A combinatorial approach of comprehensive QTL-based comparative genome mapping and transcript profiling identified a seed weight-regulating candidate gene in chickpea

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High experimental validation/genotyping success rate (94–96%) and intra-specific polymorphic potential (82–96%) of 1536 SNP and 472 SSR markers showing in silico polymorphism between desi ICC 4958 and kabuli ICC 12968 chickpea was obtained in a 190 mapping population (ICC 4958 × ICC 12968) and 92 diverse desi and kabuli genotypes. A high-density 2001 marker-based intra-specific genetic linkage map comprising of eight LGs constructed is comparatively much saturated (mean map-density: 0.94 cM) in contrast to existing intra-specific genetic maps in chickpea. Fifteen robust QTLs (PVE: 8.8–25.8% with LOD: 7.0–13.8) associated with pod and seed number/plant (PN and SN) and 100 seed weight (SW) were identified and mapped on 10 major genomic regions of eight LGs. One of 126.8 kb major genomic region harbouring a strong SW-associated robust QTL (Caq'SW1.1: 169.1–171.3 cM) has been delineated by integrating high-resolution QTL mapping with comprehensive marker-based comparative genome mapping and differential expression profiling. This identified one potential regulatory SNP (G/A) in the cis-acting element of candidate ERF (ethylene responsive factor) TF (transcription factor) gene governing seed weight in chickpea. The functionally relevant molecular tags identified have potential to be utilized for marker-assisted genetic improvement of chickpea.

Chickpea (Cicer arietinum L.), represented majorly by desi and kabuli cultivar types, is one of the most cultivated food legume crops in the world. High yield potential but low crop productivity necessitates genetic improvement of yield component and stress tolerant traits of chickpea cultivars. To expedite the marker-assisted breeding for genetic enhancement in chickpea, identification and mapping of informative markers tightly linked to the genes/QTLs (quantitative trait loci) regulating important agronomic traits is essential. In recent years, such marker-assisted trait improvement in a large chickpea genome with narrow genetic base is predominantly attributed to construction of high-resolution SNP (single nucleotide polymorphism) and SSR (simple sequence repeats) marker-based intra- and inter-specific genetic linkage maps, and subsequently fine mapping and map-based cloning of trait-governing genes/QTLs.

Until recently, about two thousand SSR and SNP markers have been validated and genotyped in diverse mapping populations using high-throughput genotyping assays to construct the high-density inter-specific genetic linkage maps in chickpea¹–¹⁰. By use of numerous such informative sequence-based codominant, multi-/bi-allelic and abundant SSR and SNP markers in combinations, the resolution of constructed inter-specific genetic linkage maps in terms of mean map-density has now increased upto 0.59–1.7 cM in chickpea¹⁰. Several efforts have also been made to construct SSR marker-based low resolution genetic linkage maps (map density ranged from 2.5-7 cM) utilizing the diverse desi and kabuli intra-specific mapping populations¹¹–¹⁷,41,48–2².
However, only single report on construction of high-density intra-specific genetic linkage maps (with map density varied from 1.74 to 2.16 cm) by high-throughput genotyping of about 1000 SSR and SNP markers in RIL (recombinant inbred lines) mapping populations using automated fragment analyzer and Illumina GoldenGate assay is available in chickpea\(^\text{49}\). Therefore, the combined use of SSR and SNP markers in large-scale validation and high-throughput genotyping of diverse mapping populations using suitable modern advanced genotyping assays can significantly enhance the resolution of intra-specific genetic linkage maps in chickpea.

Using the intra- and inter-specific genetic map information, many QTLs associated with yield component and abiotic/biotic stress tolerance traits have been identified and mapped in chickpea. It includes identification and mapping of QTLs associated with Fusarium wilt, Ascochyta blight, Botrytis gray mold and rust resistance, salinity and drought tolerance, root traits, flowering time, plant growth habit, seed size/100-seed weight, double podding, seed/pod number per plant and harvest index in chickpea\(^\text{13,16,18,21–26}\). In spite of such huge efforts on QTLs identification, most of the markers/gene harboring QTL regions have not been fine mapped and validated across diverse mapping populations and/or environments to be harnessed for efficient marker-assisted selection for chickpea genetic improvement. The available draft assemblies of genome and transcript sequences of diverse desi and kabuli chickpea have enabled to select numerous chromosome-wise well distributed and informative in silico polymorphic SSR and SNP markers for genomics-assisted breeding applications in chickpea\(^\text{47–50}\). In this perspective, large-scale validation and high-throughput genotyping of genome-wide polymorphic SSR and SNP markers and their use in construction of diverse mapping population-derived high-resolution intra-specific genetic linkage maps are now feasible in chickpea. It would also accelerate the identification, fine mapping and map-based isolation of genes/QTLs associated with traits of agricultural importance, and thereby, genetic enhancement of chickpea through marker-assisted selection.

