table 17). The selected heterozygous F_2 recombinants were self-pollinated during November 2014 at ICRISAT, Patancheru. The F_3 families derived from F_2 individuals were subjected to genotyping with newly generated KASPar markers. A total of 284 plants from 19 $F_{2:3}$ families were analyzed initially with 6 flanking markers. Among which, 52 plants showed homozygous recombination from 9 F_3 families. These lines were selected for further genotyping with newly generated 18 KASPar markers (CKAM2211, CKAM2213, CKAM2214, CKAM2215, KAM2216, CKAM2217, CKAM2218, CKAM2219, CKAM2220, CKAM2221, CKAM2222, CKAM2223, CKAM2224, CKAM2225, CKAM2226, CKAM2227, CKAM2228 and CKAM2229) to find more recombinations in the target regions. The flanking sequences and SNP information are explained in Table 17 whereas the KASPar marker profiles and genotyping data are shown in Figure 12 and Table 18 respectively. Based on the availability of seeds, 51 selected homozygous recombinants (one line was not included due to less seed number) along with 8 $F_{2:3}$ lines showing parental type alleles for the targeted region were subjected for genotyping (Table 19). The genotyping data obtained was used to construct a high resolution map for "*QTL-hotspot_a*" and "*QTL-hotspot_b*". Among 59 lines analyzed, 17 types of genotypes were observed (Figure 13).

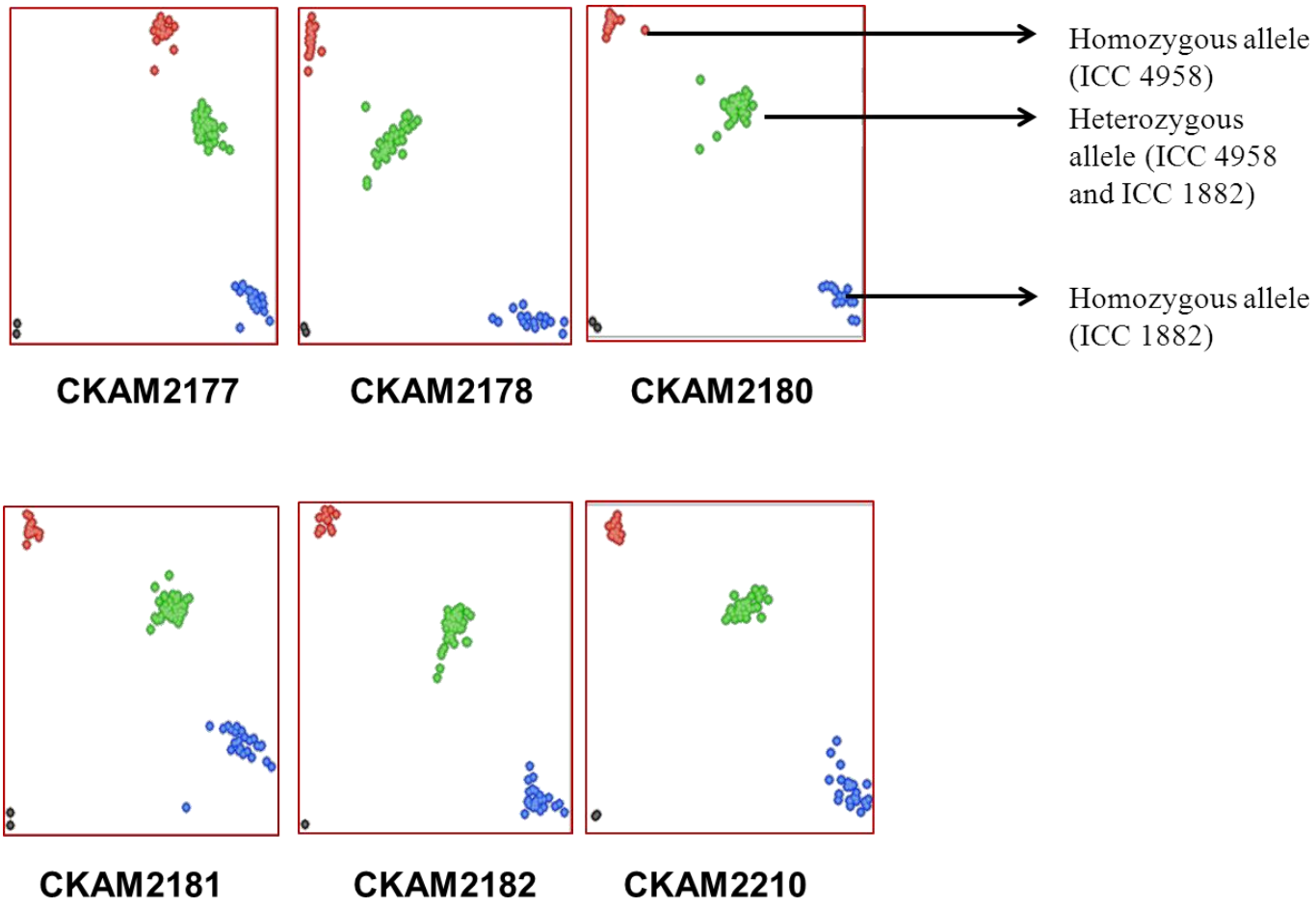

Figure 12: Snapshots showing SNP genotyping with KASPar assays. Marker genotyping data generated for each genotype were used for allele calling using the automatic allele calling option of KBiosciences-KlusterCaller software. Allelic discrimination (two alleles) for a particular marker in the genotypes examined has been shown on a scatter plot with axes 'X' and 'Y'. Genotypes showing homozygosity for ICC 4958 allele are represented by red spots; Genotypes showing homozygosity for ICC 1882 allele are represented by blue spots; whereas, genotypes of heterozogosity (having both alleles) was shown in green spots.

Table 17: List and details of KASPar markers used for screening F_3 plants

Primer ID	SNP position	Allele in ICC 4958	Allele in ICC 1882	Sequence used for primer designing
CKAM2211	13257253	A	G	GTGTTAATTATTGGATCAAAGAGATAAGAAACTTTG AGGTCTATAAGATGAGGAGCATGA[A/G]TTGTAGGT CTCATATTAGGGCATCCACAAATCTATGGACCAGTT CAGATATTGATGTTAT
CKAM2213	13272042	C	T	AAACTTGACATTTGACTTTAGATGGTCCTTAATTCA AAATAATTAAATGGTACTAATGTT[C/T]CTTGATGAC TTTGGATTTGCAATATATTAAAACAATATATGATTA GACAAAGATGAACAA
CKAM2214	13282908	C	T	TGGGTCGGTTAATGCATTACGCTGGCCTGCCCGCCC CAAGTTGAGAGTTGTCTAATGGGT[C/T]CTGGGCGA AGGCTCAATTCTCGTTGGTCCTAGACGTGAGGGCAC CGCGTGAGGCTGGCTT
CKAM2215	13297602	A	G	ATCAAGTAGGGTAAAACTATAAATCATCTAGAATC ACTTCCCTTAAAAACCAAATTTTAT[A/G]AGAGGAG CATAAATAACATTCAAAATTTATTGATGTTACTATC CTTTGTGTGAAAGGAAA
CKAM2216	13327480	A	G	ATACATTTTGTTTTTCAAACTAGCTTTTTAAAGCATA ATACTTGTTCTGCCAAAAAAGGC[A/G]CCAACATCA TTTAATGATATGCTGAAGTCACTCAGAATTAAAATG AGCAGAGCAGCAATA
CKAM2217	13331455	A	C	TCTTTACCTACCTTATCCGGTGAGACTCCATCATAG ACATTAACCTTACCACAGTAGAAA[A/C]TTGTCATTT GCCCAAATGATCCGCTAGGTGCGCACCCTCTGTCCA AATCAAATTTTCAAG
CKAM2218	13334878	A	T	AGAATGATGTGATAAGAAAATATAAATAGAGATCA AATAAAGTATTGAATGAATGTTTGG[A/T]ATTTCAA ATGTACATGTATCATTATTATTTCACAAAGTTATAA TTATAATTAGTATATGT
CKAM2219	13394035	C	T	CTTGTTCCCTTCATGATTACCAATGATTCTTTTATGT CTTTTAGAGTGCATTTCTCTGCT[C/T]TGTATGAACA ACCAACTTCTTCCAACATTCCTAGAGAAATTAAATT CCTCATCAGTTCTG
CKAM2220	13394283	C	T	GTAGTTAAGAAATCAATCTCTATTTGAGGTCATGTG AAATTAACATCCAGAGTCAAGCAC[C/T]CAGTCATC ATCCATGATTTCAGTTGAATTTTTCATGAGAACCTC TGTGTTTTCATATCCT
CKAM2221	13420967	C	G	AGGTTCATTACCAGAACAACTCGGTCTCTTAACAGA CTTGGCCCTTTTCCACATAAACTC[C/G]AATCGATTC TGCGGTTCGCTCCCATATAATTTCACCTACCTTCAA CTTCTCCATGAATTA
CKAM2222	13430655	C	T	AAACGCTCTAAAACTTAAATTGGTCGTATACGAGC AAACACAGTGAATGGTAACAACAAC[C/T]AAATGAT AATATGGTGACACTCGACTAGTGAACGATCAAATA GTTCAACTGAATAAGTTA
CKAM2223	13434985	A	T	TGAACAAAAACTTCTACGTGATCAGTTTGTCATATT TCACAAAAAAAAAAAAAAGGAATA[A/T]ATGCAAT ATATGCGGCTCAATTGGATGTTGTAACCATGGATTC TATTGATTAGTGGTCAA
CKAM2224	13436788	A	G	AATTGACAGCTCTACCCATAGGGTATGAACATGGA

				GTATTTTTTTAGATCGTGGGTATAG[A/G]AACGAGT ACTATAATATCCTATCAAACTCCACCCATTGACATC TCTACCAACTATGTATA
CKAM2225	13439534	A	G	TTTGGTAACCAATAGAATATTCACACAACCGCTAAC ACAAGACTGCATAATTGCATGTAT[A/G]AGTTAACA TTGAAAGCAACAACAAGAAAAAATCCCTTAAGAAG GGCCACTTAAACCCGAA
CKAM2226	13445618	A	T	CGCTATTAAGTACAAAAAATTGTCAAATAGCGGTT ATAGCAATCTATAGCGTTGTTGCTT[A/T]GAGGAAT ATAAATAAACCACTATTTTTCACAATCTGCGATTCA CAAAATTGGTATGTATG
CKAM2227	13510544	A	G	CGAGGCCCAAATCCAAAACCGGATTCAAATTCATTT TAAATATCCGGTTAAAATCATATG[A/G]TTATAATTT GGTTTATTTATAAACCGGTTGGATAACCACTTATGT TTTATATTTGGATTT
CKAM2228	13530557	C	G	CATCTGAAGATTATGTGCAGCTTAAGGTGTTGGCGG CAATTCAAGGGGACGCTAGTGTTT[C/G]TAAGGATG ACAAAATTGAGCATTTGTTCTTTTCCTTAATGTTTTT TCAAAAACTCTCAAT
CKAM2229	13543950	A	T	TTCATTACATCGCATATTTGAGGTTAGGATTTCTGTT TAATAATTGGACTTCAAAAATCA[A/T]GTTTCTCATA GGATTTCGATAAAGACGTGATTAGTTGATTTTCAGT GCAGGAAATTTGAA

Table 18: Genotyping data of the homozygous $F_{2:3}$ plants for flanking KASPar markers

