Genetic dissection of seed-iron and zinc concentrations in chickpea

Hari D. Upadhyaya1,*, Deepak Bajaj2,*, Shouvik Das2,*, Vinod Kumar3, C. L. L. Gowda1, Shivali Sharma1, Akhilesh K. Tyagi2 & Swarup K. Parida2

The SNP-based high-resolution QTL mapping mapped eight major genomic regions harbouring robust QTLs governing seed-Fe and Zn concentrations (39.4% combined phenotypic variation explained/PVE) on six chromosomes of an intra-specific high-density genetic linkage map (1.56 cM map-density). 24620 SNPs discovered from genome-wide GBS (genotyping-by-sequencing) and 13 known cloned Fe and Zn contents-related chickpea gene-orthologs were genotyped in a structured population of 92 sequenced desi and kabuli accessions. The large-scale 16591 SNP genotyping- and phenotyping-based GWAS (genome-wide association study) identified 16 genomic locigenes associated (29% combined PVE) with seed-Fe and Zn concentrations. Of these, 11 trait-associated SNPs in the genes linked tightly with eight QTLs were validated by QTL mapping. The seed-specific expression, including pronounced differential-regulation of 16 trait-associated genes particularly in accessions/mapping individuals with contrasting level of seed-Fe and Zn contents was apparent. Collectively, the aforementioned rapid integrated genomic strategy led to delineate novel functional non-synonymous and regulatory SNP allelic-variants from 16 known/candidate genes, including three strong trait-associated genes (encoding late embryogenesis abundant and yellow stripe-like 1 protein, and vacuolar protein sorting-associated protein) and eight major QTLs regulating seed-Fe and Zn concentrations in chickpea. These essential inputs thus have potential to be deployed in marker-assisted genetic enhancement for developing nutritionally-rich iron/zinc-biofortified chickpea cultivars.

The global population strength is fast rising in an alarming rate and anticipated to cross the mark of nine billion by 20501–2. Ensuring food and nutritional security of this fast growing population will pose a huge challenge especially in the era of climatic variability and resource scarcity. Adequate intake of nutritious food enriched with essential micronutrients is prerequisite for humans to meet their metabolic need and maintain good health. However, over three billion people, including one third of the children in developing countries are suffering from micronutrient malnutrition or hidden hunger world-wide3–5. Henceforth, micronutrient malnutrition/hidden hunger is a major global health concern and require immediate attention5. Iron (Fe) and Zinc (Zn) are the major components of micronutrient and play a crucial role in growth and development of human by acting as co-factors for several proteins, including haemoglobin, cytochrome and transcription factors. The Fe and Zn deficiencies are reportedly the most widespread and commonly observed micronutrient deficiencies in human beings7–9. Fe and Zn deficiency leads to several physiological disorders, including anaemia, tissue hypoxia, impaired physical growth, hypogonadism, dwarfism, orificial and acral dermatitis. About half of the world population is at risk for inadequate intake of Fe and Zn in their diet10. Consequently, several strategies, including mineral supplementation, dietary diversification and food fortification have been implemented to mitigate the micronutrient deficiency/hidden hunger11. Nonetheless, these attempts achieved a limited success due to lack of proper cultural awareness and inappropriate socio-economic infrastructure11.

With the advancement of cutting edge next generation sequencing (NGS)- and array-based genotyping strategies, the genomics-assisted breeding, which primarily involves identification and introgression of useful genes/ QTLs (quantitative trait loci) regulating Fe and Zn contents, can be deployed as a powerful intervention tool for biofortification of food crops11,12. The essential inputs obtained from the above-mentioned strategy will be useful to increase the bioavailable micronutrients and thus enhance the quality component traits in food crops for nutritional security. However, the accumulation of mineral nutrients, including Fe and Zn in the edible parts

1International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502324, Telangana, India.
2National Institute of Plant Genome Research (NIPGR), Aruna Asaf Ali Marg, New Delhi 110067, India.
3National Research Centre on Plant Biotechnology (NRCPB), New Delhi 110012, India. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to S.K.P. (email: swarup@nipgr.ac.in or swarupdbt@gmail.com)
(mostly seeds/grains) of crops is tightly regulated through complex cellular and genetic mechanism. The proportionate quantity of Fe and Zn present in seeds is a complex quantitative trait and governed by many major genes/QTLs. Therefore, it is highly imperative to dissect the genetic basis of variability governing Fe and Zn concentrations in seeds of food crops. Tremendous efforts have been made to delve deeper into the complex genetic architecture of this trait in several crop species, including cereals and legumes. Among legumes with much emphasis on common bean, attempts have been made to decipher the underlying genetic factors regulating Fe and Zn concentrations in seeds of *Medicago* lentil, field pea and chickpea.

Chickpea is a self-pollinated, annual diploid and highly nutritious legume with a genome size of ~740 Mbp that is considered as a staple source of protein. This legume crop ranks second in consumption and third in production among pulses grown world-wide. The genomes of two chickpea cultivars (*de**esi* and *kabuli*) with contrasting agronomic traits representing diverse gene pools, have been sequenced. Chickpea seeds constitute several nutritional composition, including protein, carbohydrate and minerals like Fe and Zn. Considering the importance of this crop as a dietary element across a major part of global population, especially in developing countries, it is desirable to understand the complex genetic inheritance pattern and gene regulatory function of seed-Fe and Zn concentrations in chickpea leading to their bio-fortification through marker-assisted genetic enhancement. To date, only one 1536 SNP markers-based trait association mapping study has been reported to identify the major genomic loci/gene regulating seed-Fe and Zn concentrations in chickpea. Unfortunately, no informative markers tightly linked to the major QTLs/gene governing seed-Fe and Zn contents are available that could be deployed in marker-assisted genetic improvement of chickpea. An integrated genomic approach involving traditional QTL mapping, association analysis and differential gene expression profiling is currently the most efficient strategy for rapid dissection of complex yield and quality component quantitative traits in diverse crop plants, including chickpea.