Keeping all above in view, the present study was undertaken to validate and genotype genome-wide physically mapped 1632 SNP and 500 SSR markers showing in silico polymorphism between ICC 4958 (desi) and ICC 12968 (kabuli) in a 190 F\(_4\) mapping population (ICC 4958 × ICC 12968) using Illumina GoldenGate assay, gel-based assay and fluorescent dye-labeled automated fragment analyzer. The marker genotyping and robust field phenotyping information of mapping individuals were utilized to develop a high-resolution intra-specific genetic linkage map for identification of major QTLs associated with pod and seed number/plant and 100-seed weight in chickpea. The relevant high-resolution QTL mapping information was integrated with comprehensive marker-based comparative genome mapping and differential expression profiling to delineate a candidate gene at one of the robust seed weight-governing major QTL region in chickpea.

**Results and Discussion**

The large-scale validation and high-throughput genotyping of genome-wide informative sequence-based robust SNP and SSR markers in advanced generation mapping populations is useful in construction of high-resolution genetic linkage maps and identification/mapping of genes/QTLs associated with important agronomic traits, which could accelerate genetic enhancement in chickpea. To expedite such process in a large chickpea genome with narrow genetic base, the use of whole genome SNP and SSR markers showing in silico polymorphism (based on repeat-unit variations) in the genomic and transcript sequences among diverse desi and kabuli genotypes could be an attractive strategy. In our study, we selected genome-wide (physically mapped on eight chromosomes) in silico polymorphic genic and genomic 1632 SNP and 500 SSR markers between desi ICC 4958 and kabuli ICC 12968 chickpea for their large-scale validation and high-throughput genotyping in 190 F\(_4\) mapping individuals using the gel-based assay, fluorescent dye-labelled automated fragment analyser and Illumina GoldenGate assay to construct a high-density intra-specific genetic linkage map in chickpea.

**Large-scale validation and high-throughput genotyping of SNP and SSR markers.** A selected set of 1632 SNPs with designability scores of \(\geq 0.8\) were included to design chickpea ‘Ga-II-OPA’ for their genotyping in 190 F\(_4\) mapping individuals (ICC 4958 × ICC 12968) and parental genotypes though Illumina GoldenGate assay. Reproducibility of genotyping assay was estimated as 100% using two parental genotypes as biological replicates. Of the 1632 SNP loci, 1587 (97.2%) could be genotyped successfully on all 192 individuals showing distinct cluster separation at \(\geq 0.3\) GenCall and GenTrain scores. After removal of missing SNP genotyping data, including monomorphic and heterozygous SNPs from parental genotypes, 1536 SNPs (Supplementary Table S1) were found relevant with overall genotyping success rate of 94.1%. Genotype polymorphic potential of SNP and SSR markers in chickpea genome analysis and molecular breeding.

**Polymorphic potential of SNP and SSR markers.** A selected 96 genome-wide well-distributed (physically mapped on eight chickpea chromosomes) SNP markers were genotyped in 92 desi and kabuli chickpea genotypes using GoldenGate assay. Ninety-two (95.8%), mean PIC: 0.43) of 96 SNP markers showed polymorphism among these genotypes (Fig. 1b). Eighty (87%), mean PIC: 0.39) of 92 SNP markers showed polymorphism between desi and kabuli, while 63 (68.5%, mean PIC: 0.32) and 43 (46.7%, 0.26) markers were polymorphic within 52 desi and 40 kabuli chickpea, respectively. A set of 96 SSR markers physically mapped on eight chickpea chromosomes were selected to evaluate their polymorphic potential among 92 desi and kabuli genotypes using gel-based assay and automated fragment analyzer. Seventy-nine (82.2%) markers of these showed polymorphism (with average PIC of 0.69) among desi and kabuli genotypes (Fig. 2c). Sixty-six (83.5%, mean PIC: 0.65) of 79 markers were polymorphic between desi and kabuli. Fifty-two (65.8%) of 79 markers showed polymorphism among 52 desi genotypes (varied from 1 to 4 alleles with mean PIC of 0.60), while 34 (43%) markers detected polymorphism among 40 kabuli genotypes (1 to 3 with 0.51). The 92 SNP and 79 SSR markers overall
Figure 1 | Example of one regulatory SNP (G/A) validated in an ERF TF gene by Illumina GoldenGate genotyping assay showing homozygous and heterozygous cluster separation for 190 mapping individuals along with two parental genotypes (a) and 92 desi and kabuli genotypes (b) based on plotting of normalised R [sum of intensities of the two channels (Cy3 and Cy5)] on the y-axis vs. normalised theta \( \left( \frac{2}{\pi} \tan^{-1} \left( \frac{Cy5}{Cy3} \right) \right) \) on the x-axis. A normalised theta value nearest 0 is homozygous for allele A (red), a theta value nearest 0.5 is heterozygote AB (violet) and a theta value nearest 1 is homozygous for allele B (blue).

Figure 2 | Validation of a representative set of 14 SSR markers (physically mapped on eight chickpea chromosomes) showing in silico fragment length polymorphism between parental genotypes (ICC 4958 and ICC 12968) of a F_4 mapping population (ICC 4958 × ICC 12968) using the gel-based assay (a) and fluorescent-dye labeled automated fragment analyzer (b). (c) Segregation pattern of one selected SSR marker in a representative set of mapping individuals. (d) Amplification and polymorphism profiles of one SSR marker in a selected set of desi and kabuli genotypes. The fragment sizes (bp) of the amplified polymorphic alleles are indicated. The identities of SSR markers with their detailed information are provided in the Supplementary Table S1. M: 50 bp DNA ladder size standard.
produced a total of 528 alleles in 92 chickpea genotypes. The number of alleles detected by these markers varied from 2 to 4 with an average of 3.1 alleles per marker.