Genotype	F_2 score	CKAM2210	CKAM2177	CKAM2178	CKAM2179	CKAM2181	CKAM2182
ICC 1882	BBBBBBB	B	B	B	B	B	B
ICC 4958	AAAAAA	A	A	A	A	A	A
ICCX-130026-P33-P84-P1	HHBBBB	A	A	B	B	B	B
ICCX-130026-P13-P3-P1	HHHHHB	A	-	A	A	A	B
ICCX-130026-P13-P3-P15	HHHHHB	A	A	A	A	A	B
ICCX-130026-P13-P3-P17	HHHHHB	A	-	A	A	A	B
ICCX-130026-P13-P3-P4	HHHHHB	A	-	A	-	A	B
ICCX-130026-P20-P12-P10	BBHHHH	B	B	A	A	A	A
ICCX-130026-P20-P12-P16	BBHHHH	B	B	A	-	A	A
ICCX-130026-P20-P12-P23	BBHHHH	B	B	A	A	A	A
ICCX-130026-P20-P12-P24	BBHHHH	B	B	A	-	A	A
ICCX-130026-P20-P12-P26	BBHHHH	B	B	A	A	A	A
ICCX-130026-P20-P12-P28	BBHHHH	B	B	A	-	A	A
ICCX-130026-P20-P12-P5	BBHHHH	B	B	A	A	A	A
ICCX-130026-P20-P12-P8	BBHHHH	B	B	A	-	A	A
ICCX-130026-P20-P12-P9	BBHHHH	B	B	A	-	A	A
ICCX-130026-P20-P40-P13	HHBBBB	A	-	B	B	B	B
ICCX-130026-P20-P40-P14	HHBBBB	A	-	B	B	B	B
ICCX-130026-P20-P40-P15	HHBBBB	A	A	B	B	B	B
ICCX-130026-P20-P40-P17	HHBBBB	A	-	B	B	B	B
ICCX-130026-P20-P40-P19	HHBBBB	A	A	B	B	B	B
ICCX-130026-P20-P40-P20	HHBBBB	A	A	B	B	B	B
ICCX-130026-P20-P40-P22	HHBBBB	A	-	B	B	B	B
ICCX-130026-P20-P40-P24	HHBBBB	A	A	B	B	B	B
ICCX-130026-P20-P40-P3	HHBBBB	A	-	B	B	B	B
ICCX-130026-P20-P40-P6	HHBBBB	A	-	B	B	B	B
ICCX-130026-P20-P47-P1	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P47-P13	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P47-P17	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P47-P18	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P47-P19	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P47-P2	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P47-P9	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P9-P10	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P9-P17	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P9-P22	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P9-P23	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P9-P29	HHHHHA	B	B	B	B	B	A
ICCX-130026-P20-P9-P30	HHHHHA	B	B	B	B	B	A

ICCX-130026-P29-P4-P1	HHHHBB	A	A	A	-	B	B
ICCX-130026-P29-P4-P11	HHHHBB	A	-	A	-	B	B
ICCX-130026-P29-P4-P12	HHHHBB	A	-	A	-	B	B
ICCX-130026-P29-P4-P19	HHHHBB	A	-	A	-	B	B
ICCX-130026-P29-P4-P8	HHHHBB	A	A	A	A	B	B
ICCX-130026-P29-P4-P9	HHHHBB	A	A	A		B	B
ICCX-130026-P6-P16-P1	HHHHHA	B	B	B	B	B	A
ICCX-130026-P6-P16-P10	HHHHHA	B	B	B	B	B	A
ICCX-130026-P6-P16-P11	HHHHHA	B	B	B	B	B	A
ICCX-130026-P6-P16-P14	HHHHHA	B	B	B	B	B	A
ICCX-130026-P6-P16-P7	HHHHHA	B	B	B	B	B	A
ICCX-130026-P6-P17-P10	HHHAAA	B	B	B	-	A	A
ICCX-130026-P6-P17-P2	HHHAAA	B	B	B	-	A	A
ICCX-130026-P6-P17-P3	HHHAAA	B	B	B	-	A	A
ICCX-130026-P6-P17-P8	HHHAAA	B	B	B	-	A	A

Table 19: Genotyping of 59 selected homozygous recombinants from $F_{2:3}$ families with newly developed KASPar markers

Marker		CKAM2210	CKAM2211	CKAM2213	CKAM2214	CKAM2215	CKAM2177	CKAM2216	CKAM2217	CKAM2218	CKAM2178	CKAM2179	CKAM2219	CKAM2220	CKAM2221	CKAM2222	CKAM2223	CKAM2224	CKAM2225	CKAM2226	CKAM2181	CKAM2227	CKAM2228	CKAM2229	CKAM2182
$F_{2:3}$ recombinants	Family																								
ICCX-130026-P13-P3-P1	BC_6F_3_1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A	B
ICCX-130026-P13-P3-P4		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A	B
ICCX-130026-P13-P3-P15		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A	A	B	A	A	B
ICCX-130026-P13-P3-P17		A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	A	A	A	-	A	A	B
ICCX-130026-P17-P21-P5	BC_6F_3_2	A	A	A	A	A	A	B	B	A	A	A	A	A	A	A	A	B	A	B	A	A	B	A	A
ICCX-130026-P17-P21-P6		A	A	A	A	A	A	B	B	A	A	A	A	A	A	A	A	B	A	B	A	A	B	A	A
ICCX-130026-P17-P21-P8		A	A	A	A	A	A	B	B	A	A	A	A	A	A	A	A	B	A	B	A	A	B	A	A
ICCX-130026-P17-P21-P10		A	A	A	A	A	A	B	B	A	A	A	A	-	A	A	A	B	A	B	A	A	B	A	A
ICCX-130026-P20-P40-P3	BC_6F_3_3	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B
ICCX-130026-P20-P40-P6		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	A	B	B	-	B	B
ICCX-130026-P20-P40-P13		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	A	B	-	-	B	B
ICCX-130026-P20-P40-P14		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	A	B	B	-	B	B
ICCX-130026-P20-P40-P15		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	B	B	B	A	B	B
ICCX-130026-P20-P40-P17		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	-	B	B	A	B	B
ICCX-130026-P20-P40-P19		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	A	B	B	A	B	B
ICCX-130026-P20-P40-P20		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	A	B	-	A	B	B
ICCX-130026-P20-P40-P22		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	A	B	B	A	B	B
ICCX-130026-P20-P40-P24		A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	A	A	A	B	B	A	B	B
ICCX-130026-P29-P4-P1	BC_6F_3_4	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	B	A	B	A	B
ICCX-130026-P29-P4-P8		A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	B	A	B	A	B
ICCX-130026-P29-P4-P9		A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	B	A	B	A	B
ICCX-130026-P29-P4-P11		A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	B	A	B	A	B
ICCX-130026-P29-P4-P12		A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	B	A	B	A	B
ICCX-130026-P29-P4-P19		A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A	B	A	A	B	A	B	A	B
ICCX-130026-P20-P12-P28		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	B	A	B	A	A	B	A	A
ICCX-130026-P20-P12-P5	BC_6F_3_5	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P12-P8		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P12-P9		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P12-P10		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P12-P16		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P12-P23		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P12-P24		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P12-P26		B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P6-P17-P2	BC_6F_3_6	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P6-P17-P3		B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P6-P17-P8		B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P6-P17-P10		B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ICCX-130026-P20-P47-P1	BC_6F_3_7	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	A
ICCX-130026-P20-P47-P2		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	A
ICCX-130026-P20-P47-P9		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	A
ICCX-130026-P20-P47-P13		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	A
ICCX-130026-P20-P47-P17		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	A
ICCX-130026-P20-P47-P18		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	A
ICCX-130026-P20-P47-P19		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	A
ICCX-130026-P13-P3-P5	BC_6F_3_8	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	B	B	A	B	B
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ICCX-130026-P20-P9-P23		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	B	B	A	B	B	A
ICCX-130026-P20-P9-P29		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	B	B	A	B	B	A
ICCX-130026-P20-P9-P30		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	B	B	A	B	B	A
ICCX-130026-P6-P16-P1	BC_6F_3_10	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B	B	B	A	B	B	A
ICCX-130026-P6-P16-P7		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	A
ICCX-130026-P6-P16-P10		B	H	H	H	H	B	H	H	H	B	B	H	H	H	H	H	A	H	H	B	H	H	H	A
ICCX-130026-P6-P16-P11		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	B	B	B	B	B	B	A
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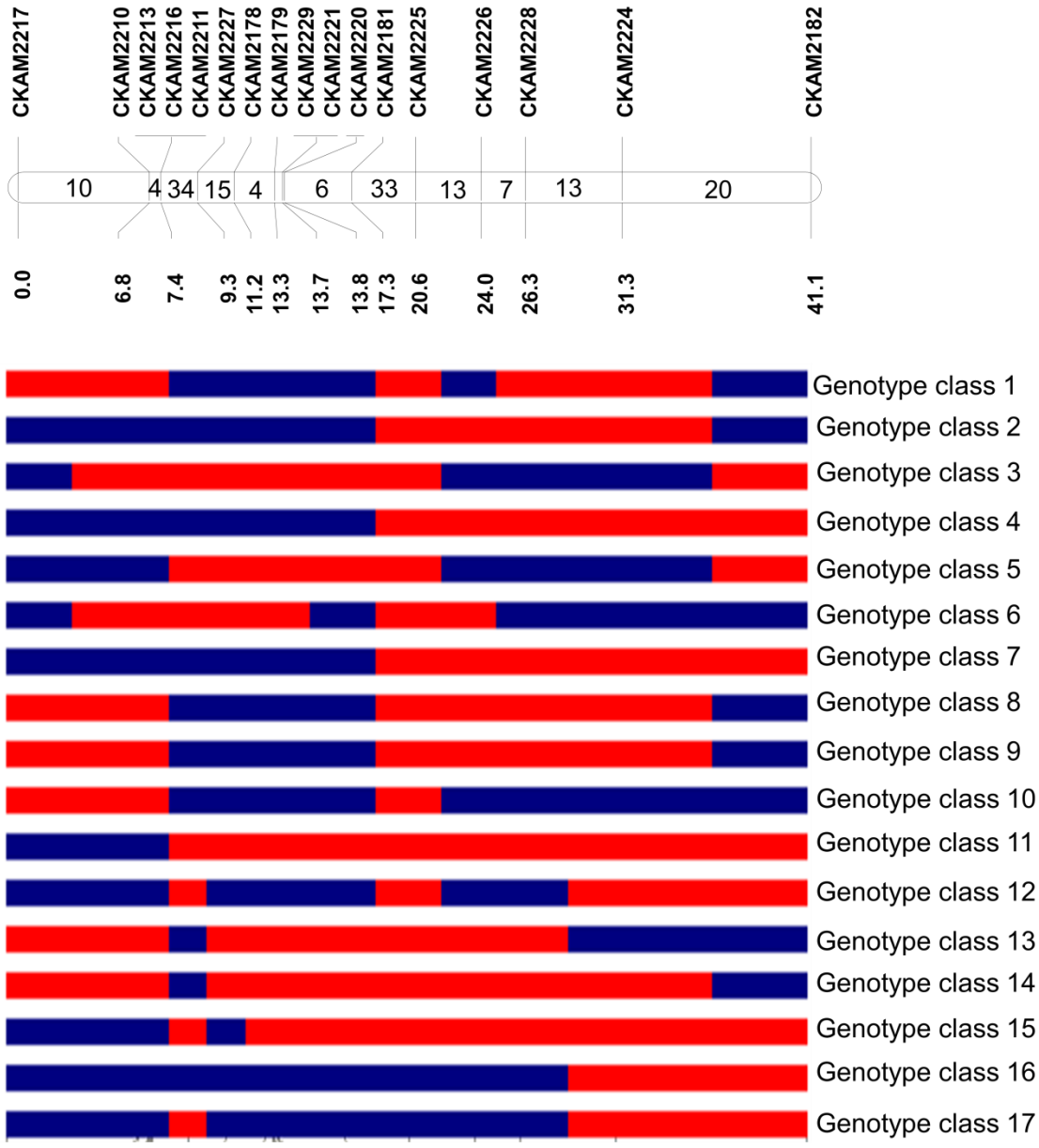


Figure 13: Genotype classes of $F_{2:3}$ fine mapping lines using additional KASPar markers. The selected $F_{2:3}$ recombinant lines (no=59) were further genotyped with newly generated 18 KASPar markers. Genotyping of these $F_{2:3}$ lines with additional KASPar markers resulted in 17 genotype classes based on the recombination identified in this region.

4.7.5 Identification of QTL using F_3 data

The genetic linkage map constructed for fine genetic mapping of "*QTL-hotspot*" comprised 21 markers and the total map distance was 41.09 cM. The high resolution map along with the phenotyping data were used for QTL-fine mapping. Two QTLs were identified for 100-SDW, of which one QTL was flanked by CKAM2210 and CKAM2213 with the phenotypic variation of 56.12% and the LOD of 11.95 whereas the other QTL was flanked by CKAM2225 and CKAM2226 with the LOD of 3.64 and PVE of 11.42% (Figure 14, Table 20).