In this aspect, the current study has made an effort to integrate QTL mapping with genome-wide association study (GWAS), candidate gene-based association mapping and expression profiling using large-scale natural germplasm lines and mapping populations to delineate functionally relevant molecular tags (markers, genes, QTLs and alleles) regulating seed-Fe and Zn concentrations, with an ultimate objective of genetic enhancement in chickpea.

**Results and Discussion**

Genome-wide distribution of SNPs. The *kabuli* reference genome (16376)- and *de novo* (8029)-based GBS (genotyping-by-sequencing) assays were employed to mine 24405 high-quality SNPs from 92 *de**esi* and *kabuli* chickpea accessions (Table S1). The GBS assay is advantageous in rapid large-scale mining and high-throughput genotyping of high-quality accurate genome-wide SNPs simultaneously for high-resolution genetic and association mapping. Henceforth, this NGS-based strategy has potential to identify genes/QTLs regulating important agronomic traits in chickpea. A diverse array of known cloned genes involved in homeostasis, including uptake, translocation, regulation, signaling, storage and compartmentalization of Fe and Zn-contents have been isolated and characterized in multiple crop plants, including dicots. Therefore, it would be interesting to assess the association potential of these known cloned genes for seed-Fe and Zn contents in chickpea. This can be simply achieved by large-scale genotyping of novel coding and regulatory SNP allelic variants mined from the seed-Fe and Zn contents-related known cloned genes in a diverse set of phenotypically well-characterized natural germplasm lines. With a prime objective to discover gene-based SNPs, the high-quality cloned amplicon sequences of 13 known Fe and Zn content-regulating gene orthologs of chickpea (involved in transport and signalling pathways of Fe and Zn contents in crop plants) were compared initially among 12 representative *de**esi* and *kabuli* accessions. The subsequent high-throughput genotyping of these mined SNPs in all 92 *de**esi* and *kabuli* accessions by Illumina GoldenGate assay identified 215 coding and regulatory SNPs in 13 genes (46421 bp) with a mean density of one SNP per 215.9 bp (Table 1). Altogether, *kabuli* reference genome- and *de novo*-based GBS (24405) assay as well as known gene-derived (215) SNP genotyping assay discovered 24620 SNPs (Fig. 1A). This includes 16591 reference genome-based SNPs, of which 14330 and 2280 SNPs were physically mapped across eight chromosomes (mean map density: 24.2 kb) and unanchored scaffolds of *kabuli* genome, respectively.

The structural annotation of 16591 SNPs, including 16376 *kabuli* reference genome- and 215 gene-based SNPs was performed. This revealed presence of 5720 and 10871 SNPs in the intergenic regions and 4656 genes, respectively (Fig. 1B). The annotation of SNPs within the genes exhibited the occurrence of a highest and lowest number of 6267 (6183 genome-wide and 84 gene-based SNPs) and 17 SNPs in the exons/CDS (coding sequence) and DRRs (downstream regulatory regions), respectively. The coding SNPs comprised of 4259 synonymous and 2008 non-synonymous (missense and nonsense) SNPs. The regulatory SNPs (143, including 12 genome-wide and 131 gene-based SNPs) mostly derived from the URRs (upstream regulatory regions) of *kabuli* chickpea genes (Fig. 1B). The GBS-based genome-wide and known gene-derived novel SNP allelic variants discovered among a diverse set of *de**esi* and *kabuli* chickpea accessions can be deployed for various high-throughput genetic analysis particularly in rapid identification of genes (alleles)/QTLs governing complex Fe and Zn-content traits in chickpea.

Construction of a high-density intra-specific chickpea genetic linkage map. For generating an intra-specific high-resolution genetic linkage map, 536 SNPs (529 genome-wide GBS- and 7 known gene-based SNPs) exhibiting polymorphism between parental accessions (*de**esi* accession ICC 4958 and *kabuli* accession ICC 8261) were genotyped in 277 individuals of a RIL (recombinant inbred line) mapping population (ICC 4958 x ICC 8261). The use of these SNP genotyping data in linkage analysis mapped 533 SNPs onto eight LGs (linkage groups) of an intra-specific genetic map of chickpea (Table S2, Fig. 2). The genetic map consisting of eight LGs covered a total map length of 831.2 cM with a mean inter-marker distance of 1.56 cM. The LG4 had longest map length spanning 168.5 cM, whereas LG8 exhibited shortest map length of 57.9 cM (Table S2, Fig. 2). A highest
Characterized known cloned genes