The intra-specific polymorphic potential detected by SNP (95.8%) and SSR (82.3%) markers among 92 desi and kabuli chickpea genotypes is much higher compared to that estimated using in silico polymorphic SSR markers (50–60%). Remarkably, such intra-specific marker polymorphic potential was comparatively much higher than that estimated with random genome-wide SSR markers among desi and kabuli chickpea genotypes (~35%) [6, 8, 9]. Therefore, about 2000 highly informative in silico polymorphic SNP and SSR markers developed in our study at a genome-wide scale have utility in various high-throughput genotyping applications in chickpea. Furthermore, these polymorphic markers have practical significance in detecting a higher intra-specific polymorphic potential among desi and kabuli chickpea genotypes than any other random and sequence-based markers and thus, will serve as a valuable resource for expediting the genomics-assisted breeding applications in chickpea.

Construction of a high-resolution intra-specific chickpea genetic linkage map. To construct a saturated intra-specific genetic linkage map, 2008, including 1536 SNP (Fig. 1a) and 472 SSR (Fig. 2d) markers showing parental polymorphism between ICC 4958 and ICC 12968 were genotyped among 190 individuals of a mapping population (ICC 4958 × ICC 12968). The linkage analysis using 2008 marker genotyping data mapped 2001 (1536 SNP and 465 SSR markers) marker loci onto eight LGs of an intra-specific genetic map of chickpea (Table 1, Fig. 3, 4). This integrated high-density intra-specific genetic map comprising of eight LGs constructed by us supports the previous similar documentation [11–17, 4, 18–23]. The genetic map comprising eight LGs covered a total map length of 1888.86 cM with an average inter-marker distance of 0.94 cM (Table 1). Longest map length spanning 316.55 cM was observed in LG4, while LG6 showed shortest map length of 195.57 cM. Maximum (282 markers) numbers of markers were mapped on LG4, followed by LG3 (266) and minimum on LG8 (234). The LG6 had the most saturated genetic map (varying from 0.68 to 0.94 cM with an average inter-marker distance 0.82 cM), while LG4 contained the least saturated map (0.91 to 1.21 cM with an average of 1.12 cM) (Table 1).

The average inter-marker distance (0.94 cM) obtained in the presently constructed intra-specific genetic linkage map was much lower and thus highly saturated in contrast to that reported (2.5–7 cM) using diverse desi and kabuli intra-specific mapping populations [11–17, 4, 18–22]. This intra-specific genetic linkage map has remarkably higher map density compared to one of the integrated SSR and SNP marker-based intra-specific genetic maps (1.74–2.16 cM) of chickpea [22]. Therefore, we constructed a more advanced and highly saturated intra-specific genetic linkage map in contrast to all other intra-specific genetic maps reported so far in chickpea. Henceforth, this integrated high-density intra-specific genetic linkage map would be useful for mapping the whole genome and rapid targeted mapping of genes/QTLs governing important agronomic traits in chickpea as well as comparative mapping across legumes.

Identification and mapping of QTLs associated with agronomic traits in chickpea. We observed a significant difference of three quantitative agronomic traits, PN [37.1–119.0 with 76% broad-sense heritability (H2)], SN (43.9–146.4 with 72% H2) and SW (20.4–44.5 g with 89% H2) in 190 mapping individuals (ICC 4958 × ICC 12968) and two parental genotypes across two years based on ANOVA (Supplementary Table S2). ANOVA results indicated highly significant differences (P < 0.0001) among mapping individuals (RILs) for all three traits despite significant environmental (years) effects on these traits in both seasons (Supplementary Table S3). A significant interaction between genotypes (G) and environment (E) for PN, SN and SW traits was also observed. However, the G × E (58% lower than total mean squares) and E (26.3% lower) variances were found to be smaller for SW compared with PN and SN at significance level of P < 0.001 (Supplementary Table S3).

The normal frequency distribution of three agronomic traits in mapping individuals and parental accessions was observed across two years (Supplementary Fig. S1). Remarkably, bi-directional transgressive segregation of traits beyond that of parental genotypes in mapping population was evident. A highly significant positive correlation between PN and SN (r = 0.96, P < 0.0001) and negative correlation of PN and SW with SW (~0.26, P < 0.001) based on Pearson’s correlation coefficient estimation was observed (Supplementary Fig. S2). A significant phenotypic variation and normal frequency distribution of three quantitative agronomic traits (PN, SN and SW) among 190 mapping individuals along with parental genotypes indicates the involvement of multiple genes for regulation of these traits, and thereby, suggests the utility of developed mapping population (ICC 4958 × ICC 12968) in QTL mapping.