4.7.6 Fine mapping of the QTL for 100-SDW

In order to refine the identified QTL region, progeny testing was carried out on the $F_{2:3}$ recombinant lines. These F_3 lines were grouped into 10 families (BC_6F_3_1 to BC_6F_3_10) each family represents one F_2 individual from which the F_3 lines were derived. These groups carried the homozygous allele of ICC 4958 and homozygous allele of ICC 1882 for the tested markers located in "*QTL-hotspot_a*" and "*QTL_hotspot_b*". Genotypes of these lines were determined and the recombination around "*QTL-hotspot_a*" and "*QTL_hotspot_b*" were identified. This analysis clearly showed that the QTL identified earlier within the "*QTL-hotspot*" region, ('*QR3100sdw03*' from the current study, see chapter 4.3.4, Table 7) for 100-SDW was mapped between CKAM2215 (13297602 bp) and CKAM2217 (13331455 bp) on CaLG04 (Figure 14) which covers a region of 33.85 kb. Due to the deviation observed between genetic and physical map, fine mapped region was considered from CKAM2210 (13221840 bp) and CKAM2217 (13331455 bp). This region spans 113.03 kb on pseudomolecule 4 of chickpea physical map.

Table 20: QTLs identified from F_3 fine mapping population

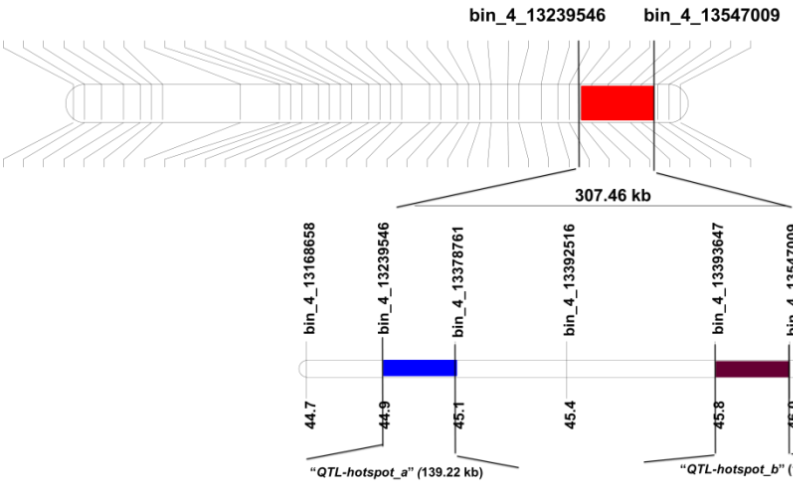
Trait name	Linkage group	Position (cM)	Left marker	Right marker	LOD#	PVE* (%)	Additive effect	Dominance effect
100-SDW	4	7	CKAM2210	CKAM2213	11.96	56.12	2.61	6.52
100-SDW	4	24	CKAM2225	CKAM2226	3.65	11.42	-0.55	-8.55

LOD# - logarithm of odds (base 10); PVE* - phenotypic variation explained.

Table 21: Genes identified from progeny testing of F_3 homozygous lines

Gene	Start	End	Predicted function
Ca_04546	13238649	13241556	stem-specific protein tsjt1-like
Ca_04547	13249157	13251234	probable inactive serine threonine-protein kinase lvsg-like
Ca_04548	13252578	13253678	transcription initiation factor tfiid subunit 8-like
Ca_04549	13258318	13268884	probable inactive serine threonine-protein kinase lvsg-like
Ca_04550	13277417	13279562	coated vesicle membrane
Ca_04551	13283454	13287152	nad -linked oxidoreductase superfamily protein isoform 1
Ca_04552	13288811	13289685	early light-induced-like protein
Ca_04553	13291505	13297363	dymeclin-like isoform x1
Ca_04554	13306199	13306531	heat stress transcription factor a-6b-like
Ca_04555	13311000	13314040	-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2-like
Ca_04556	13318116	13320581	PREDICTED: uncharacterized protein LOC101502642 isoform X1
Ca_04557	13323350	13324896	-dihydroxy-3-keto-5-methylthiopentene dioxygenase 1-like
Ca_04558	13328534	13333733	divergent cct motif protein
Ca_04559	13354281	13354692	epidermal patterning factor-like protein 4-like
Ca_04560	13374809	13377721	emb: isoform 1

a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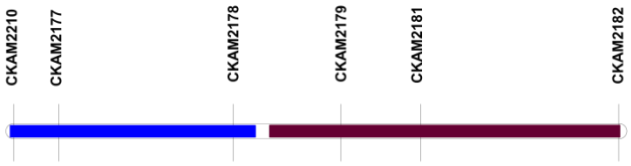
b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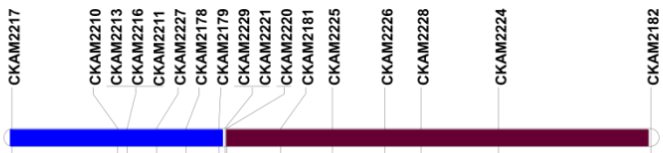
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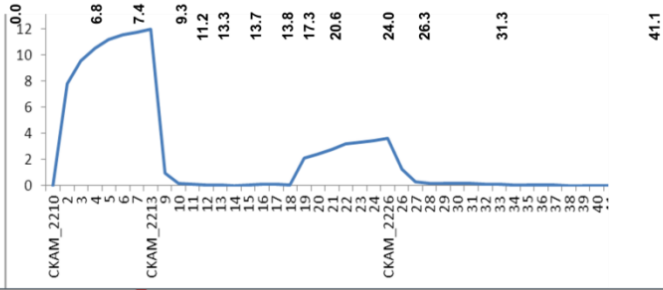
CKAM2182

d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CKAM_2213
CKAM_2226

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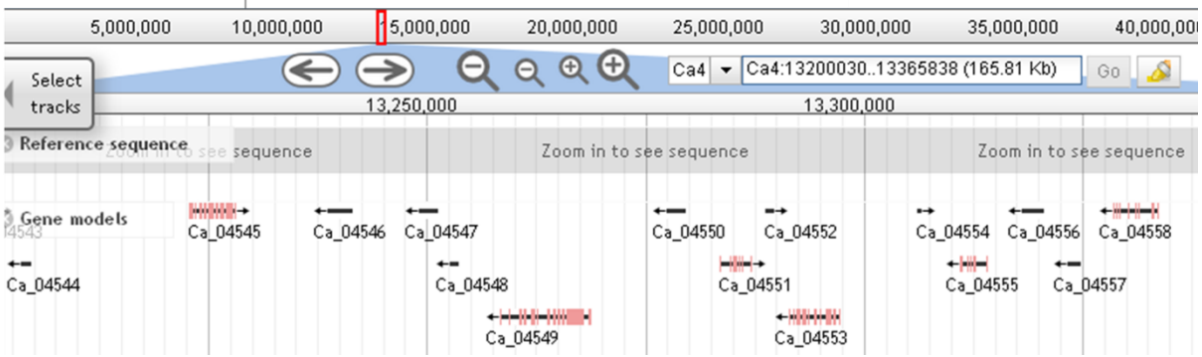
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Ca_04557
Ca_04549
Ca_04553

f

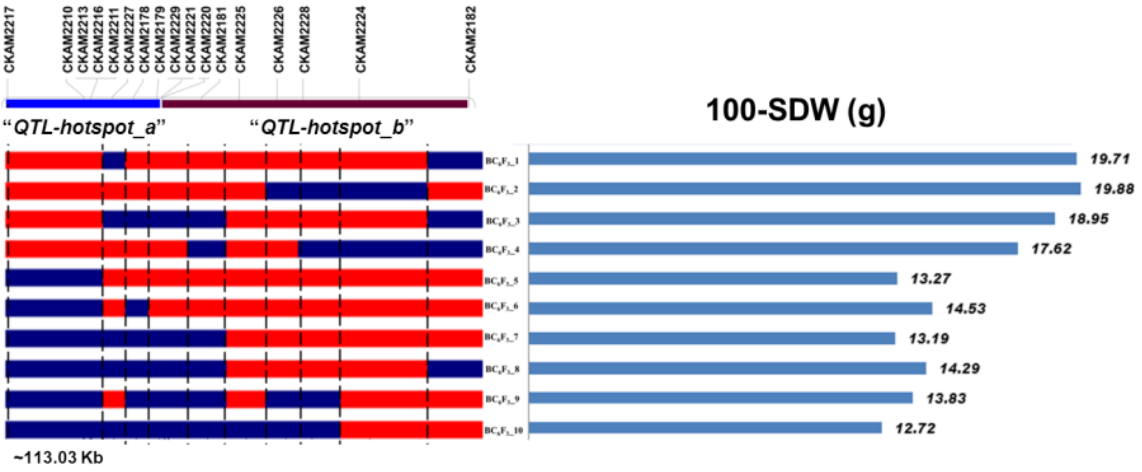

Figure 14: Fine mapping of '*QR3100sdw03*' through progeny testing. a) Bin map of CaLG04. "*QTL-hotspot*" region reported earlier was refined through skim sequencing based SNP analysis and highlighted in red colour which covers a shortest physical length of ~ 307.46 kb. This region was delimited into two regions namely, "*QTL-hotspot_a*" and "*QTL-hotspot_b*" on CaLG04; b) Map generated on F_2 population. Flanking SNP markers of "*QTL-hotspot_a*" and "*QTL hotspot-b*" were converted into KASPar markers and screened on F_2 population. Linkage map was constructed using JoinMap V4.0 which covered a length of 17.39 cM; c) Map generated on F_3 population. In order to identify more recombinants, all the SNPs located in "*QTL-hotspot_a*" and "*QTL-hotspot_b*" were converted to KASPar markers and screened on F_3 lines. Genotyping data was used to construct refined genetic map using JoinMap V4.0. This map spanned 41.09 cM; d) QTL identified on F_3 population. QTL analysis using QTL IciMapping Version 3.3 identified QTLs within "*QTL_hotspot_a*" for 100-SDW e) Jbrowse view of "*QTL_hotspot_a*". A total of 15 genes were located in "*QTL_hotspot_a*" which are shown in the figure; f) Progeny testing with the selected informative $F_{2:3}$ recombinants. Progeny testing on F_3 lines using genotyping and phenotyping data clearly indicated the role of "*QTL_hotspot_a*" in controlling 100-SDW.

Figure 15: Field view of fine mapping population in F_1, F_2 and F_3 generations. F_1 and F_3 lines were generated at ICRISAT, Patancheru during October 2013 and October 2014 respectively, whereas F_2 lines were generated at Indian Institute of Pulses Research-Regional Research Station cum Off-season Nursery, Dharwad, Karnataka during June 2014.

4.8 Candidate Genes in the Refined Regions

The delimited region of 113.03 kb (13221840 bp to 13331455 bp) from "*QTL-hotspot_a*" was examined for candidate gene identification. A total of 15 genes were identified in this region. Gene ontology study was conducted for all these genes. enes and their functional role have been given in Table 21. Among these, few genes were annotated to be stress specific. For instance, Ca_04547, Ca_04548, Ca_04549, Ca_04551 and Ca_04554 codes for probable inactive serine threonine-protein kinase lvsg-like, transcription initiation factor tfiid subunit 8-like, probable inactive serine threonine-protein kinase lvsg-like, nad -linked oxidoreductase superfamily protein isoform and heat stress transcription factor a-6b-like respectively. Additionally, few other important genes from "*QTL-hotspot_b*" also were noted like Ca_04561, Ca_04564, Ca_04567 and Ca_04570 coding for e3 ubiquitin-protein ligase rnf128-like, leucine-rich repeat extensin-like protein 6-like, serine threonine-protein kinase ht1-like isoform x1 and vicilin 47 kDa protein respectively.