| Crop plants | Gene identities with functions | Gene accession IDs | Gene accession IDs | Chromosomes | Sequenced gene amplicon size (bp) | Coding and upstream regulatory SNPs mined |
|-------------|--------------------------------|-------------------|-------------------|-------------|-----------------------------------|------------------------------------------|
| Arabidopsis | AtNRAMP1 Natural resistance-associated macrophtage protein 1 | At1g08030 | Cal02923 | Ca_Kabuli_Chr01 | 3485 | 13 |
| Arabidopsis | AtZIP2 Zinc/iron permease Zinc/iron regulated transporter-related protein 2 | At5g59520 | Cal02630 | Ca_Kabuli_Chr01 | 3005 | 12 |
| Arabidopsis | AtFER3 Ferritin 3 | At3g56090 | Cal12453 | Ca_Kabuli_Chr02 | 2765 | 16 |
| Arabidopsis | AtFER1 Ferritin 1 | At5g01600 | Cal09416 | Ca_Kabuli_Chr03 | 2777 | 14 |
| Arabidopsis | AtYSL1 Yellow stripe-like 1 Oligopeptide transporter OPT superfamily | At4g24120 | Cal03842 | Ca_Kabuli_Chr04 | 3923 | 20 |
| Arabidopsis | AtIRT3 Iron-regulated transporter 3 | At1g60960 | Cal01633 | Ca_Kabuli_Chr05 | 3224 | 15 |
| Arabidopsis | AtYSL2/3 Yellow stripe-like 2/3 Oligopeptide transporter OPT superfamily | At5g24380 | Cal04020 | Ca_Kabuli_Chr06 | 3863 | 18 |
| Arabidopsis | AtHMA2/4 Heavy metal ATPases 2/4 ATPase, FAD-transporting proton pump | At4g30110 At2g19110 | Cal03227 | Ca_Kabuli_Chr07 | 5099 | 25 |
| Arabidopsis | AtZIP1 Zinc/iron permease Zinc/iron regulated transporter-related protein 1 | At3g12750 | Cal07626 | Ca_Kabuli_Chr08 | 3044 | 14 |
| Arabidopsis | AtYSL2/3 Yellow stripe-like 2/3 Oligopeptide transporter OPT superfamily | At5g24380 | Cal04020 | Ca_Kabuli_Chr06 | 3863 | 18 |
| Arabidopsis | AtHMA2/4 Heavy metal ATPases 2/4 ATPase, F-type, H+ transporting proton pump | At4g30110 At2g19110 | Cal03227 | Ca_Kabuli_Chr07 | 5099 | 25 |
| Arabidopsis | AtZIP1 Zinc/iron permease Zinc/iron regulated transporter-related protein 1 | At3g12750 | Cal07626 | Ca_Kabuli_Chr08 | 3044 | 14 |
| Arabidopsis | AtFRO2 Ferric chelate reductase 2 FAD-binding 8 | At1g01580 | Cal13723 | Ca_Kabuli_Chr07 | 4151 | 21 |
| Arabidopsis | AtNRAMP2 Natural resistance-associated macrophtage protein 3 | At2g23150 | Cal09322 | Ca_Kabuli_Chr07 | 3533 | 12 |
| Arabidopsis | AtFRO2 Ferric chelate reductase 2 FAD-binding 8 | At1g01580 | Cal13723 | Ca_Kabuli_Chr07 | 4151 | 21 |
| Arabidopsis | AtNRAMP4 Natural resistance-associated macrophtage protein 4 | At5g67330 | Cal02191 | Ca_Kabuli_Chr08 | 3521 | 16 |
| Arabidopsis | AtYSL4/6 Yellow stripe-like 4/6 Oligopeptide transporter OPT superfamily | At5g41000 At5g27020 | Cal27338 | Ca_Kabuli_Scaffold537 | 4031 | 19 |

Table 1. Thirteen chickpea gene orthologs known to regulate signalling and transport of Fe and Zn contents in crop plants selected for trait association mapping.

Number of SNPs were mapped on LG4 (124 markers), followed by LG3 (118) and lowest on LG8 (30). The most saturated genetic map was observed in LG4 (a mean inter-marker distance of 1.36 cM), while LG8 (1.93 cM) had the least saturated map (Table S2, Fig. 2). Overall, an intra-specific genetic linkage map constructed in our study had a comparable/higher mean map-density (an average inter-marker distance of 1.56 cM) than that recently documented in diverse microsatellite and SNP markers-anchored intra- and inter-specific genetic maps of chickpea35,36,38,39,41,49–55.

Molecular mapping of QTLs associated with seed-Fe and Zn concentrations. A highly significant difference (P < 0.001) of seed-Fe (51.3 ± 5.0, 40.3–67.0 ppm with 81% H2) and Zn (36.0 ± 3.8, 27.9–48.5 ppm with 83% H2) concentrations in two parental accessions and 277 individuals of a RIL mapping population [ICC 4958 (Fe: 43.0 ± 2.9 and Zn: 30.2 ± 2.5 ppm) × ICC 8261 (Fe: 64.8 ± 3.3 and Zn: 42.0 ± 2.7 ppm)] across two years based on ANOVA was observed (Table S3, Fig. 3A,B). Especially, ANOVA results inferred a highly significant environmental (years and geographical locations) effects on these traits. A significant interaction between genotypes (G)/accessions and environment (E) for seed-Fe and Zn concentrations traits was evident. However, G × E and E-variances (% of total mean squares) was found to be almost equal between seed-Fe and Zn concentrations at a significant level of P < 0.001. The normal frequency distribution, including bi-directional transgressive segregation of two target traits among mapping individuals and parental accessions was evident (Fig. 3A,B). The Pearson’s correlation coefficient estimation revealed a significant positive correlation (0.53–0.68 with mean r: 0.61, P < 0.0001) between seed-Fe and Zn contents among individuals of a mapping population.

The use of genotyping data of 533 SNPs mapped on an intra-specific genetic linkage map and phenotyping data of 277 RIL mapping individuals in QTL mapping identified eight major genomic regions underlying eight robust QTLs (6.0–8.8 LOD) significantly associated with seed-Fe and Zn concentrations in chickpea (Table 2, Fig. 2). These validated QTLs exhibited consistent phenotypic expression (>10% phenotypic variation explained) across environments (years and geographical locations) at a higher LOD (>6.0) and H2 (>90%) were considered as robust QTLs governing chickpea seed-Fe and Zn contents. Eight major genomic regions harbouring eight robust QTLs (CaqFe1.1, CaqZn2, CaqFe3.1, CaqFe4.1, CaqZn3.1, CaqFe4.1, CaqZn3.1, and CaqFe7.1) were mapped on six chromosomes (except chromosomes 6 and 8) (Table 2, Fig. 2). These QTL intervals spanning in the range from 2.2 cM on chromosome 2 to 4.8 cM on chromosome 1 covered with a total of 27 mapped SNP loci. The proportion of phenotypic variation explained (PVE) by individual QTL ranged from 16.9 to 23.6%. The combined PVE measured for all eight robust QTLs was 39.4%.