The QTL mapping using the genotyping information of 2001 SNP and SSR markers mapped on an intra-specific genetic linkage map (Fig. 3, 4) and field phenotyping data of 190 mapping population identified and mapped 18 major genomic regions underlying 28 significant (LOD: 4.6–13.8) QTLs associated (R2: 6.8–25.8%) with PN, SN and SW on eight LGs of chickpea (Table 2, Fig. 3, 4). It includes 10 major genomic regions harboring 15 PN, SN and SW-associated QTLs, which were validated and showed consistent phenotypic expression at higher LOD (7.0–13.8) across two years/seasons. These were considered as robust QTLs for controlling PN, SN and SW in chickpea (Table 2). Ten major genomic regions underlying robust QTLs covered (1.7 cm on LG8 to 3.5 cm on LG4) with 37 SNP and SSR markers were mapped on eight LGs (Table 2, Fig. 3, 4). The proportion of phenotypic variation explained (PVE) by individual robust QTL (R’) varied from 8.8–25.8%. The combined PVE estimated for all 15 robust QTLs was 31.7%. Ten QTLs associated with multiple traits (PN and SN) were mapped on the five different

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Table 1 | Markers mapped on eight LGs of an integrated high-density intra-specific genetic linkage map of chickpea

| Linkage groups [LGs] | Genomic and genic SSR + SNP markers mapped | Map length covered [cM] | Inter-marker distance [cM] |
|---------------------|------------------------------------------|-------------------------|---------------------------|
|                     |                                          |                         | Minimum                   |
|                     |                                          |                         | Maximum                   |
|                     |                                          |                         | Average                   |
| LG1                 | 57 + 192 = 249                          | 210.80                  | 0.75                      |
| LG2                 | 59 + 192 = 251                          | 225.37                  | 0.85                      |
| LG3                 | 74 + 192 = 266                          | 261.13                  | 0.90                      |
| LG4                 | 90 + 192 = 282                          | 316.55                  | 0.91                      |
| LG5                 | 47 + 192 = 239                          | 262.20                  | 0.97                      |
| LG6                 | 45 + 192 = 237                          | 195.57                  | 0.68                      |
| LG7                 | 51 + 192 = 243                          | 213.99                  | 0.81                      |
| LG8                 | 42 + 192 = 234                          | 203.25                  | 0.83                      |
Figure 3 | Eight major genomic regions underlying 11 robust QTLs (PVE: 8.5–25.8%, LOD: 6.5–13.8) associated with three agronomic quantitative traits (PN, SN and SW) identified and mapped on four LGs (CaLG01-CaLG04) using a 190 F4 mapping population (ICC 4958 × ICC 12968) of chickpea. The genetic distance (cM) and identity of the marker loci integrated on the chromosomes are indicated on the left and right side of the LGs, respectively. Red, green and blue boxes indicate the QTLs regulating PN, SN and SW mapped on eight LGs, respectively. For clear visibility, the individual LG has been divided into four parts; [1], [2], [3] and [4] based on lower to higher genetic positions of mapped markers.
Figure 4 | Two major genomic regions underlying four robust QTLs (PVE: 8.8–14.7%, LOD: 7.3–9.6) associated with two agronomic quantitative traits (PN and SN) identified and mapped on four LGs (CaLG05–CaLG08) using a 190 F4 mapping population (ICC 4958 × ICC 12968) of chickpea. The genetic distance (cM) and identity of the marker loci integrated on the chromosomes are indicated on the left and right side of the LGs, respectively. Red and green boxes indicate the QTLs regulating PN and SN mapped on eight LGs, respectively. For clear visibility, the individual LG has been divided into four parts; [1], [2], [3] and [4] based on lower to higher genetic positions of mapped markers.
genomic regions with similar marker intervals of LGs (Fig. 3, 4). The remaining five QTLs associated only with single SW trait were mapped on five different genomic regions of LGs. The mapping and clustering of multiple QTLs controlling PN and SN particularly on a single major genomic region of eight LGs gave clues for pleiotropy and complex genetic inheritance patterns of target traits in chickpea. For PN and SN, five major genomic regions underlying traits identified in our study are novel and may show population-specific genomic distribution on eight LGs/chromosomes. These 12 novel and robust QTLs underlying seven major genomic regions covered with different informative genomic and gene-based SNP and SSR markers, once successfully validated in diverse genetic backgrounds of populations and/or fine mapped, can be utilized for marker-assisted genetic improvement of chickpea.

Integration of QTL mapping with comparative genome mapping and differential expression profiling to delineate candidate gene(s) at SW-influencing QTL interval. One thousand six hundred fifty-seven of 2001 SNP and SSR markers genetically mapped on eight LGs of an intra-specific genetic map were physically mapped on eight *C. arietinum* chromosomes with an average map density of 75.0 kb (varied from 41.9 kb in chromosome 7 to 106.8 kb in chromosome 3) (Supplementary Fig. S3). Maximum number of markers were physically mapped on *C. arietinum* chromosome 3 (219 markers, 13.2%) and least on chromosome 6 (201, 12.1%) (Supplementary Table S4). The marker–based comparative genomics is useful for evolutionary studies and for transferring information from model crop species to related orphan species. The integration of markers into the genetic linkage map of chickpea is expected to serve as a reference for comparative genomics in legumes as inferred from their synteny and conservation of gene order. The comparative mapping of 2001 SNP and SSR marker loci genetically and/or