DISCUSSION

5. DISCUSSION

Terminal drought is a major constraint to chickpea production and productivity, especially in arid and semi-arid regions. The changes in global climate are predicted to aggravate losses due to drought in particular. In this context, identification of candidate genes for drought tolerance will help to define breeding strategies and provide molecular markers to improve selection efficiency and therefore mitigate yield losses in chickpea. Drought will continue to be a serious constraint to chickpea production. Owing to its complex nature, the genetic dissection of drought tolerance into component traits has been challenging. However, comprehensive insights have been provided into component traits by few recent studies (Hamwieh et al. 2013; Varshney et al. 2014). Varshney and colleagues (2014) reported a promising "*QTL-hotspot*" region on linkage group 4 spanning about 29 cM and harbours QTLs for several drought tolerance related traits. In this present study several approaches were adopted to fine map this genomic region for further use in chickpea improvement programs.

5.1 SNP Discovery and Refining "*QTL-hotspot*" Region

5.1.1 Linkage map with GBS-derived SNP markers

To date, SSR markers have been extensively used for linkage mapping on chickpea intra-specific populations. Although availability of genomic resources have reduced the SSR marker identification span, polymorphism study and further screening is still a time consuming and labor intensive process. As a result, most genetic maps remain limited to only a few hundred markers (Radhika et al. 2007; Jamalabadi et al. 2013; Varshney et al. 2014). In this study, GBS approach was followed which has the advantage of simultaneous SNP identification and genotyping. As a result, 828 novel SNPs were identified on ICC 4958 × ICC 1882 RIL population. Thus a greater number of markers are now available for this intra-

specific population. As compared to GBS studies in other plant species, SNP markers identified in the present study were less (Poland et al. 2012; Sonah et al. 2013). This may be due to variable number of reads generated per RIL (0.28 – 19.23 million reads) resulting in more missing data points or very stringent SNP calling criteria adopted in this study, for instance SNPs present in < 50% RILs were excluded.

A total of 1,146 markers (828 SNPs, 279 SSRs, 21 DArT, 14 EST-SSR and 4 GMM) were used for linkage map construction, out of which 1,007 (87.87%) markers were mapped which spanned 727.29 cM. This saturated map has ~4 fold more markers and increased the marker density from 0.50 to 1.30 per cM as compared to the 241 loci map reported earlier (Varshney et al. 2014). Nearly, 94.60% (228) markers from the earlier study (Varshney et al. 2014), were mapped on the respective linkage groups in the new map, reflecting the higher level of conservation in marker order between the maps. Interestingly, 46% of the SNP markers were mapped on CaLG04. This may be due to high repeat rich regions in the case of Ca4 pseudomolecule and it was evident from the earlier studies (Varshney et al. 2013a), that the average SNP density per kb (7.6) is higher in the case of chickpea "Ca4" psuedomolecule i.e., the CaLG04. Further, the study also indicated higher diversity level in elite cultivars of chickpea in the case of "Ca4" pseudomolecule (θ_π =2.8180; θ_w =2.2377). High θ_π and θ_w are usually associated with repeat rich regions in genome (Varshney et al. 2013a).

While undertaking this study, genetic maps with only few hundreds of markers especially with SSR markers were available for chickpea. Even though few high density linkage maps were reported in recent past, these maps were constructed for inter specific crosses (Nayak et al. 2010, Gujaria et al. 2011, Thudi et al. 2011, Hiremath et al. 2012). Up to date, no studies have reported high density linkage map for intra specific crosses (Radhika et al. 2007, Garg et al. 2011, Jamalabadi et al. 2013, Varshney et al. 2014). This study reports high density linkage map with 1,007 loci including NGS based SNP markers.

5.1.2 Developing breeder friendly markers from the "*QTL-hotspot*" region

This study integrated 49 new markers in the "*QTL-hotspot*" region thereby enriching the same from 7 markers to 55 markers. Integration of these 49 markers has refined the "*QTL-hotspot*" region from 29 cM to 14 cM. Several fine mapping studies earlier have shown that the integration of additional markers have narrowed down the QTL interval. For instance, in the case of rice, Yu et al. (2011) demonstrated that mapping of additional SNP markers not only detected new QTLs but also increased the resolution of the QTL. Similarly, Silvar et al. (2012) fine mapped the QTLs for powdery mildew resistance by integrating 32 markers in the QTL region in Spanish barley. Likewise, in case of basmati rice, the "aro3-1" QTL was narrowed down to an interval of 390 kb from the earlier reported interval of 8.6 Mb and "aro8-1" QTL was narrowed down to a physical interval of 430 kb (Singh et al. 2007).

QTL analysis was performed for 20 different traits and 164 robust M-QTLs were detected for 16 traits which included all 14 reported traits from Varshney et al. (2014). More than 50% (91) of QTLs were located on CaLG04 and all were detected in the "*QTL-hotspot*" region which highlights the importance of this region for drought tolerance mechanism in chickpea. Additionally, the current study also identified new QTLs for PBS and DSI which were not detected or reported earlier. Furthermore, some QTLs which were unstable, inconsistent in the earlier study (Varshney et al. 2014) were identified to be stable and consistent. For instance, five additional QTLs were identified in the case of PHT and one additional QTL each for SDW, DF, BM, POD, SPD and yield (Table 9). Comparatively the PVE observed for most of the traits were significantly high, indicating robustness of the identified QTLs.

In order to enhance the introgression of "*QTL-hotspot*" into molecular breeding program, SNP markers were converted into CAPS/dCAPS. As the SSR markers from the "*QTL-hotspot*" showed less/no polymorphism between ICC 4958 and few recurrent chickpea elite

cultivars (Thudi et al. 2014), these CAPS and dCAPS markers would be of interest to breeders in marker assisted breeding programs to introgress the "*QTL-hotspot*" region.

5.1.3 Pinpointing candidate genes in the refined "*QTL-hotspot*" region

Functional annotation of the candidate genes identified in the "*QTL-hotspot*" region revealed their role in various abiotic and biotic stress tolerance mechanisms. For instance, dehydration responsive element binding protein (DREB) which is a well-known transcription factor involved in abiotic stress including drought tolerance (Liu et al. 1998; Lata and Prasad 2011) was identified in the "*QTL-hotspot*" region. Similarly, thiamine thiazole synthase, which was reported to be involved in stress related mechanisms (Rapala-Kozik et al. 2012) was also identified. In addition to these, few trait specific genes like e3 ubiquitin-protein ligase and TIME FOR COFFEE (TIC) were also identified. The e3 ubiquitin-protein ligase activity has been reported to be involved in grain width and weight in rice (Song et al. 2007) while TIC protein has been reported to play role in plant growth, development and circadian clock (Hall et al. 2003; Sanchez et al. 2011; Shin et al. 2013). Shin et al. (2012) has reported a role of TIC in jasmonic acid signaling pathways and in the control of root meristem size in *Arabidopsis*. Loss of this gene was reported to result in reduced root meristem length and cell number (Hong et al. 2014). Therefore further fine mapping and cloning of genes underlying QTL would unravel the genetics behind drought tolerance in chickpea.

In summary, this study identified and implemented GBS based SNP markers for developing a high density linkage map from an intra-specific population in chickpea. The map contains 1,007 loci spanning 727.29 cM and enriching the "*QTL-hotspot*" region from 7 markers to 55 markers. Also this study had refined the "*QTL-hotspot*" region from 29 cM to 14 cM on a genetic map corresponding to ~3 Mb on the physical map. The current study also identified the presence of several stress related candidate genes including DREB in the "*QTL-hotspot*".

Further characterization of these genes will help in identifying the mechanisms of drought tolerance in chickpea. In addition, the CAPS/dCAPS markers developed from this study can be used in marker assisted breeding program for introgressing the "*QTL-hotspot*" into elite cultivar.

5.2 Marker Saturation in "*QTL-hotspot*" region through Skim Sequencing

NGS technologies have revolutionized the methods of identifying candidate genes for crop improvement. In this study, a skim sequencing approach was used and more than 53,000 SNPs were identified in a RIL population of ICC 4958 and ICC 1882 cross, which represents the largest number of markers used for QTL mapping in chickpea to date.

5.2.1 High density bin map for chickpea

A sliding window based bin mapping approach identified an average of 35 bins per RIL. Theoretically, each RIL contains 3-5 recombination events on each chromosome (Lai et al. 2010). The results obtained in the current study correlates these assumptions (4 × 8 = 32). The average bin size was 210.60 kb and >90% of bins were less than 1 Mb in size, suggesting that majority of the recombinations were captured in this study. Moreover, ~68% bins contained ≤ 10 genes, indicating a high resolution of the bin map. All 1,610 recombination bins were used as markers to construct a linkage map of 973.94 cM. The average inter-bin distance (0.66 cM) observed in this study was lower than other studies conducted on intra-specific mapping populations (0.94 - 7 cM) in chickpea (Kujur et al. 2015c) indicating that the current map is highly saturated. Such a highly saturated map can be used for targeted QTL mapping, QTL cloning and for identification of candidate genes for important agronomic traits in chickpea. A high correlation between bin orders and their positions on the genome sequence was observed for linkage groups 4 and 8. The interchanging of bin positions observed on other linkage groups could be due to misassembled portions of

chickpea genome assembly. Such misassembled regions in chickpea genome have recently been reported (Bayer et al. 2015; Ruperao et al. 2014).

5.2.2 Fine mapping of "*QTL-hotspot*" region

Majority of QTLs identified in the present study are in concordance with earlier study (Varshney et al. 2014). For instance, in all the three studies (Varshney et al. 2014; current study; chapter 4.2, chapter 4.5.5), QTLs for 100-SDW, RLD, PHT, POD, RTR and SDW were observed on CaLG04, while a QTL for DF was reported to be present on CaLG08 indicating the quality and accuracy of the linkage map. The "*QTL-hotspot*" region of 7.74 Mb identified in earlier study (Varshney et al. 2014) had 7 SSRs and was further refined to ca. 3 Mb by integration of 49 SNP markers as described in section 5.1.2. The skim sequencing approach based QTL analysis, integrated 1,421 SNPs and identified 38 recombination breakpoints within this region, thereby splits the "*QTL-hotspot*" into two subregions viz. "*QTL-hotspot_a*" (139.22 kb; 0.23 cM) and "*QTL-hotspot_b*"(153.36 kb; 0.22 cM), and thus further narrowed down the earlier reported "*QTL-hotspot*" region. This demonstrates that whole genome resequencing (WGRS) approach helps to significantly improve the accuracy and resolution of QTL mapping which was observed in several studies in other crops. For example, in soybean, QTLs of sizes 7.90 to 340 kb were identified for root knot nematode resistance using ~100 thousand SNPs and 242 RILs (Xu et al. 2013), while in another study QTLs of 176 kb to 1.28 Mb sizes were identified using 1,798,504 SNPs and 96 ILs for salt tolerance (Qi et al. 2014). This resolution is equivalent to traditional fine mapping studies. For instance, a grain-weight QTL, *gw*3.1 in rice was narrowed down to 93.8 kb using fine mapping procedures (Li et al. 2004). These studies suggest that the bin mapping approach can be used for candidate gene identification using a RIL population and thus avoiding the laborious time required for traditional fine mapping.

Annotation of the 26 genes located within the refined "*QTL-hotspot_a*" and "*QTL-hotspot_b*" identified several important genes, including e3 ubiquitin-protein ligase, leucine-rich repeat extensin-like protein, serine threonine-protein kinase, homocysteine s-methyltransferase, vicilin, which have been reported to play a role in biotic and abiotic stress tolerance were identified (Osakabe et al. 2013; Ruiz et al. 2002; Song et al. 2007; Wang et al. 2012) and differential gene expression analysis proved their involvement in the drought tolerance mechanism in chickpea. 'Homocysteine s-methyltransferase' gene was also reported to be upregulated in drought tolerant chickpea cultivar 'Xj-209' under stress condition (Wang et al. 2012) and could be one of the important genes involved in drought tolerance mechanism in chickpea. Proteomic analysis of rice seedlings also reported involvement of 'Homocysteine s-methyltransferase' in regulating cold stress (Hashimoto et al. 2007). The Ca_04567 gene was annotated as 'LRR receptor like serine threonine-protein kinases' which are the well characterized membrane proteins known for their roles in stress response and abscisic acid (ABA) regulation **(**Osakabe et al. 2013).