For seed-Fe concentration, three major genomic regions harbouring three robust QTLs (21.1–23.4% PVE with 8.0–8.8 LOD) were mapped on three chromosomes (1, 3 and 4) (Table 2, Fig. 2). The combined PVE of all three robust QTLs was 34.6%. These QTL regions covered (3.2 cM on chromosome 3 to 4.8 cM on chromosome 1) with 11 SNPs, were genetically mapped on chromosomes. The identified major QTLs exhibited positive additive gene effect for increasing seed-Fe concentration with higher effective allelic contribution from ICC 8261. For seed-Zn concentration, two major genomic regions underlying two robust QTLs were identified and mapped on two chromosomes (2 and 3) with 18.7–21.8% PVE (7.0–8.3 LOD) (Table 2, Fig. 2). These QTL intervals covered...
(2.2 cM on chromosome 2 to 3.3 cM on chromosome 3) with seven SNPs, were genetically mapped on chromosomes. The combined PVE for all two robust QTLs was 33.9%. These QTLs revealed positive additive gene effect for increasing seed-Zn concentration with major allelic contributions from ICC 8261. For both seed-Fe and Zn concentrations, three major genomic regions harbouring three robust QTLs were identified and mapped on three chromosomes (4, 5 and 7) with 16.9–18.5% PVE (6.0–6.7 LOD) (Table 2, Fig. 2). The combined PVE of all three robust QTLs was 36.4%. These QTL regions covered (2.9 cm on chromosome 5 to 4.1 cM on chromosome 7) by nine genetically mapped SNP loci, were exhibited positive additive gene effect for increasing both seed-Fe and Zn concentrations with maximum effective allelic contribution from ICC 8261. All 11 informative SNP loci tightly linked to the robust eight major QTLs governing seed-Fe and Zn concentrations are provided in the Table 2.

The 11 SNP loci linked to the robust eight major QTLs governing seed-Fe and Zn concentrations were compared/correlated with that of an only previous association mapping study on similar traits available till date in chickpea\(^1\). This revealed correspondence of two SNP loci (SNP53 and SNP317) in the genes (encoding elongation factor and I-Q domain like protein) linked tightly with two major QTLs (CaqFe1.1 and CaqFe4.1) between previous and present study. Molecular mapping of QTLs associated with any of such traits pertaining to seed-Fe and Zn concentrations has not been reported so far in chickpea. In this context, the informative

Figure 1. (A) Genomic distribution of 24620 SNPs discovered from the reference kabuli genome (eight chromosomes and scaffolds)- and de novo-based GBS (24405 SNPs) assays as well as by combining the Fe and Zn contents-related known cloned chickpea gene orthologs-led amplicon resequencing assay with a Illumina GoldenGate (215 SNPs) SNP genotyping assay. (B) Structural annotation of SNPs in the diverse non-coding (intron, URR and DRR) and coding (synonymous and non-synonymous) sequence components of genes and intergenic regions of kabuli genome. The CDS (coding sequences), URR (upstream regulatory region) and DRR (downstream regulatory region) of genes (mentioned in the Parentheses) were demarcated according to gene annotation information of reference kabuli genome (Varshney et al.\(^3\)). The digits mentioned within/above the bars indicate the structural annotation of genome-wide GBS and candidate gene-based SNPs.
SNPs especially derived from the major known/candidate genes identified and mapped at robust seed-Fe and Zn concentrations-associated QTL regions in our study seem quite relevant. The gene-based SNPs tightly linked with major QTLs identified, can be utilized in marker-assisted genetic improvement for developing cultivars with high level of Fe and Zn contents in the seeds of chickpea.

**Association mapping of seed-Fe and Zn concentrations.** The implication towards integrating GWAS with candidate gene-based association analysis for efficient quantitative dissection of diverse complex agronomic traits have been realized currently in chickpea\(^8,39,56,57\). Considering this, an integrated high-resolution association mapping strategy involving GWAS and candidate gene-based association analysis has been deployed in our study to narrow-down the potential genomic loci/genes governing quantitative traits of seed-Fe and Zn concentrations.
at a genome-wide scale in chickpea. For GWAS and candidate gene-based association mapping, the genotyping information of 16591 informative SNPs (differentiating the 92 desi and kabuli chickpea accessions) mined through genome-wide GBS (16376 SNPs)- and known/candidate gene (215 SNPs)-based SNP genotyping assays were utilized (Fig. 1A). The classification of 92 chickpea accessions into two distinct population groups (POP I and POP II) based on neighbour-joining phylogenetic tree, high-resolution population genetic structure and principal component analysis (PCA) using 16591 SNP genotyping data was apparent 37. The determination of LD pattern using the genotyping data of 14330 genome-wide SNPs (physically mapped on eight kabuli chromosomes) in 92 desi and kabuli chickpea accessions exhibited 200–250 kb LD decay across chromosomes. The normal frequency distribution along with a wider phenotypic variation for seed-Fe (63.3 ± 13.3, 40.2–91.0 ppm with 80% H2) and Zn (46.2 ± 9.1, 26.8–61.8 ppm with 82% H2) concentrations among 92 desi and kabuli chickpea accessions was observed (Table S3, Fig. 3C). A higher significant positive Pearson correlation (0.71–0.86 with mean r: 0.80, P < 0.0001) between these two target traits in 92 chickpea accessions was apparent (Fig. 3D). By comparing the multi-location field phenotyping information of seed-Fe and Zn concentrations observed in our constituted natural and mapping populations, a broader target trait variation in 92 natural germplasm lines than that of 277 RIL individuals of an intra-specific mapping population (ICC 4958 × ICC 8261) was observed. This infers that the natural germplasm lines representing 16 diverse geographical regions of the world selected by us are rich in allelic diversity for seed-Fe and Zn content traits. Therefore, the screened germplasm lines can serve as a useful genetic resource for mining novel functional allelic variants to accelerate trait association mapping at a genome-wide and/or gene-level in chickpea.