Table 2 | Significant QTLs associated with pod and seed number/plant and seed weight identified and mapped on eight chickpea LGs/chromosomes using an intra-specific mapping population (ICC 4958 × ICC 12968)

| QTLs       | LGs/chromosomes | Marker intervals with genetic positions (cM) | Markers associated with QTLs | 2012 | 2013 |
|------------|-----------------|---------------------------------------------|-------------------------------|------|------|
| Caq’PN1.1 & Caq’SN1.1 | CaLG(Chr01) | Ca-IISNP18 (16.8) to Ca-IISNP20 (18.6) | Ca-IISNP18 | NS   | NS   | NS   | NS   | 4.6 | 12.7 | 8.9 |
| Caq’PN1.2 & Caq’SN1.2 | CaLG(Chr01) | Ca-IISNP32 (29.5) to Ca-IISNP35 (32.1) | Ca-IISNP34 | 5.1 | 7.8 | 4.5 | 8.5 | NS | NS |
| Caq’PN1.3 & Caq’SN1.3 | CaLG(Chr01) | Ca-IISNP116 (127.6) to Ca-IISNP119 (130.9) | Ca-IISNP116 | 8.5 | 12.4 | 6.3 | 7.8 | 14.7 | 5.4 |
| Caq’PN2.1 & Caq’SN2.2 | CaLG(Chr02) | Ca-IISNP330 (154.9) to Ca-IISNP332 (157.5) | Ca-IISNP331 | 10.5 | 10.8 | 11.4 | 9.8 | 12.4 | 8.9 |
| Caq’PN2.1 & Caq’SN3.1 | CaLG(Chr03) | Ca-IISNP398 (24.7) to Ca-IISNP401 (27.7) | Ca-IISNP399 | 6.4 | 9.4 | 10.9 | NS | NS | NS |
| Caq’PN4.1 & Caq’SN4.1 | CaLG(Chr04) | Ca-IISNP649 (12.7) to Ca-IISNP651 (123.1) | Ca-IISNP649 | 11.4 | 19.8 | 12.9 | 10.2 | 18.5 | 9.5 |
| Caq’PN5.1 & Caq’SN5.1 | CaLG(Chr05) | Ca-IISNP831 (89.1) to Ca-IISNP834 (92.5) | Ca-IISNP832 | 8.5 | 12.5 | 6.5 | 7.3 | 14.7 | 5.7 |
| Caq’PN6.1 & Caq’SN6.1 | CaLG(Chr06) | Ca-IISNP1104 (154.8) to Ca-IISNP1108 (157.6) | Ca-IISNP1106 | NS | NS | NS | 4.8 | 8.5 | 5.1 |
| Caq’PN7.1 & Caq’SN7.1 | CaLG(Chr07) | Ca-IISNP1332 (199.5) to Ca-IISNP1335 (202.2) | Ca-IISNP1335 | 5.7 | 6.8 | 10.2 | NS | NS | NS |
| *Caq’PN8.1 & Caq’SN8.1 | CaLG(Chr08) | Ca-IISNP1487 (152.9) to Ca-IISNP1489 (154.6) | Ca-IISNP1487 | 9.6 | 9.5 | 3.1 | 8.5 | 8.8 | 2.5 |

*Caq’PN1.1 (C. arietinum QTL for pod number on chromosome 1 number 1), Caq’SN1.1 (C. arietinum QTL for seed number on chromosome 1 number 2) and Caq’SW2.1 (C. arietinum QTL for 100-seed weight on chromosome 2 number 1), PVE: Percentage of phenotypic variation explained by QTLs, A: Additive effect; positive additive effect infers alleles from ICC 4958 with increasing trait values.
physically mapped (including 1657 markers) on eight LGs (chromosomes) of desi chickpea with their physical positions (bp) on the pseudomolecules of kabuli chickpea, M. truncatula, G. max, L. japonicus and C. cajan chromosomes revealed a significant conserved syntenic relationships among five legume genomes (Fig. 5). Maximum proportion of markers revealed a high-degree of homology with kabuli chickpea (98.9%), followed by M. truncatula (44.7%), G. max (43%), L. japonicus (10.3%) and minimum with C. cajan (9.6%) chromosomes (Supplementary Table S3–S9), which gave clues to their origin from a common ancestor. A high degree of marker-based conserved syntenic relationships and collinearity among eight chromosomes of desi and kabuli chickpea genomes was evident (Fig. 5). However, the desi chickpea chromosomes 1, 3, 4, 5 and 7 showed conserved collinear synteny with Medicago chromosomes 2, 7, 1, 3 and 4, respectively (Fig. 5). The integration of genetic/physical map with comparative genome maps identified many conserved collinear and duplicated chromosomal regions among desi and kabuli chickpea, Medicago, Glycine, Lotus and Cajanus.

The observed syntenic relationships among the chromosomes of five legume species are similar to the previous marker-based comparative genome mapping studies. Striking synteny between chickpea and Medicago chromosomes is expected keeping in view their evolutionary closeness as they belong to the same clade Galegoideae. As compared to Medicago, the chromosome of Glycine showed a lesser degree of synteny with chickpea, which reemphasizes their distant phylogenetic relationship as Glycine belongs to separate clade Phaseoloid. The lowest degree of marker-based synteny among chickpea, Lotus and Cajanus genomes is expected. The comparative genome maps constructed among the chromosomes of five legume crop species thus would guide cloning and mapping of trait-regulatory genes/QTLs in the draft genome sequenced chickpea using the positional information of candidate genes/QTLs from completely sequenced model legume species like Medicago and Glycine.