In addition to this, the Ca_04561 gene was predicted to be an 'e3 ubiquitin-protein ligase'. Ubiquitination is an important post translational modification to regulate growth and development in all eukaryotes. Various studies in plants (Pokhilko et al. 2011; Sonoda et al. 2009; Thomann et al. 2005) reported the role of the ubiquitin 26S proteasome system (UPS) in regulating fundamental processes such as embryogenesis, photomorphogenesis, and organ development. E3 ubiquitin ligase, which determines substrate specificity, is an important enzyme of the ubiquitination pathway (Lee et al. 2011; Lyzenga et al. 2012). Recent studies (Ju et al. 2013; Kim and Kim 2013a; Li et al. 2013) reported the involvement of e3 ubiquitin ligase in the regulation of abiotic stress tolerance. A study in *Arabidopsis (*Kim and Kim 2013b) showed that suppression of one of RING e3 ubiquitin ligase (*AtATL78*) gene was responsible for increasing tolerance to cold stress while decreasing drought stress tolerance.

Regulation of this gene could be responsible for determining drought sensitivity/resistance in chickpea. A loss of function of RING-type e3 ubiquitin ligase was reported to be responsible for enhanced grain width, weight and yield in rice (Song et al. 2007). This indicates that 'e3 ubiquitin-protein ligase' could be responsible for only abiotic stress tolerance in chickpea while a different mechanism might be present for controlling seed size. Ca_04564 gene was identified as 'leucine-rich repeat extensin-like protein'. The role of extensin like genes in seed germination has already been reported in *Arabidopsis* (Dubreucq et al. 2000). They found extensin like genes are expressed specifically in endosperm and might play a role in cell wall modification. This result suggests a detailed study of such genes in the context of seed size/weight is must.

5.3 High Resolution Mapping Population and Progeny Testing

Though a large number of (6,000) progenies were raised, due to unfavorable weather, only around 2,000 plants survived. Usually any fine mapping studies accounts for large population by selecting 1,000 plants per cM distance. In this case, as the QTL was refined to 0.1 - 0.4 cM, theoretically around 10,000 lines might result in identifying useful recombination and might have helped to place the QTL regions into gene level. However we used 1,911 plants for fine mapping study. This may be the reason for identifying less number of recombination during progeny testing. However the resolution of progeny testing has allowed mapping the 100-SDW QTL ("*R3100sdw03*") region into "*QTL-hotspot_a*". Ca_04546 was annotated to be stem-specific protein tsjt1-like, stem specific protein are reported to play a role in dehydration stress (Lata et al. 2010). Therefore the role of Ca_04546 may contribute to seed weight and thus controlling seed weight under drought conditions. Ca_04558 was annotated to divergent cct motif protein. Cct motif was reported to control heading date and flowering time in rice (Zhang et al. 2015; Lee et al. 2015; Song et al. 2013). Other important candidate genes like Ca_04547, Ca_04548, Ca_04549, Ca_04551, Ca_04554 coding for probable

inactive serine threonine-protein kinase lvsg-like, transcription initiation factor tfiid subunit 8-like, probable inactive serine threonine-protein kinase lvsg-like, nad -linked oxidoreductase superfamily protein isoform 1 and heat stress transcription factor a-6b-like respectively.

5.4. Candidate genes for future studies

Further evaluation of the fine mapping population on F_4 lines for all the traits will help in delimiting the regions into finer kbs for each trait. The genes pinpointed from "*QTL-hotspot_a*" through progeny testing in the current study can be focused further for confirming their role in drought tolerance related traits in chickpea. This can be achieved through gene expression studies using RT-PCR, transformation and validation of transgenic lines and knockout studies.

The genes reported from the current study are described as below. These genes can be focused towards cloning in future studies.

5.4.1 Transcription factors

5.4.1.1 Transcription initiation factor TFIID subunit 8-like

Ca_04548 was annotated to transcription initiation factor TFIID subunit 8-like. Earlier studies have reported the role of transcription initiation factor TFIID subunit in drought tolerance mechanism in cotton (Kebede et al. 2015). TFIID has been popularly known for its role in transcription initiation of RNA polymerase II by specifically binding to promoter region. TFIID is not only involved in promoter recruitment but also plays a role in gene activation and it remains associated with the promoter even after promoter is active state, however TFIID is not required for maintaining the transcription but only for initiation (Cler et al. 2009; Papai et. al. 2011). Expression studies for TFIID may reveal its role in drought tolerance in chickpea.

5.4.1.2 Heat stress transcription factor a-6b-like

Ca_04554 annotated to code for heat stress transcription factor a-6b-like. Heat stress transcription factors (Hsfs) are vast studied in plants and reported to play very important role in signal transduction during various biotic and abiotic stresses like heat, drought, heavy metals, cold, salt, metabolic inhibitors, pathogen attack and oxidative stress. Expression of heat shock proteins (HSP) is controlled by heat Hsfs. Plants respond to high temperature by producing isoforms of heat stress proteins (Baniwal et al. 2004; Nover et al. 2001; Scharf et al. 1998; Doring et al. 2007). Up regulation of heat stress transcription were observed in many plant species like Arabidopsis, rice, tomato, pigeonpea, etc. (Schramm et al. 2006; Wang et al. 2009; Mishra et al. 2002; Maibam et al. 2015). It is always understood that the stress tolerance mechanism of plants for various stresses are interlinked. For instance, DREB2A CA of Arabidopsis was found to induce not only drought and salt related genes but also heat shock related genes. Collectively it was reported that DREB2A functions in both heat and drought conditions (Sakuma et al. 2006). These findings supports to our current identification of Hsfs in the "*QTL-hotspot*" region for drought tolerance. This gene can be further targeted for expression and further validation studies.

5.4.1.3 Early light-induced-like protein

Ca_04552 annotated to code for early light-induced-like protein. Early light inducing proteins (ELIP) play important role during high light stress and transcriptional activation of ELIPs has been observed along with other important proteins. In addition to light stress, up regulation of ELIPs were also observed in wide range of stresses like drought, exposure to ultra violet radiation, cold and freezing stress (Bartels et al. 1992; Hayami et al. 2015). Recently it was reported that multi stress signals merge at a single transcriptional unit of ELIP2 promoter to

give the multi stress response. This results in one of the promoter element to unify multi stress signals.

5.4.2 Signal transduction

5.4.2.1 Inactive serine threonine-protein kinase lvsg-like protein

Ca_04547 and Ca_04549 were annotated to code for probable inactive serine threonine-protein kinase lvsg-like protein and Ca_04567 codes for serine threonine-protein kinase ht1-like isoform x1. Many studies have reported the role of serine threonine-protein kinases in signaling during biotic and abiotic stresses (Osakabe et al. 2013; Halford and Hardie 1998). Wang et al. (2012) has studied transcriptomic profiling of unigenes in drought stressed root and leaves in chickpea, where the role of serine threonine-protein kinase have been reported to play role in calcium signaling during drought stress. Serine threonine-protein kinases were found to be up regulated after 96 h of dehydration stress in chickpea while studying for the changes in nuclear proteome during drought stress (Pandey et al. 2008). In another study Serine threonine-protein kinases were reported to be expressed in both abiotic and biotic stress conditions for salinity and *Ascochyta* blight respectively (Mantri et al. 2010). It was reported to regulate both negatively and positively the cell response to stress conditions (Davies et al. 1999). Up regulation of ser/thr protein kinases during dehydration stress was also reported in chickpea by Boominathan et al. (2004). Recently Meena et al. (2015) have reported the role calcium sensors (Calcium B-like-CBL) which regulate a group of ser/thr protein kinases called CBL-interacting protein kinases (CIPKs) during dehydration stress in chickpea. Additionally, it reports the expression of CaCIPK25 gene increased at different stresses like salt, dehydration and different hormonal treatments. Over expression of CaCIPK25 in tobacco enhanced the tolerance of the transgenic plants to water deficit and salt stress. This gene was found to be located in linkage group 4 on chickpea genome. This

supports our assumption that the ser/thr protein kinase gene identified within the refined "*QTL-hotspot*" regions might be one of the possible candidate genes for drought tolerance in chickpea.

5.4.3 Protein degradation

5.4.3.1 E3 ubiquitin-protein ligase rnf128-like

Ca_04561 codes for e3 ubiquitin-protein ligase rnf128-like. Ubiquitination is an important post translational modification to regulate growth and development in all eukaryotes. Various studies in plants (Pokhilko et al. 2011; Sonoda et al. 2009; Thomann et al. 2005) reported the role of the ubiquitin 26S proteasome system (UPS) in regulating fundamental processes such as embryogenesis, photomorphogenesis, and organ development. E3 ubiquitin ligase, which determines substrate specificity, is an important enzyme of the ubiquitination pathway (Lee et al. 2011; Lyzenga et al. 2012). Recent studies reported the involvement of e3 ubiquitin ligase in the regulation of abiotic stress tolerance (Ju et al. 2013; Kim et al. 2013a; Li et al. 2013). A study in *Arabidopsis* showed that suppression of one of RING e3 ubiquitin ligase (*AtATL78*) gene was responsible for increasing tolerance to cold stress while decreasing drought stress tolerance (Kim et al. 2013b). Regulation of this gene could be responsible for determining drought sensitivity/resistance in chickpea. A loss of function of RING-type e3 ubiquitin ligase was reported to be responsible for enhanced grain width, weight and yield in rice (Song et al. 2007). This indicates that 'e3 ubiquitin-protein ligase' could be responsible for only abiotic stress tolerance in chickpea while a different mechanism might be present for controlling seed size.

5.4.4 Cell defense

5.4.4.1 Leucine-rich repeat extensin-like protein 6-like

Ca_04564 gene was identified as 'leucine-rich repeat extensin-like protein'. The role of extensin like genes in seed germination has already been reported in *Arabidopsis* (Dubreucq et al. 2000). They found extensin like genes are expressed specifically in endosperm and might play a role in cell wall modification. In *Arabidopsis*, LRX (leucine-rich repeat extensin) genes were reported for their role in cell wall development and influencing the plant growth especially in root hair development (Draeger et al. 2015). Baumberger et al. (2001) had reported the role of LRX1, an *Arabidopsis* gene which codes for leucine-rich repeat/extension protein. This study explains the role of LRX1 in root morphogenesis.

5.4.5 Metabolism

5.4.5.1 Stem-specific protein tsjt1-like

Ca_04546 gene codes for stem-specific protein tsjt1-like. Perlikowski et al. (2014) observed stem specific protein to be differentially accumulated during slow and long drought experiments in Lolium and Festuca hybrids. This protein was reported to be involved in hormone metabolism under flooding condition in soybean (Komatsu et al. 2012). This protein was also reported in tomato roots during *fusarium* (FORL) infection (Mazzeo et al. 2014).

5.4.5.2 Nad-linked oxidoreductase superfamily protein isoform 1

Ca_04551 codes for nad-linked oxidoreductase superfamily protein isoform 1. Nicotinamide adenine dinucleotide (NAD) and its derivatives are well known for redox reactions in living organism. They play important role in signaling by generation and scavenging of reactive oxygen species (ROS) and in controlling adaptation to abiotic stresses like heat, salt, drought and biotic stresses (Hashida et al. 2009). NADPH oxidoreductase homolog was identified in

the cDNA library of infected chickpea leaves for ascochyta blight and was also identified in chickpea root transcriptome for fusarium wilt (Ichinose et al. 2000). NAD(P)H-dependent oxidoredcutase protein was also identified in roots of chickpea affected by *Fusarium oxysporum* and *Meloidogyne artiellia* (Palomares-Rius et al. 2011).

5.4.6 Storage proteins

5.4.6.1 Vicilin 47 kDa protein

Ca_04570 was annotated to code for vicillin 47 kDa protein. Vicillin is a seed storage protein reported in several plants, such as soybean, pea, faba bean, chickpea, tomato (Boulter et al. 1984; Salgado et al. 2002). Seed storage protein has been reported to play role in seed weight. Johnson et al. (1996) observed a consistent association between seed storage protein, phaseolin (*Phs*) and seed weight in common bean. This study reports *Phs* as candidate gene for seed weight QTL in common bean. So according to the findings from the current study, we may assume this Ca_04570 can be one of the probable candidate genes for seed weight in chickpea.