The CMLM and P3D/EMMAX model-based association mapping approach (FDR cut-off ≤ 0.05) identified 16 genomic loci (gene-based SNPs) exhibiting significant association (P ≤ 10^−7) with seed-Fe and Zn concentrations in chickpea (Table 3, Fig. 4). Twelve SNPs of these, were scanned from the genome-wide GBS information, whereas four SNPs derived from the known cloned kabuli chickpea genes regulating Fe and Zn contents. All 16 trait-associated SNPs were physically mapped on six chromosomes (except chromosomes 6 and 8) of kabuli genome (Table 3, Fig. 4). A highest number of five trait-associated genomic loci was represented from diverse coding (five synonymous and four non-synonymous SNPs) and non-coding (four intronic and three URRs) sequence components of 16 genes, respectively (Table 3, Fig. 4). The phenotypic variation for seed-Fe and Zn concentrations explained by 16 known/candidate gene-derived maximum effect genomic SNP loci varied from 6 to 14% (P: 1.2 × 10^−6 to 1.0 × 10^−8) among 92 chickpea accessions (Table 3, Fig. 4). The combined phenotypic variation for seed-Fe and Zn concentrations explained by all significant 16 genic SNP loci was 29% (Table 3, Fig. 4). Specifically, six gene-based SNP loci, including one of each non-synonymous and regulatory SNPs were significantly associated (6–12% R² with P: 3.2 × 10^−6 to 1.3 × 10^−9) with seed-Fe concentration in chickpea (Table 3, Fig. 4). For seed-Zn concentration, six, including two non-synonymous genic SNPs revealing significant association (7–14% R² with P: 1.0 × 10^−7 to

Figure 3. Frequency distribution of seed-Fe and Zn concentration traits variation estimated among 277 individuals and parental accessions of an intra-specific F7 RIL mapping population (ICC 4958 × ICC 8261) (A, B) as well as in a structured population of 92 desi and kabuli chickpea accessions (C) depicted a goodness of fit to the normal distribution. (D) Correlation based on Pearson's coefficient estimated between seed-Fe and Zn concentrations among 92 chickpea accessions.
QTLs | LGs/chromosomes | SNP marker intervals with genetic positions (cM) | Traits associated | SNP markers tightly linked with QTLs | 2012 | 2013 |
|---|---|---|---|---|---|
| CaqFe1.1 | Ca_Kabuli_Chr01 | SNP53(91.2)-SNP55(96.0) | Seed-Fe concentration | SNP53 and SNP55 | 8.6 | 23.6 | 6.5 | 8.5 | 22.6 | 6.8 |
| CaqZn2.1 | Ca_Kabuli_Chr02 | SNP109(82.8)-SNP111(85.0) | Seed-Zn concentration | SNP110 | 8.3 | 21.8 | 5.4 | 8.2 | 20.5 | 5.5 |
| CaqFe1.1 | Ca_Kabuli_Chr03 | SNP158(43.9)-SNP161(47.1) | Seed-Fe concentration | SNP159 | 8.5 | 20.8 | 4.8 | 8.0 | 21.1 | 4.3 |
| CaqZn3.1 | Ca_Kabuli_Chr03 | SNP206(112.4)-SNP209(115.7) | Seed-Zn concentration | SNP208 | 7.3 | 19.6 | 5.1 | 7.0 | 18.7 | 5.5 |
| CaqFZ4.1 | Ca_Kabuli_Chr04 | SNP299(87.5)-SNP302(91.4) | Seed-Fe and Zn concentration | SNP300 | 6.5 | 17.7 | 4.3 | 6.7 | 18.5 | 4.7 |
| CaqFZ7.1 | Ca_Kabuli_Chr07 | SNP470(25.5)-SNP472(29.6) | Seed-Fe and Zn concentration | SNP471 and SNP472 | 6.3 | 16.9 | 8.5 | 6.0 | 17.8 | 8.7 |

Table 2. Significant QTLs associated with seed-iron and zinc concentrations identified and mapped on the chromosomes of a high-density intra-specific chickpea genetic linkage map (ICC 4958 × ICC 8261).

Validation of trait-associated genes through expression profiling. A set of 16 trait-associated known/candidate genes with SNPs, including 11 validated by both QTL and association mapping were analysed to determine the differential expression profile and functional regulatory pattern of these genes governing seed-Fe and Zn concentrations in chickpea. The differential expression analysis (by quantitative RT-PCR assay) of these trait-associated genes in the leaves and seed maturation developmental stages of five chickpea accessions/parents and four homozygous mapping individuals with contrasting level of low (ICC 4958 and ICC 8933 with 42–43 ppm Fe and 27–30 ppm Zn) and high (ICC 8261, ICC 6013 and ICC 13077 with 65–90 ppm Fe and 42–62 ppm Zn) seed-Fe and Zn concentrations was performed. All 16 trait-associated known/candidate genes (11 genes validated by QTL mapping) exhibited seed-specific expression (>4-fold) as compared to leaf tissues of parental accessions and homozygous mapping individuals with low and high level of Fe and Zn concentrations (Fig. 5). Of these, twelve and four genes gave pronounced differential up (>7-fold, P ≤ 0.0001)- and down (~5-fold, P ≤ 0.0001)–regulation, respectively in the seeds of parental accessions/mapping individuals with high level of Fe and Zn concentrations as compared to that of parental accessions/individuals with low Fe and Zn contents (Fig. 5). Collectively, 16 differentially expressed potential genes with SNPs governing seed-Fe and Zn concentrations by integrating GWAS and candidate gene-based association analysis with expression profiling and 11 genes of these, by inclusion of QTL mapping with aforesaid strategy, were delineated in chickpea (Table 3).

The seed-specific expression, including a pronounced upregulation and enhanced transcript accumulation of three strong seed-Fe and Zn contents-associated known/candidate genes [encoding LEA, YSL1 and vacuolar protein sorting-associated proteins] have ascertained significant involvement of these genes in storing high level of Fe and Zn contents in the maturing seeds of multiple crop (legumes) accessions (46–48,58–60). These genes with useful properties of Fe/Zn-binding, are known to be involved in micronutrients trafficking, signalling and storage, especially overall homeostasis of Fe and Zn contents in seeds of crop plants. Recently, novel
Table 3. Sixteen genes with SNPs regulating seed-Fe and Zn concentrations delineated by integrating GWAS and candidate gene-based association analysis with QTL mapping and expression profiling.