Considering the comparative genome mapping potential of SNP and SSR markers, one strong (PVE 25.8% with highest LOD 13.8) SW-associated robust QTL (Caq'SW1.1) region [Ca-II-SNP151 (169.1 cM) to Ca-II-SNP154 (171.5 cM)] genetically mapped on desi LG1 (Fig. 3, 4, Table 2), revealing conserved collinear syntenic relationships with Medicago chromosome 2 (Fig. 6), was selected to delineate candidate gene(s) regulating seed weight in chickpea. The integration of genetic linkage map information of markers flanking the Caq'SW1.1 QTL with that of physical map of desi chickpea genome defined a 126.8 kb genomic region (spanning 7550973–7677748 bp) harbouring such major QTL on chromosome 1 (Fig. 6A and B). This target 126.8 kb Caq'SW1.1 QTL interval in desi chromosome 1 corresponding to 13.9 Mb (spanning 23.8–37.7 Mb) and 11 Mb (15.6–26.6 Mb) conserved collinear genomic regions of kabuli chromosome 1 and Medicago chromosome 2, respectively (Fig. 6C and D) was structurally and functionally annotated. Five candidate protein-coding desi chickpea genes identified in the Caq'SW1.1 QTL region showed conserved collinear syntenic relationships with five and four gene orthologs annotated that from kabuli and Medicago genomes, respectively (Fig. 6C and D). The detailed SNP and SSR marker-based gene synteny in the Caq'SW1.1 QTL interval among desi and kabuli chickpea chromosomes 1 and Medicago chromosome 2 was performed to narrow-down the possible candidate gene(s) regulating seed weight in chickpea. One SNP (G/A) (Ca-II-SNP152) in the cis-acting dehydration-responsive element (DRE) (ACCGAC) binding site of upstream regulatory region of AP2-domain containing ERF (ethylene-responsive factor) transcription factor (TF) desi gene (Ca00596) (Fig. 7) showing tight linkage with SW-governing Caq'SW1.1 QTL (based on high-resolution QTL mapping, Table 2) and orthology with that of kabuli (Ca19297) and Medicago (MEDTR2G043020) ERF genes (known to regulate seed development and seed size/weight in crop plants, including dicots) was primarily selected (Fig. 6C and D) as potential candidate for seed weight regulation in chickpea. Interestingly, this identified SNP showing transition substitution of 'G' nucleotide in the cis-acting element (ACCGAC) of ERF TF gene of a high seed weight mapping parental genotype (ICC 4958 with SW: 35.4 g) by another nucleotide ‘A’ resulted in creation of the non-functional cis-element (ACCAAC) in the corresponding ERF gene of a low seed weight mapping parent (ICC 12968, 20.8 g). To understand the differential regulation pattern of upstream regulatory SNP-carrying ERF TF gene, the expression profiling of five selected desi chickpea genes (including ERF gene) annotated in the 126.8 kb major genomic region harboring robust Caq'SW1.1 QTL was performed. The RNA isolated from three different vegetative tissues (root, shoot and leaf) and two seed developmental stages (early cell division and late maturation phase occurring at 10–20 and 21–30 days after podding, respectively) of eight low [kabuli: ICC 12968 (SW: 20.8 g), desi: ICCX-810800 (11 g), desi: ICC 4926 (7.4 g) and desi: ICC 12654 (8.9 g)] and high [desi: ICC 4958 (SW: 35.4 g), kabuli: ICC 20268 (47 g), desi: ICC 7410 (32.5 g) and desi: ICC 6121 (30.7 g)] seed weight contrasting chickpea genotypes as well as parents of mapping population was amplified using the gene-based primers through semi-quantitative and quantitative RT-PCR assays (Supplementary Fig. S4). An ERF gene of these selected five genes in the Caq'SW1.1 QTL region showed seed-specific expression as well as pronounced up-regulated expression (~4-fold) in seed developmental stages as compared to vegetative tissues (root, shoot and leaf) of all eight low and high seed weight chickpea genotypes and mapping parents (Supplementary Fig. S4, Fig. 8). Notably, the ‘G’ allele-containing cis-acting element (ACCGAC) of ERF TF gene exhibited its pronounced up-regulated (~6.5 fold) pattern of expression specifically in seed developmental stages of three high seed weight desi and kabuli chickpea genotypes (ICC 4958, ICC 20268 and ICC 7410). In contrast the ‘A’ allele-carrying cis-element (ACCAAC) of ERF TF gene revealed its ~3-fold lower differential up-regulation in seed developmental stages of three low seed weight desi and kabuli chickpea genotypes (ICC 12968, ICC 4926 and ICC 12654) compared to that of high seed weight genotypes. However, no significant differential expression of the ‘G’ and ‘A’ SNP alleles-containing cis-acting elements of ERF genes in remaining two low (ICCX-810800) and high (ICC 6121) seed weight desi chickpea genotypes, respectively during seed development was observed. The seed-specific pronounced differential up-regulation of this ERF TF gene expression particularly in high seed weight contrasting chickpea genotypes than that of low seed weight genotypes during seed development further ascertained its potential as candidates controlling seed weight in chickpea.
Figure 5 | Comparative genome mapping of 2001 SNP and SSR markers genetically/physically mapped on eight desi chickpea LGs/chromosomes with their physical position on the pseudomolecules of kabuli chickpea (A), M. truncatula (B), G. max (C), L. japonicus (D) and C. cajan (E) chromosomes depicted conserved syntenic relationships among five legume genomes, which are depicted in the Circos circular ideogram. A high-degree of conserved collinear synteny among the chromosomes of desi and kabuli chickpea and Medicago genomes was evident. The outermost circles represent the LGs/chromosomes of five legume genomes coded with different colours. The syntenic relationships of each LGs/chromosomes between two legume species are marked individually with different coloured lines.
Figure 6 | Integration of genetic (A) and physical (B) map identified and mapped one robust SW-governing major *Caq*′SW1.1 QTL on 126.8 kb genomic region of desi chickpea chromosome 1. The marker-based comparative genome mapping revealed a high-degree of conserved collinear syntenic relationships of five candidate protein-coding desi genes annotated at this target genomic sequence interval with *kabuli* chickpea chromosome 1 (C) and *Medicago* chromosome 2 (D). A regulatory SNP (G/A) (Ca-II-SNP152) in a ERF TF gene showing strong linkage with *Caq*′SW1.1 QTL and conserved synteny with ERF orthologous genes annotated from *kabuli* chromosome 1 (C) and *Medicago* chromosome 2 (D), was selected as potential candidate for seed weight regulation in chickpea. The genetic (cM)/physical (bp) distance and identity of the markers mapped on the chromosomes are indicated on the left and right side of the chromosomes, respectively. Red and blue dotted lines represent the gene- and marker-based syntenic relationships, respectively among *desi* and *kabuli* chickpea and *Medicago* chromosomes.