SUMMARY

6. SUMMARY

The current study entitled "Fine mapping of the "*QTL-hotspot*" region for drought tolerance in chickpea (*Cicer arietinum* L.)" was proposed to understand the molecular mechanism behind drought tolerance in chickpea.

Salient achievements of this study are listed as below:

1. In total 6.24 Gb data from ICC 4958, 5.65 Gb data from ICC 1882 and 59.03 Gb data from ICCRIL03 was generated, which identified 828 novel SNPs for genetic mapping. Together with these new markers, a high-density intraspecific genetic map was developed that comprises 1,007 marker loci spanning a distance of 727.29 cM.
2. QTL analysis using the extended genetic map along with precise phenotypic data for 20 traits collected over 1-7 seasons identified 49 SNP markers in the "*QTL-hotspot*" region. These efforts resulted in re-estimating the "*QTL-hotspot*", which narrowed down the QTL region from 29 cM to 14 cM. In total, 164 main-effect QTLs (M-QTLs) were identified.
3. The refined "*QTL-hotspot*" spanned 14 cM on genetic map corresponding to ~3 Mb on the physical map. A total of 286 genes were identified from this refined "*QTL-hotspot*" including several stress related genes.
4. In addition, a total of 15 novel genic (14 CAPS and 1 dCAPS) markers were newly developed from the SNPs integrated into the "*QTL-hotspot*". These markers can be used in marker assisted breeding program for introgressing the "*QTL-hotspot*" into elite cultivar.

   This work (mentioned in points 1-4) has been published in Molecular Genetics and Genomics in 2015 (Jaganathan et al. 2015).

5. A total of 82,127 SNPs were identified by employing skim sequencing approach on the RIL population (ICC 4958 × ICC 1882). Of which, 62,370 SNPs located on pseudomolecules were considered for bin construction. A total of 53,169 SNPs were selected after filtering.
6. Sliding window approach was followed to construct bins on the 53,169 SNPs which are derived from skim sequencing approach along with 54 GBS-based SNPs which were integrated into the "*QTL-hostpot*" from objective 1. A total of 1,610 recombination breakpoints were identified and used for linkage map construction. A linkage map spanning 973.94 cM with 1,557 bins was constructed.
7. The QTL study identified 71 major QTLs and 63 minor QTLs. Major QTLs were identified for 11 traits (RLD, RTR, SDW, PHT, PBS, DF, DM, POD, 100-SDW, HI and DC) and minor QTLs were identified for all the analyzed traits.
8. The high density map delineates the "*QTL-hotspot*" region from ~3 Mb to two sub-QTL regions viz. "*QTL-hotspot_a*" of 139.22 kb and "*QTL-hotspot_b*" of 153.36 kb sizes.
9. A total of 15 genes were identified in "*QTL-hotspot_a*" and 11 genes were identified in "*QTL-hotspot_b*". Some of the identified genes such as e3 ubiquitin ligase, serine threonine protein kinases, homocysteine s-methyltransferase were annotated as candidate drought tolerance genes, and expression profiling needs to be carried out to understand their involvement in drought tolerance in chickpea.

   This work (mentioned in points 5-9) has been published in Nature Scientific Reports in 2015 (Kale and Jaganathan et al. 2015).
10. For further fine mapping of QTL sub regions, a high resolution mapping population was developed by crossing a NIL^{+} line (introgression line with "*QTL-hotspot*" from

ICC 4958) with ICC 1882 genetic background. A total of 6,000 F_2 plants were generated. Of which 1, 911 lines were used for selecting the recombinant lines.

11. KASPar assays were developed for 6 SNPs (two flanking and one within the target regions of each sub regions). Genotypic analysis of F_2 lines revealed 42 recombinants.
12. Progeny testing on the selected recombinants in F_3 generation confirmed the role of "*QTL-hotspot_a*" in controlling 100- seed weight trait.
13. GBS and skim sequencing based SNP marker integration has narrow down the "*QTL-hotspot*" region into two sub-regions of 26 candidate genes.
14. Among the two sub regions, "*QTL-hotspot_a*" was identified to play role in controlling 100-seed weight.
15. Few candidate genes like Ca_04546, Ca_04547, Ca_04548, Ca_04549, Ca_04551, Ca_04554 and Ca_04558 coding for stem-specific protein tsjt1-like, probable inactive serine threonine-protein kinase lvsg-like, transcription initiation factor tfiid subunit 8-like, probable inactive serine threonine-protein kinase lvsg-like, nad -linked oxidoreductase superfamily protein isoform 1, heat stress transcription factor a-6b-like and divergent cct motif protein respectively were identified in this region.

Future Needs

Although this study provides some basic information on candidate genes, in order to reach prominent conclusions on the role of these genes in drought tolerance, following steps are required. Progeny testing of all the reported major traits identified within the "*QTL-hotspot_a*" and "*QTL-hotspot_b*" in the successive generations will reveal the candidate genes for the respective drought component traits. Therefore further phenotyping for the selected traits and genotyping on the next generation i.e F_4 is prerequisite for identifying more recombinations within these two regions. Additionally, detailed analysis of such identified genes through sequence level variation studies, gene expression, complement

testing, knock down experiments and proteomic analysis will be required to further validate these genes in elucidating drought tolerance mechanism in chickpea.

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7. REFERENCES

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APPENDICES

APPENDICES

Appendix 1: List of CAPS/dCAPS markers developed from the "*QTL-hotspot*" region

SNP ID	Primer ID	CAPS candidates*	Marker type	Enzyme	Forward sequence (5'- 3')	Reverse sequence (5'- 3')	Amplification*	Product size (bp)	ICC 4958 × PI 489777#	ICC 4958 × ICC 1882	ICC 283 × ICC 8261
Ca4_12558541	CaLG040001	CGMM139	CAPS	*Mnl*I	GGGGGACCAA GGAATAAAAA	CACCCCTCTCC ATGCATATT	A	320	M	P	P
Ca4_13724666	CaLG040002	-	dCAPS	*Bsr*I	CCTACTAGCTG CTTCTGACTG	TTGCCAACTCC AATATGTCG	NA	350	-	-	-
Ca4_13588956	CaLG040003	-	dCAPS	*Dpn*I	AAGTTGTTGTT ATGGCATGGA T	TCACATATGGT CCCACCTCA	NA	351	-	-	-
Ca4_13699663	CaLG040004	-	dCAPS	*Dpn*I	CAGCAGATGC TTTCAACTTCC	TTTGGCTTGGA T	NA	408	-	-	-
Ca4_13840484	CaLG040005	-	dCAPS	*Kpn*I	ACATGGGCTG GTATTGGAAA	TACCCCCTGGA GCGGT	NA	408	-	-	-
Ca4_13840251	CaLG040006	CGMM140	CAPS	*Mbo*II	GGAATTTGCTG CGTTTGAAT	AGCACGGACC AACTTTCATC	A	391	P	P	P
Ca4_11276225	CaLG040007	CGMM141	CAPS	*Aci*I	GATGCACTTGC ATTTCTTGC	TAAGCAGCTTC CCAAATGGT	A	403	M	P	P
Ca4_11398889	CaLG040008	-	dCAPS	*Dpn*I	TTTTCCTCTGT GACGAGCAG	ATCAACTAGTC TCAGGTTCGA	NA	367	-	-	-
Ca4_11276413	CaLG040009	-	CAPS	*Dpn*I	CAGCAAGCAG GTTGTTTTCA	TCCACTGTAGC CGCAAAAAT	A	397	-	-	-
Ca4_11234130	CaLG040010	-	dCAPS	*Mbo*II	AAGAGAGCTT AAGCCTTGAA	CAGCTGGAGC ATTGACAGAT	NA	374	-	-	-
Ca4_11441735	CaLG040011	-	dCAPS	*Ava*I	ATGTAATATTA ATAAGCACCC	TGCATATGCCT GTGGATTTG	NA	363	-	-	-
Ca4_11435380	CaLG040012	CGMM142	CAPS	*Rsa*I	GCCACATGCTT GTTGCTGTA	ATGTCCGAATC CTTTGAAGC	A	397	P	P	P
Ca4_11435651	CaLG040013	CGMM143	dCAPS	*Hpa*II	GATCTACTCAA AGAGATTGAG GCC	CAAGATGAAA CAACAGTTAC AATGC	A	338	M	P	P
Ca4_11343257	CaLG040014	-	dCAPS	*Dpn*I	ATTTTAGATTG	CACATTGTTCC	NA	387	-	-	-

					ATGTGGCGAT	TTCGAAAACC					
Ca4_11343230	CaLG040015	-	dCAPS	*Dpn*I	TCATGGAGGC TATTTGCATTC	TCTAAAATAA GAT	A	388	-	-	-
Ca4_11547141	CaLG040016	CGMM144	CAPS	*Hph*I	TTTGCAGCCTA CATGACTCG	TGGTTAGCCCA AAATATGGA	A	626	P	P	P
Ca4_11246164	CaLG040017	-	dCAPS	*Bsr*I	CTTTTCTAATT TTTGTACTG	TCCACCAGAG AGGGAAATTG	NA	409	-	-	-
Ca4_11398699	CaLG040018	-	dCAPS	*Mbo*II	GAGTAAACCC TAAGAAG	ATGGGTTACA GCCGAAACTT	NA	389	-	-	-
Ca4_11334350	CaLG040019	CGMM145	CAPS	*Mbo*II	AAAGCAGAAG TTCCTGCACAA	TGCATGGGGA TTGTTGTAGA	A	614	P	P	P
Ca4_11246093	CaLG040020	CGMM146	CAPS	*Ava*I	TCAGTGCCTAG CTTGTCTCGA	TGCTACCTCAA ATCAAAGAAC AA	A	404	P	P	P
Ca4_11273405	CaLG040021	-	dCAPS	*Mse*I	TATATTAGAA GTTCAAATTA	CCCAAAACAT CATCAAAGAG AA	NA	326	-	-	-
Ca4_11398682	CaLG040022	-	dCAPS	*Ava*I	TACATCAATTA GAAAATCCYC	ATGGGTTACA GCCGAAACTT	NA	410	-	-	-
Ca4_11517582	CaLG040023	CGMM147	CAPS	*Hae*III	ATTTTCCTGGG CTTTGAGGT	CAACGCCAAT TCGACAATAC	A	556	M	P	P
Ca4_11246173	CaLG040024	-	dCAPS	*Hae*III	TTTGTTATGGT GTTAGGC	TCCACCAGAG AGGGAAATTG	NA	398	-	-	-
Ca4_11517517	CaLG040025	-	dCAPS	*Dpn*I	AAGAAATTCTT GGCAGGAT	CGAACCTTGA GATGGTCTCC	NA	414	-	-	-
Ca4_11277574	CaLG040026	-	dCAPS	*Bsr*I	TATTTACAAGG ATTCACTG	GCGCAGAAGT GCTTTCAGAG	NA	404	-	-	-
Ca4_11491864	CaLG040027	-	dCAPS	*Mse*I	CTTAATATATC ACCAAATT	TTGAGCCCTGG ATAAGTTGC	NA	379	-	-	-
Ca4_11273328	CaLG040028	-	dCAPS	*Mbo*II	TGTCTGATCTT CACCGAACTG	ATTTTCAGCGT GAA	NA	365	-	-	-
Ca4_11490100	CaLG040029	-	dCAPS	*Dpn*I	GGTGCAGGTC CTGTGAT	ACTAGAGGCC CACGAAGGAT	A	392	-	-	-
Ca4_11334343	CaLG040030	-	dCAPS	*Rsa*I	CCTCCTGCTCA GATTTTTCCGT	CCACAAAAAG TCTAAGTGCTT GAA	NA	414	-	-	-
Ca4_11490496	CaLG040031	-	dCAPS	*Bsr*I	CCTAGGACATT CAACTCACT	ATCGGAAAGC TTAGGTACGG	NA	387	-	-	-