*Validated by QTL mapping and †differential expression profiling.

| SNP IDs | Kabuli chromosomes | SNP physical positions (bp) | SNPs | Gene accession IDs | Structural annotation | Known/putative functions | Association analysis |
|---------|-------------------|-----------------------------|------|-------------------|------------------------|------------------------|---------------------|
| ^1SNP1426/SNP53 | Ca_Kabuli_chr01 | 16379258 | [C/T] | Ca06927 | Intron | Ubiquitin-associated/translation elongation factor EF1A | 1.5 × 10^-4 | 10 Seed Fe concentration |
| ^1SNP1628/SNP55 | Ca_Kabuli_chr01 | 24071014 | [T/G] | Ca19289 | Intron | AUX/IAA (auxin/multiple-3-acetic acid) protein | 1.9 × 10^-4 | 10 Seed Fe concentration |
| ^1SNP14941/SNP110 | Ca_Kabuli_chr02 | 96532 | [G/T] | Ca27126 | Intron | Vacular protein sorting-associated protein | 1.0 × 10^-4 | 14 Seed Zn concentration |
| ^1SNP159 | Ca_Kabuli_chr03 | 20101590 | [G/A] | Ca09416 | URR | Ferritin 1 (FER1) | 2.0 × 10^-4 | 10 Seed Fe concentration |
| ^1SNP4409/ SNP186 | Ca_Kabuli_chr03 | 30837284 | [C/T] | Ca12181 | CDS(Nsyn) | Zinc/iron permease (ZIP) 1.0 | 1.2 × 10^-7 | 6 Seed Fe and Zn concentrations |
| ^1SNP4476/SNP208 | Ca_Kabuli_chr03 | 31631451 | [A/C] | Ca12262 | CDS(Nsyn) | RNA recognition motif domain | 2.0 × 10^-4 | 12 Seed Zn concentration |
| ^1SNP4678/SNP216 | Ca_Kabuli_chr03 | 36050319 | [T/C] | Ca00903 | CDS(Syn) | SWAP (suppressor-of-white-apricot)/Surp domain-containing protein | 1.2 × 10^-7 | 9 Seed Zn concentration |
| ^1SNP4740/SNP229 | Ca_Kabuli_chr03 | 36387718 | [C/T] | Ca00947 | CDS(Nsyn) | WD40 [tryptophan-aspartic acid (W-D) dipeptide] repeat-containing protein | 1.0 × 10^-7 | 8 Seed Zn concentration |
| ^1SNP300 | Ca_Kabuli_chr04 | 4138693 | [C/T] | Ca03842 | URR | Yellow stripe-like 1 (YSL1) | 1.0 × 10^-4 | 12 Seed Fe and Zn concentrations |
| ^1SNP6693/SNP315 | Ca_Kabuli_chr04 | 30258596 | [G/C] | Ca14189 | CDS(Syn) | Late embryogenesis abundant (LEA) protein | 1.3 × 10^-4 | 12 Seed Fe concentration |
| ^1SNP7405/SNP317 | Ca_Kabuli_chr04 | 44814982 | [A/G] | Ca09416 | Intron | IQ-domain like protein | 2.5 × 10^-9 | 11 Seed Fe concentration |
| ^1SNP15585/SNP380 | Ca_Kabuli_chr05 | 43372 | [C/T] | Ca22196 | CDS(Syn) | Ubiquitin | 1.1 × 10^-5 | 8 Seed Zn concentration |
| ^1SNP15145/SNP396 | Ca_Kabuli_chr05 | 82342 | [C/T] | Ca24399 | CDS(Nsyn) | Unknown expressed gene | 1.3 × 10^-7 | 7 Seed Zn concentration |
| ^1SNP9048/SNP413 | Ca_Kabuli_chr05 | 4100035 | [C/T] | Ca07626 | CDS(Syn) | Zinc/iron permease (ZIP1) | 1.0 × 10^-8 | 11 Seed Fe and Zn concentrations |
| ^1SNP1677/SNP471 | Ca_Kabuli_chr07 | 781409 | [A/G] | Ca03400 | CDS(Nsyn) | Oticocaspases/Phox/Bem1p (PB1) domain-containing protein | 3.2 × 10^-2 | 6 Seed Fe concentration |
| ^1SNP472 | Ca_Kabuli_chr07 | 2515209 | [A/G] | Ca03227 | URR | Heavy metal ATPases 2/4 (HMA2/4) | 3.5 × 10^-7 | 10 Seed Fe and Zn concentrations |

Methods

Discovery and genotyping of SNPs. The structural/functional annotation, including genotyping information of kabuli reference genome- and de novo-based SNPs discovered from the sequenced 92 (39 desi and 53 kabuli) diverse chickpea accessions through a GBS-assay, were acquired.

For large-scale discovery and genotyping of gene-derived SNPs in chickpea, a selected set of 30 cloned genes known to be involved in regulation, transport and signalling pathways of Fe and Zn contents in crop plants, including dicots were obtained. To find out the best possible chickpea gene orthologs, the coding sequence of these known genes were BLAST homology functional allelic variants scanned from the selected candidate genes that encode elongation factor, ubiquitin family [SWAP (suppressor-of-white-apricot)/Surp domain-containing protein] and IQ-domain like protein as well as unknown expressed protein, are found to be associated with seed-Fe and Zn concentrations based on 1536 SNP markers-led trait association mapping in chickpea. A number of DUF 828 (domain of unknown function 828), WD40 [tryptophan-aspartic acid (W-D) dipeptide] repeat and RNA recognition motif domain-containing protein-coding genes belonging to Zn-finger family identified in our study are known to reflect molecular response towards Zn homeostasis in plant species. The essential role of a diverse array of vacuolar protein sorting genes, including HMA2/4 (heavy metal ATPases 2/4) (ATPase/P-type/H^+ transporting proton pump) in regulation of seed Fe and Zn-concentrations suggests the critical involvement of these genes in translocation, remodeling and storage of Fe and Zn-contents in crop plants. These vacuolar protein-encoding genes thus can be selected as potential candidates in deciphering the functional regulatory pathways underlying Fe and Zn content-accumulation in seeds of chickpea. Additionally, an YSL gene representing oligopeptide transporter OPT superfamily and ferritin (FER) gene regulating Fe translocation and storage, respectively have been isolated and characterized in higher plants. A zinc/iron permease (ZIP1) gene encodes zinc/iron regulated transporter-related protein belonging to a novel metal transporter ZIP gene family, is known to regulate transport of a wide-range of cations, including Fe and Zn for retaining their cellular homeostasis in plants.