Figure 7 | Structural annotation of one candidate SW-associated AP2-domain-containing ERF TF gene delineated at a major *Caq*′SW1.1 QTL interval by integrating QTL mapping with comparative genome mapping and differential expression profiling. Diverse coding (functional domain) and non-coding upstream (URR) and downstream (DRR) regulatory regions of gene are highlighted. One functionally relevant SNP (G/A) identified in the DRE cis-acting element (ACCGAC) of ERF gene possibly involved in transcriptional regulation of this gene for seed weight and development in chickpea is indicated. CDS: coding sequences.
positions of markers flanking/tightly linked to the QTLs) with one of our earlier mapped CaqSW1.1 QTL on the chromosome 1 of chickpea.

Collectively, the integration of QTL mapping with comparative genome mapping and expression profiling were able to delineate one regulatory SNP (G/A)-containing candidate ERF TF gene in a major SW-governing robust QTL (Caq’SW1.1) region for controlling seed weight in chickpea. Such integrated approach of high-resolution genetic/QTL mapping and marker-based comparative genome mapping (specifically between chickpea and Medicago) for narrowing down the QTL region into specific functionally relevant candidates have been recently implemented in chickpea for isolation/fine-mapping of a nodulation gene64. Three TF genes harboring a known major QTL (CaqSW1.1) regulating 100-seed weight mapped on chromosome 1 (on which Caq SW1.1 QTL identified in the present study) have been validated recently by integrating association analysis with QTL mapping, differential expression profiling and gene-based molecular haplotyping in chickpea65. The identified regulatory SNP-containing ERF gene harboring a major SW-regulating robust QTL has significance in controlling diverse transcriptional functions during seed development and determining the seed size/weight in crop plants, including legumes66–69. The SNP marker-based allelic variations in the upstream cis-acting element of ERF gene is significant for understanding the seed weight regulation in chickpea. The validation of this candidate TF gene delineated at trait-influencing QTL interval is required through fine mapping and map-based cloning for its subsequent use in marker-assisted genetic improvement of chickpea.
weight candidate gene in chickpea can be applied to diverse crop plants for narrowing-down the trait-specific QTL intervals and in rapid isolation/positional cloning of functionally relevant candidate genes regulating many useful agronomic traits for crop genetic enhancement.

In conclusion, high experimental validation, genotyping success rate (94–96%) and intra-specific polymorphic potential (82–96%) of 1536 SNP and 472 SSR markers showing in silico polymorphism between ICC 4958 (desi) and ICC 12968 (kabuli) in 190 advanced generation mapping population (ICC 4958 × ICC 12968) as well as 92 diverse desi and kabuli genotypes have suggested their immense use in large-scale genotyping applications of chickpea. An intra-specific 2001 marker-based genetic linkage map comprising of eight LGs constructed by us is highly saturated (mean map density: 0.94 cM) in contrast to previous documentation of intra-specific genetic maps in chickpea. Fifteen robust QTLs harbouring 10 major genomic regions associated with three agronomic traits, PN, SN and SW (PVE: 8.8–25.8% with LOD: 7.0–13.8) were identified and mapped on eight chickpea chromosomes. Positive additive effects of all these QTLs for high seed and pod number and seed weight were evident. An integrated approach of high-resolution QTL mapping, comprehensive marker-based comparative genome mapping and differential expression analysis have been utilized to delineate one of the strong SW-associated major genomic region (126.8 kb) underlying robust QTL (Çağ SW 1.1). This led to identify one potential SNP (G/A) in the cis-acting element region of a gene encoding an ethylene responsive factor, which presumably regulate seed weight in chickpea. The functionally relevant molecular tags (markers, intra-specific genetic linkage map, high-resolution PN, SN and SW QTLs, and gene-novel allele(s) regulating seed weight) identified have immense utility in diverse genomics-assisted breeding applications for chickpea genetic improvement.