Ca4_11274281	CaLG040032	-	dCAPS	*Rsa*I	TCCTCCATTTG CTTCTCTGA	TTCCCATCAAG AGGCGT	NA	384	-	-	-
Ca4_11244334	CaLG040033	CGMM148	CAPS	*Mse*I	TGTGCTGTCTT CTTAGCATGG	CCATGCGTTGC TCCATTAG	A	602	M	P	P
Ca4_11244395	CaLG040034	-	dCAPS	*Mbo*II	TACCCCTCGGG TGAA	GCATTTAAAA ATAAGAAATG GGAGA	NA	384	-	-	-
Ca4_11276484	CaLG040035	-	dCAPS	*Mbo*II	GATGCAGCTTC CTAGAAATTG G	TTCTGGACCAT GAAG	NA	386	-	-	-
Ca4_11275171	CaLG040036	-	dCAPS	*Mse*I	CACACATATTC ATGGCACTTG AT	AGAGCTGGTA ATCCATT	NA	378	-	-	-
Ca4_11465075	CaLG040037	CGMM149	CAPS	*Bsa*BI	TCTAAGCAAG CCGCAGGTAT	TCGTGACGGT ATCGAACAGA	A	598	P	P	P
Ca4_11465057	CaLG040038	-	CAPS	*Dpn*I	TCAACCAATTC GCTCATCAC	CATTGGGGAA TCAAAGGAGT	A	589	-	-	-
Ca4_11441673	CaLG040039	CGMM150	CAPS	*Eco*RI	CAAGAAACCA TTCACATGCAG	GGATGGCAAG TTTTCTCAGG	A	600	M	P	P
Ca4_11441604	CaLG040040	-	dCAPS	*Mse*I	TTATATGACAT ATCTGTATTTG ATGATTTA	TGGTGAGCTTA GTGCTGCTG	NA	431	-	-	-
Ca4_12982420	CaLG040041	-	dCAPS	*Rsa*I	TGATCTCAAA ATGGTGATGA GG	TCACAACACT GGGTA	NA	414	-	-	-
Ca4_13787649	CaLG040042	-	dCAPS	*Mse*I	TCTGCAGATA AGAGATGGAG AGG	GAAAGAGTTT GCTTA	NA	381	-	-	-
Ca4_13787448	CaLG040043	-	dCAPS	*Mse*I	TTGTTGAATTT TTATTATTCAT TA	AACCCACTTG GTTTCCCAAC	A	425	-	-	-
Ca4_13841340	CaLG040044	-	dCAPS	*Dpn*I	ACCGGTATAG CTGCAGAAGG	CAATAATCTTG AT	NA	411	-	-	-
Ca4_13840227	CaLG040045	-	dCAPS	*Dpn*I	CTCCAGCACC AGACGTGAT	TGCCATGTTTC CTGGTCATA	NA	391	-	-	-
Ca4_13838796	CaLG040046	CGMM151	CAPS	*Mnl*I	TGGAGAGTTCT TGGCTTGCT	TATGCCTACTG CCATCAACG	A	616	M	M	M
Ca4_13839288	CaLG040047	CGMM152	CAPS	*Mnl*I	CGGAATTGGG	GTGAAACTTCT	A	609	P	P	P

					AAACTTGTTG	CCAGCGTTTG					
Ca4_13839294	CaLG040048	CGMM153	CAPS	*Mse*I	GCTGAAGAAT TTGCTGCTGA	TGCCAATGCA GACACCTAAC	A	591	M	P	P
Ca4_13687456	CaLG040049	-	dCAPS	*Dpn*I	GCAGGGACGA CAACTTGTTT	CTTGTGTTTGA GA**G**A**T**	NA	397	-	-	-

Appendix 2: QTLs identified for all the traits and two drought indices phenotyped over 1-5 locations and 1-5 seasons in India from the RIL population of the ICC 4958 × ICC 1882 cross using high density bin map

TraitID	Trait Name	Year	Location[a]	Environment[b]	Position (cM)	Markers flanked	LOD	PVE (%)[#]
Root traits								
Root length density (RLD, cm cm^{-3})	RLD	2007	PAT	CC	45	bin_4_13239546 - bin_4_13378761	5.23	10.36
Root surface area (RSA, cm^2)	RSA	2007	PAT	CC	1	bin_8_16021376 - bin_8_16020537	3.70	7.40
Root Volume (RV, cm^3)	RV	2007	PAT	CC	1	bin_8_16021376 - bin_8_16020537	3.57	7.14
R-T ratio (RTR, %)	RTR	2007	PAT	CC	46	bin_4_13393647 - bin_4_13547009	10.57	20.09
	RTR	2005	PAT	CC	46	bin_4_13393647 - bin_4_13547009	5.89	11.82
Morphological traits								
Shoot dry weight (SDW, g)	SDW	2007	PAT	RF	46	bin_4_13393647 - bin_4_13547009	13.78	25.22
Plant height (PHT, cm)	PHT	2009	HIR	RF	45	bin_4_13239546 - bin_4_13378761	24.34	41.76
	PHT	2009	PAT	RF	45	bin_4_13239546 - bin_4_13378761	21.97	36.68
	PHT	2009	PAT	IR	45	bin_4_13239546 - bin_4_13378761	15.17	28.45
	PHT	2008	PAT	RF	45	bin_4_13239546 - bin_4_13378761	13.99	24.83
	PHT	2009	HIR	IR	45	bin_4_13239546 - bin_4_13378761	11.73	21.62
	PHT	2008	NDL	RF	45	bin_4_13239546 - bin_4_13378761	10.17	19.03
	PHT	2007	PAT	RF	45	bin_4_13239546 - bin_4_13378761	9.02	17.96
	PHT	2008	PAT	RF	52	bin_8_3018346 - bin_8_3013230	8.23	15.73

PHT	2009	NDL	RF	45	bin_4_13239546 - bin_4_13378761	7.81	14.97
PHT	2006	PAT	RF	46	bin_8_6034209 - bin_8_5984553	6.78	14.27
PHT	2009	PAT	RF	7	bin_6_3613490 - bin_6_3632957	6.43	12.55
PHT	2005	PAT	RF	8	bin_6_3677353 - bin_6_3683132	5.78	11.34
PHT	2007	PAT	RF	21	bin_6_6943324 - bin_6_6999653	4.65	10.58
PHT	2009	HIR	RF	24	bin_6_6943324 - bin_6_6999653	4.53	9.85
PHT	2009	PAT	RF	49	bin_8_3992475 - bin_8_3962288	4.68	9.28
PHT	2005	PAT	RF	42	bin_4_12586721 - bin_4_12650792	4.40	8.79
PHT	2006	PAT	RF	46	bin_4_13393647 - bin_4_13547009	4.10	8.16
PHT	2009	DUG	IR	37	bin_4_11210420 - bin_4_11690035	4.03	8.10
PHT	2008	NDL	RF	8	bin_6_3677353 - bin_6_3683132	3.96	7.91
PHT	2007	PAT	RF	41	bin_8_6034209 - bin_8_5984553	3.19	7.83
PHT	2009	NDL	RF	48	bin_8_4015752 - bin_8_3992475	3.56	7.35
PHT	2009	HIR	IR	21	bin_6_6943324 - bin_6_6999653	3.29	6.94
PHT	2006	PAT	RF	28	bin_6_7491206 - bin_6_7526573	3.24	6.51
PHT	2009	PAT	RF	99	bin_4_45192563 - bin_4_45238182	3.93	5.53
PHT	2009	PAT	IR	80	bin_4_40000893 - bin_4_40023390	3.33	5.36
PHT	2008	PAT	RF	123	bin_4_48635948 - bin_4_48732122	3.03	4.75

Primary branches (PBS)	PBS	2006	PAT	RF	47	bin_8_6034209 - bin_8_5984553	5.70	11.27
	PBS	2005	PAT	RF	37	bin_8_6167474 - bin_8_6034209	3.91	7.82
	PBS	2006	PAT	RF	102	bin_1_1824635 - bin_1_1821759	3.46	6.97
	PBS	2007	PAT	RF	89	bin_7_4537517 - bin_7_4519483	3.21	6.69
Secondry branches (SBS)	SBS	2007	PAT	RF	46	bin_4_13393647 - bin_4_13547009	4.18	8.63
	SBS	2006	PAT	RF	93	bin_1_4992190 - bin_1_4870121	3.85	7.68
	SBS	2006	PAT	RF	52	bin_8_3018346 - bin_8_3013230	3.38	6.79
Phenological traits								
Days to 50% flowering (DF)	DF	2009	HIR	RF	46	bin_8_6034209 - bin_8_5984553	27.66	48.53
	DF	2009	HIR	IR	47	bin_8_6034209 - bin_8_5984553	28.19	44.76
	DF	2006	PAT	RF	136	bin_1_1216139 - bin_1_45888678	3.12	38.44
	DF	2009	PAT	IR	47	bin_8_6034209 - bin_8_5984553	22.99	38.44
	DF	2005	PAT	RF	46	bin_8_6034209 - bin_8_5984553	17.88	36.72
	DF	2009	PAT	RF	47	bin_8_6034209 - bin_8_5984553	21.30	36.00
	DF	2007	PAT	RF	47	bin_8_6034209 - bin_8_5984553	20.09	34.71
	DF	2006	PAT	RF	47	bin_8_6034209 - bin_8_5984553	19.84	34.05
	DF	2009	DUG	RF	47	bin_8_6034209 - bin_8_5984553	18.38	32.06
	DF	2009	NDL	RF	47	bin_8_6034209 - bin_8_5984553	17.89	31.20
	DF	2008	PAT	RF	46	bin_8_6034209 - bin_8_5984553	15.03	29.74

	DF	2009	NDL	IR	47	bin_8_6034209 - bin_8_5984553	8.98	17.18
	DF	2008	NDL	RF	47	bin_8_6034209 - bin_8_5984553	8.36	16.06
	DF	2009	SEH	RF	47	bin_8_6034209 - bin_8_5984553	7.04	13.77
	DF	2008	NDL	RF	65	bin_5_45473300 - bin_5_45530795	3.80	8.70
	DF	2009	HIR	IR	66	bin_5_45473300 - bin_5_45530795	3.70	8.69
	DF	2009	HIR	RF	67	bin_5_45473300 - bin_5_45530795	3.59	8.66
	DF	2009	PAT	RF	69	bin_5_45473300 - bin_5_45530795	3.41	8.12
	DF	2005	PAT	RF	70	bin_5_45473300 - bin_5_45530795	3.54	8.06
	DF	2006	PAT	RF	65	bin_5_45473300 - bin_5_45530795	3.24	7.65
	DF	2007	PAT	RF	56	bin_5_42079654 - bin_5_42142721	3.32	6.66
	DF	2008	SEH	RF	50	bin_8_3992475 - bin_8_3962288	3.09	6.37
	DF	2005	PAT	RF	19	bin_6_5555667 - bin_6_6943324	3.11	6.28
Days to maturity (DM)	DM	2009	HIR	RF	41	bin_7_12870961 - bin_7_12856579	19.53	45.38
	DM	2009	PAT	RF	47	bin_8_6034209 - bin_8_5984553	21.37	36.11
	DM	2009	NDL	RF	47	bin_8_6034209 - bin_8_5984553	20.66	35.09
	DM	2009	DUG	RF	47	bin_8_6034209 - bin_8_5984553	17.97	31.47
	DM	2008	PAT	RF	46	bin_8_6034209 - bin_8_5984553	12.26	24.79
	DM	2009	HIR	RF	48	bin_8_4015752 - bin_8_3992475	11.14	21.33

DM	2009	HIR	IR	48	bin_8_4015752 - bin_8_3992475	10.60	20.17
DM	2009	PAT	IR	48	bin_8_4015752 - bin_8_3992475	10.40	19.61
DM	2008	NDL	RF	48	bin_8_4015752 - bin_8_3992475	9.14	17.54
DM	2009	DUG	IR	47	bin_8_6034209 - bin_8_5984553	8.60	16.47
DM	2009	SEH	RF	46	bin_8_6034209 - bin_8_5984553	7.18	15.38
DM	2008	SEH	RF	45	bin_4_13239546 - bin_4_13378761	7.74	14.90
DM	2006	PAT	RF	46	bin_8_6034209 - bin_8_5984553	6.99	14.51
DM	2007	PAT	RF	47	bin_8_6034209 - bin_8_5984553	6.08	11.93
DM	2008	PAT	RF	68	bin_5_45473300 - bin_5_45530795	4.27	10.51
DM	2005	PAT	RF	48	bin_8_4015752 - bin_8_3992475	5.08	10.14
DM	2009	PAT	IR	8	bin_6_3677353 - bin_6_3683132	3.99	7.96
DM	2009	SEH	RF	77	bin_2_30506636 - bin_2_30527477	3.67	7.41
DM	2009	HIR	IR	20	bin_6_6943324 - bin_6_6999653	3.51	7.40
DM	2009	DUG	RF	68	bin_5_45473300 - bin_5_45530795	3.04	7.36
DM	2008	PAT	RF	43	bin_7_12389184 - bin_7_12378886	3.60	7.34
DM	2009	DUG	IR	71	bin_5_45473300 - bin_5_45530795	3.25	6.81
DM	2009	HIR	RF	30	bin_6_8283807 - bin_6_8299950	3.21	6.52
DM	2008	SEH	RF	118	bin_5_30232785 - bin_5_30210335	3.20	6.44