The aforementioned previous reports altogether suggests that the novel functional SNP allelic variants in the known/candidate genes and high-resolution major QTLs regulating seed-Fe and Zn concentrations delineated in our study, by deploying a combinatorial genomics-assisted breeding approach (QTL mapping, GWAS, candidate gene-based association analysis and differential expression profiling) seems much relevant towards rapid quantitative dissection of complex seed quality component target traits in chickpea. These informative tags thus have potential to expedite marker-assisted genetic enhancement for developing iron/zinc-biofortified cultivars of chickpea.
Figure 4. (A) GWAS-based Manhattan plot exhibiting significant P-values (measured by CMLM and P3D/EMMAX model) associated with seed-Fe and Zn concentrations using 16591 genome-wide GBS- and candidate gene-based SNPs in chickpea. The x-axis illustrates the relative density of reference genome-based SNPs physically mapped on eight chromosomes and scaffolds of kabuli genome. The y-axis displays the -log_{10}(P)-value for significant association of 16 SNP loci with seed-Fe and Zn concentrations. The SNPs revealing significant association with seed-Fe and Zn concentrations at cut-off P value \( \leq 10^{-8} \) are marked with dotted lines. (B) Quantile-quantile plot illustrating the comparison between expected and observed -log_{10}(P)-values with a FDR cut-off < 0.05 to detect significant genomic loci (genes) associated with seed-Fe and Zn concentrations in chickpea.