Methods

Development of an intra-specific chickpea mapping population and their phenotyping. An intra-specific F₂ mapping population (consisting of 190 segregating individuals) derived from the bi-parental crosses between desi ICC 4958 [high pod (101.6 ± 2.2) and seed (137.2 ± 2.1) number/plant and high 100-seed weight (35.4 g ± 2.2)] and kabuli ICC 12968 [low pod (46.7 ± 2.3) and seed (54.2 ± 1.8) number/plant and low 100-seed weight (20.8 g ± 2.1)] chickpea genotypes was generated by single seed descent method. The mapping populations along with their parental genotypes were grown (planted in a single row with 35 × 10 cm spacing) in the experimental field according to randomized complete block design (RCBD) with at least two replications for two consecutive years (2012 and 2013) during crop season at New Delhi (latitude 28.6 N and longitude 77.2 E). The parental genotypes (ICC 4958 and ICC 12968) sown after every 10 rows of the RILs served as reference in experimental field according to randomized complete block design (RCBD) with at least two replications for two consecutive years (2012 and 2013) during crop season at New Delhi (latitude 28.6 N and longitude 77.2 E). The SNP and SSR markers were designated (LG1 to LG8) based on the corresponding marker physical positions (bp) at the centiMorgan (cM) genetic distances and an intra-specific genetic map was constructed using MapChart v2.2. The LGs with genetically mapped markers were designated (LGI to LG8) based on the corresponding marker physical positions (bp) on the chromosomes.

QTL mapping. For QTL mapping, the genotyping data of SSLN and SSR markers genetically mapped on eight LGs of chickpea and field phenotypic data (SN, PN and SW) of 190 mapping individuals and parental genotypes were correlated using single marker analysis, interval mapping and composite interval mapping functions of QTL Cartographer. The LOD threshold (1-LOD) was set at 4.0 at 1000 permutations was considered significant (p < 0.05) to identify and map the major QTLs on LGs governing PN, SN and SW traits in chickpea. The positional genetic effects and phenotypic variation explained (PVE) by QTLs were evaluated at significant LOD. The multiple-trait composite interval mapping (MTCIM) of QTL Cartographer was employed to detect pleiotropic QTLs. The additive effect of marker loci harboring the QTLS was determined using QTL Network v2.0. The confidence interval (CI) of each significant major QTL peaks was measured by using ±1-LOD support intervals (95% CI).

Comparative genome mapping. The genomic and transcript sequences flanking the SNP and SSR markers that were genetically mapped on the eight LGs of chickpea were BLAST searched (e ≤ 10^{-10}) against the pseudomolecules of desi chickpea (*Medicago truncatula*), *Glycine max*, *Lotus japonicus* and *Cajanus cajan* chromosomes. Reciprocal best hit method (RBH) of OrthoMCL was used to define orthologous relationships of marker sequences among five dicot genomes. The
marker-based syntenic relationships among chickpea and five other dicot genomes were visualized with visualization blocks using Circos 0.55.

Differential expression profiling. To determine the differential expression patterns of genes annotated at the SW-regulating major genomic region harboring a robust QTL, suitable primer pairs from these genes were designed for expression profiling. The gene-based primers along with internal control elongation factor 1-alpha (EF1α) were amplified using cDNA isolated from three different vegetative tissues (shoot, root and leaf) and two seed developmental stages [early cell division at 10–20 days after paddling (DAP) and late maturation phases 21–30 DAP as defined by Kujur et al.19] of eight low [kabuli: ICC 12968 (SW: 20.8 g), desi: ICCX-810800 (11 g), desi: ICC 4926 (7.4 g) and desi: ICC 12656 (9.6 g)] and high [desi: ICC 4958 (35.4 g), kabuli: ICC 2628 (47.5 g), desi: ICC 7410 (32.5 g) and desi: ICC 6212 (30.7 g)] seed weight contrasting chickpea genotypes as well as parents of mapping populations using semi-quantitative and quantitative RT-PCR assays. The expression level of genes was compared with each other and along with control (vegetative tissues of respective genotypes) following Kujur et al.19.

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**Author contributions**

D.B. conducted all experiments and drafted the manuscript. D.B., Y.K., S.D., S.B., T.S. and V.K. involved in genotyping, sequencing and data analysis. H.D.U., S.T., C.G., S.S. and Sh.S. helped in constitution of association panel and mapping population, and performed their phenotyping. S.K.P., D.C. and A.K.T. conceived and designed the study, guided data analysis and interpretation, participated in drafting and correcting the manuscript critically and gave the final approval of the version to be published. All authors have read and approved the final manuscript.

**Additional information**

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