	DM	2009	DUG	IR	0	bin_7_29276309 - bin_7_29967944	3.01	6.05
Yield related traits								
Pods/plant (POD)	POD	2009	PAT	RF	45	bin_4_13239546 - bin_4_13378761	9.82	16.66
	POD	2007	PAT	RF	50	bin_4_15082304 - bin_4_15250027	5.91	11.96
Seeds/pod (SPD)	SPD	2007	PAT	RF	11	bin_5_20457969 - bin_5_7323627	3.17	6.66
	SPD	2006	PAT	RF	27	bin_8_8297568 - bin_8_8296790	3.00	6.04
100-seed weight (100SDW, g)	100SDW	2009	DUG	RF	45	bin_4_13239546 - bin_4_13378761	43.56	59.83
	100SDW	2009	HIR	RF	45	bin_4_13239546 - bin_4_13378761	39.20	55.99
	100SDW	2009	HIR	IR	45	bin_4_13239546 - bin_4_13378761	35.73	52.74
	100SDW	2008	PAT	RF	45	bin_4_13239546 - bin_4_13378761	34.64	51.41
	100SDW	2009	NDL	RF	45	bin_4_13239546 - bin_4_13378761	33.93	50.89
	100SDW	2009	PAT	IR	45	bin_4_13239546 - bin_4_13378761	33.21	50.16
	100SDW	2009	NDL	IR	45	bin_4_13239546 - bin_4_13378761	32.47	49.34
	100SDW	2009	DUG	IR	45	bin_4_13239546 - bin_4_13378761	27.30	43.68
	100SDW	2007	PAT	RF	45	bin_4_13239546 - bin_4_13378761	22.96	36.91
	100SDW	2008	NDL	RF	46	bin_4_13393647 - bin_4_13547009	18.31	31.66
	100SDW	2006	PAT	RF	45	bin_4_13239546 - bin_4_13378761	16.20	28.55
	100SDW	2005	PAT	RF	45	bin_4_13239546 - bin_4_13378761	15.75	27.96
	100SDW	2009	PAT	IR	107	bin_1_1202614 - bin_1_1195249	9.13	17.34

	100SDW	2009	PAT	RF	106	bin_1_1244015 - bin_1_1229294	7.11	13.71
	100SDW	2008	PAT	RF	107	bin_1_1202614 - bin_1_1195249	6.70	12.98
	100SDW	2009	HIR	IR	107	bin_1_1202614 - bin_1_1195249	6.02	11.89
	100SDW	2005	PAT	RF	107	bin_1_1202614 - bin_1_1195249	5.47	10.78
	100SDW	2009	HIR	RF	107	bin_1_1202614 - bin_1_1195249	4.91	9.81
	100SDW	2009	PAT	IR	78	bin_1_7476098 - bin_1_7412354	4.00	7.10
	100SDW	2009	NDL	IR	107	bin_1_1202614 - bin_1_1195249	3.45	6.96
	100SDW	2009	NDL	RF	107	bin_1_1202614 - bin_1_1195249	3.39	6.85
Biomass (BM, g)	BM	2009	PAT	RF	58	bin_1_45827489 - bin_1_39391172	4.09	8.32
	BM	2009	DUG	RF	44	bin_4_13048918 - bin_4_13097584	3.90	7.85
	BM	2009	PAT	IR	99	bin_7_3102566 - bin_7_3050024	3.65	7.33
Harvest index (HI, %)	HI	2009	PAT	IR	47	bin_8_6034209 - bin_8_5984553	8.01	15.42
	HI	2009	HIR	IR	19	bin_6_5555667 - bin_6_6943324	6.17	12.14
	HI	2009	PAT	RF	43	bin_4_12935549 - bin_4_12982420	5.52	10.81
	HI	2008	PAT	RF	49	bin_8_3992475 - bin_8_3962288	4.64	9.19
	HI	2009	PAT	RF	47	bin_8_6034209 - bin_8_5984553	4.12	8.27
	HI	2009	PAT	IR	4	bin_6_2804634 - bin_6_2917216	3.32	6.71
	HI	2008	SEH	RF	51	bin_8_3962288 - bin_8_3135881	3.18	6.46

	HI	2009	PAT	IR	11	bin_7_31651413 - bin_7_31652416	3.16	6.43
Yield (YLD, g)	YLDG	2007	PAT	RF	110	bin_4_47375605 - bin_4_47453959	4.36	8.74
	YLDG	2009	DUG	RF	45	bin_7_12083724 - bin_7_11984393	3.53	7.09
	YLDG	2009	SEH	IR	19	bin_3_35078988 - bin_3_35056553	3.18	6.38
Transpiration related traits								
Delta carbon ratio ($\delta^{13}C$)	DC	2008	DUG	RF	45	bin_4_13239546 - bin_4_13378761	6.11	11.90
	DC	2008	PAT	RF	2	bin_4_1710658 - bin_4_1713134	4.44	8.36
	DC	2008	DUG	RF	86	bin_4_42549627 - bin_4_43207105	3.33	6.32
Drought indices								
Drought tolerance indices (DTI)	DTI	2009	NAN	RF	46	bin_8_6034209 - bin_8_5984553	3.40	7.38
	DTI	2009	PAT	RF	92	bin_7_4171826 - bin_7_4156233	3.92	7.33
	DTI	2009	PAT	RF	63	bin_1_22810338 - bin_1_22349608	3.69	6.87
	DTI	2009	PAT	RF	16	bin_7_32532047 - bin_7_32710042	3.26	6.05
Drought susceptibility indices (DSI)	DSI	2009	NAN	RF	47	bin_8_6034209 - bin_8_5984553	4.31	8.64

Note

[a]PAT - Patancheru; NDL - Nandyal; sehore - Sehore; DUG - Durgapura; HIR - Hiriyur

[b]RF - Rainfed; IR - Irrigated; CC - Cylider culture

phenotypic variation explained

Major QTLs are shown in green colour.

Appendix 3: List of BC_6F_1 lines screened for the selection of parental line to be used in fine mapping population development

Lines	TAA170	ICCM0249	GA24	NCPGR21	NCPGR127
ICCX-110125-P18-P1	H	H	H	A	H
ICCX-110125-P18-P2	A	A	H	H	H
ICCX-110125-P18-P3	A	H	H	B	A
ICCX-110125-P18-P4	H	NA	A	H	A
ICCX-110125-P18-P5	H	A	A	B	A
ICCX-110125-P18-P6	A	NA	NA	A	B
ICCX-110125-P18-P7	H	NA	H	NA	H
ICCX-110125-P18-P8	H	B	H	H	B
ICCX-110125-P18-P9	A	A	A	A	A
ICCX-110125-P18-P10	H	H	H	H	H
ICCX-110125-P18-P11	H	H	H	H	A
ICCX-110125-P18-P12	B	H	B	B	B
ICCX-110125-P18-P13	A	A	H	A	A
ICCX-110125-P18-P14	B	B	B	B	B
ICCX-110125-P18-P15	H	H	A	A	B
ICCX-110125-P18-P16	A	NA	A	A	A
ICCX-110125-P18-P17	B	B	NA	B	B
ICCX-110125-P18-P18	H	NA	H	H	H
ICCX-110125-P18-P19	B	NA	NA	B	H
ICCX-110125-P18-P20	B	A	NA	B	A
ICCX-110125-P18-P21	H	H	H	H	H
ICCX-110125-P18-P22	B	NA		B	B
ICCX-110125-P18-P23	A	NA	A	H	A
ICCX-110125-P18-P24	H	NA	H	H	A
ICCX-110125-P18-P25	A	H	H	A	B
ICCX-110125-P18-P26	H	NA	H	H	A
ICCX-110125-P18-P27	H	NA	B	B	A

ICCX-110125-P18-P28	A	A	A	A	A
ICCX-110125-P18-P29	A	A	A	A	A
ICCX-110125-P18-P30	H	H	A	A	H
ICCX-110125-P18-P31	A	A	A	A	B
ICCX-110125-P18-P32	H	NA	A	H	H
ICCX-110125-P18-P33	H	H	A	H	H
ICCX-110125-P18-P34	A	A	H	A	A
ICCX-110125-P18-P35	B	B	H	H	B
ICCX-110125-P18-P36	B	B	H	H	B
ICCX-110125-P18-P37	A	A	A	A	A
ICCX-110125-P18-P38	A	A	NA	H	A
ICCX-110125-P18-P39	H	B	H	H	A
ICCX-110125-P18-P40	H	H	A	A	H
ICCX-110125-P18-P41	H	H	H	H	H
ICCX-110125-P18-P42	A	A	NA	B	A
ICCX-110125-P18-P43	H	H	H	H	B
ICCX-110125-P18-P44	H	H	H	H	H
ICCX-110125-P18-P45	H	H	H	H	H
ICCX-110125-P18-P46	H	NA	H	H	B
ICCX-110125-P18-P47	H	H	H	H	A
ICCX-110125-P18-P48	B	NA	NA	B	H
ICCX-110125-P18-P49	H	H	H	H	H
ICCX-110125-P18-P50	H	H	H	H	A
ICCX-110125-P18-P51	B	NA	NA	B	A
ICCX-110125-P18-P52	H	H	NA	B	A
ICCX-110125-P18-P53	NA	B	NA	B	B
ICCX-110125-P18-P54	A	A	H	H	A
ICCX-110125-P18-P55	H	NA	H	H	A
ICCX-110125-P18-P56	H	NA	H	H	H
ICCX-110125-P18-P57	B	NA	H	B	B

ICCX-110125-P18-P58	A	A	A	A	A
ICCX-110125-P18-P59	B	B	NA	B	B
ICCX-110125-P18-P60	H	NA	H	H	H
ICCX-110125-P18-P61	H	H	H	H	H
ICCX-110125-P18-P62	A	A	A	A	A
ICCX-110125-P18-P63	B	NA	NA	B	B
ICCX-110125-P18-P64	H	H	H	H	A
ICCX-110125-P18-P65	B	H	NA	B	A
ICCX-110125-P18-P66	H	NA	H	H	H
ICCX-110125-P18-P67	H	NA	H	H	H
ICCX-110125-P18-P68	H	NA	H	H	H
ICCX-110125-P18-P69	H	NA	H	H	H
ICCX-110125-P18-P70	H	NA	NA	H	H
ICCX-110125-P18-P71	B	NA	NA	B	B
ICCX-110125-P18-P72	H	NA	NA	B	H
ICCX-110125-P18-P73	NA	H	A	A	H
ICCX-110125-P18-P74	H	H	NA	B	A
ICCX-110125-P18-P75	H	NA	H	H	H
ICCX-110125-P18-P76	H	H	NA	H	A
ICCX-110125-P18-P77	A	A	A	A	A
ICCX-110125-P18-P78	H	NA	H	H	B
ICCX-110125-P18-P79	A	NA	A	A	H
ICCX-110125-P18-P80	NA	NA	NA	B	A
ICCX-110125-P18-P81	NA	NA	H	H	H
ICCX-110125-P18-P82	A	A	A	A	A
ICCX-110125-P18-P83	NA	NA	H	H	H
ICCX-110125-P18-P84	NA	NA	H	H	H
ICCX-110125-P18-P85	NA	NA	NA	B	B
ICCX-110125-P18-P86	NA	NA	H	H	A

NA- not amplified; Lines highlighted in blue colour are homozygous for "*QTL-hotspot*" region and selected for F_1 generation of fine mapping population.