Figure 5. Hierarchical cluster display depicting the differential expression profiles of 16 seed-Fe and Zn concentrations-associated genes (including 11 validated by QTL mapping) with SNPs in the leaves and seed maturation developmental stages of five chickpea accessions/parents and four representative homozygous mapping individuals (ICC 4958 × ICC 8261) with contrasting level of low (ICC 4958 and ICC 8933) and high (ICC 8261, ICC 6013 and ICC 13077) seed-Fe and Zn concentrations. The average log signal expression value of genes in leaves and seed developmental stages are represented at the top with a colour scale; in which green, black and red color denote low, medium and high level of expression, respectively. The genes showing seed-specific expression, including pronounced differential down-regulation are highlighted with black boxes, respectively. The structural and functional annotation of 16 genes with SNPs are mentioned in the Table 3. The tissues/stages and genes utilized for expression profiling are provided on the right and upper side of expression map, respectively. Digits mentioned in the vertical and horizontal bars illustrate the range (minimum, optimum and maximum) of correlation coefficient varying among tissues/stages of diverse contrasting accessions/mapping individuals and across trait-associated genes, respectively. LFeH: low seed-Fe concentration homozygous mapping individual and LZnH: low seed-Zn concentration homozygous mapping individual. HFeH: high seed-Fe concentration homozygous mapping individual and HZnH: high seed-Zn concentration homozygous mapping individual.
were digested initially at 120 °C for one h, followed by digestion at 230 °C for about 2 h to get clear and colourless
in the ratio of 10:0.5:2 (v/v)] and the contents left overnight for cold digestion in a digestion chamber. The samples
et al. generated among accessions was utilized for trait association mapping (following Bajaj
of 92 chickpea accessions using Illumina GoldenGate assay. Subsequently, the genotyping information of SNPs
USA). The high-quality SNPs detected from the gene sequences were further genotyped in the genomic DNA
(12 chickpea accessions) (G), environments (experimental years and/or geographical locations) (E) and G
among 92 chickpea accessions were obtained from the previous study37. The population structure ancestry coef-
PCA and population structure) and genome-wide/population-specific LD patterns (LD estimates and LD decay)
75 ml, and shaken well. Aliquots were taken from the digests and analyzed for Fe and Zn concentrations (mg kg
Further, the seed samples were washed with distilled water and oven-dried at 60 °C for 48 h before grinding.
seed samples kept overnight in an oven at 60 °C. The standards and samples were digested simultaneously
Fe and Zn concentrations was genotyped in 277 RIL mapping individuals and
sequenced twice in
41). The concentrations of Fe and Zn were esti-
expressed as mg kg⁻¹ (ppm) seed. The seed-Fe and Zn contents of accessions were measured on a plot basis and their mean values were used for statistical analysis.
were then powdered in a mill with Teflon chambers and
Fe and Zn concentrations (mg kg⁻¹) calculated with appropriate blanks in duplicate (two independent analyses). One g of ground sample was transferred to a
digestion tube [75 ml capacity and 10 ml of tri-acid mixture consisting of nitric acid, sulfuric and perchloric acid in the ratio of 10:0.5:2 (v/v)] and the contents left overnight for cold digestion in a digestion chamber. The samples were
digested initially at 120 °C for one h, followed by digestion at 230 °C for about 2 h to get clear and colourless
digests. Following cooling of the digests, the contents were dissolved in distilled water and volume made up to 75 ml and shaken well. Aliquots were taken from the digests and analyzed for Fe and Zn concentrations (mg kg⁻¹)
by atomic absorption spectrophotometry (Varian Spectra AA 2063). The concentrations of Fe and Zn were esti-
son of observed and expected -log10(P)-value relative distribution for each trait-associated genomic loci based on
searched (cut-off E-value ≤ 1e–41 and bit score ≥ 500) against the CDS of kabuli genes. The CDS and 2000-bp
URRs of these identified chickpea gene orthologs were retrieved. These coding and regulatory sequences were
used to design multiple overlapping forward and reverse primers with expected amplification product size of 400
to 500bp per primer. The gene-based primers were amplified using the genomic DNA of 12 chickpea accessions
(0.05, Benjamini and Hochberg 1995) were performed following Kujur
parameters previously determined 64)/EMMAX (efficient mixed model association expedited 65) approaches of
mixed model (P
+ 10⁻⁴) and highest R² (degree of SNP marker-trait association) were iden-
tified in chickpea.
Phenotyping for seed-Fe and Zn contents. During normal crop season, 92 desi and kabuli chickpea
accessions, and 277 individuals of an intra-specific F1 RIL mapping population (desi traditional cultivar/landrace
ICC 4958 x kabuli landrace ICC 8261) were grown in the field (following randomised complete block design with at least two replications) for two consecutive years (2012 and 2013) at two diverse geographical locations (New Delhi: 28° 4'N/77° 2'E and Patancheru, Hyderabad: latitude 17° 3'N/longitude 77° 2'E) of India. The mature seeds of each accession and mapping individual (2-4 representative plants from each accession/individual) were col-
lected separately during harvesting stage. The healthy mature seeds (20 g) of chickpea accessions were analyzed at
Central Analytical Services Laboratory, ICRISAT, Patancheru to measure their Fe and Zn contents. Precautions were
avoided the contamination of seeds with dust and metal particles during harvest and while preparing them for analysis.
QTL mapping. The multi-location/years replicated field phenotyping data for seed-Fe and Zn contents
(ppm) of parental accessions and 277 individuals derived from a RIL mapping population (ICC 4958 x ICC
8261) was obtained. A selected set of parental polymorphic GBS-based (obtained from Kujur et al.37) and known
gene-derived SNPs physically mapped on eight chromosomes was genotyped in 277 RIL mapping individuals and
parental accessions using an Illumina GoldenGate SNP genotyping assay (as per Bajaj et al.41). The SNP geno-
typing data of a RIL mapping population were utilized to construct an intra-specific high-density genetic linkage
map. Subsequently, this was correlated with the aforementioned phenotyping data of RIL mapping individuals
and parents for QTL mapping [composite interval mapping at significant LOD (logarithm of odds) threshold
>4.0 at a p < 0.05] as per Bajaj et al.41 and Saxena et al.42. Accordingly, identification and molecular mapping of
the major genomic regions underlying robust QTLs associated with seed-Fe and Zn concentrations were per-
formed in chickpea.
Association mapping. For GWAS, the information regarding the genetic diversity (phylogenetic tree,
PCA and population structure) and genome-wide/population-specific LD patterns (LD estimates and LD decay)
among 92 chickpea accessions were obtained from the previous study37. The population structure ancestry coeffi-
cient (Q), kinship matrix (K) and PCA (P) data as well as the genome-wide and candidate gene-based SNP geno-
typing and phenotyping information (seed-Fe and Zn concentrations) of 92 accessions were analysed through
mixed model (P + K, K and Q + K)-based CMLM (compressed mixed linear model) and P3D (population
parameters previously determined44) approaches of GAPIT (following48,66). To ascertain the accuracy and robustness of SNP marker-trait association, the compari-
sion of observed and expected -log10(P)-value relative distribution for each trait-associated genomic loci based on
quantile-quantile plot and correction of their adjusted P-value threshold of significance for multiple comparison
by false discovery rate (FDR cut-off < 0.05, Benjamini and Hochberg 1995) were performed following Kujur
et al.38 and Kumar et al.66. The degree of association of SNP loci with seed-Fe and Zn concentration traits was estimated
by the R² using a model with the SNP and adjusted P-value following a FDR-controlling method. The SNP loci
in the genomic (gene) regions revealing significant association with seed-Fe and Zn concentrations at the lowest
FDR adjusted P-values (threshold P < 10⁻⁴) were searched (cut-off E-value ≤ 1e–41 and bit score ≥ 500) against the CDS of kabuli genes. The CDS and 2000-bp
URRs of these identified chickpea gene orthologs were retrieved. These coding and regulatory sequences were
used to design multiple overlapping forward and reverse primers with expected amplification product size of 400
to 500bp per primer. The gene-based primers were amplified using the genomic DNA of 12 chickpea accessions
(0.05, Benjamini and Hochberg 1995) were performed following Kujur
parameters previously determined 64)/EMMAX (efficient mixed model association expedited 65) approaches of
mixed model (P
+ 10⁻⁴) and highest R² (degree of SNP marker-trait association) were iden-
tified in chickpea.
Expression profiling. To determine the regulatory pattern of candidate genes associated (validated by QTL mapping and association analysis) with seed-Fe and Zn concentrations, the differential expression profiling of these genes was performed using the quantitative RT-PCR assay. The leaves and late seed maturation developmental stages [21–30 days after podding (DAP)] of five chickpea accessions as well as parents and four homoygous individuals of a RIL mapping population (ICC 4958 × ICC 8261) with contrasting level of low and high seed-Fe and Zn contents (ppm), were used for RNA isolation. In the RT-PCR assay, RNA isolated from three independent biological replicates of each sample and two technical replicates of each biological replicate with no template and primer as control were utilized. In addition, an internal control gene elongation factor 1-alpha (EFlα) for normalization of expression value and gene-specific primers for determining the differential gene expression profile across tissues of accessions and mapping parents/individuals were used (as per Bajaj et al.67). The significant difference of gene expression among accessions and mapping parents/individuals was determined by constructing a heat map through TIGR MultiExperiment Viewer (MeV, http://www.tm4.org/mev).

References

1. Godfray, H. C. J. Food security: the challenge of feeding 9 billion people. Science 327, 812–818 (2010).
2. Bohra, A. et al. Genetics- and genomics- based interventions for nutritional enhancement of grain legume crops: status and outlook. J. Appl. Genet. 56, 151–161 (2015).
3. Welch, R. M. Breeding strategies for biofortified staple plant foods to reduce micronutrient malnutrition globally. J. Nutr. 132, 4955–4995 (2002).
4. Welch, R. M. & Graham, R. D. Breeding for micronutrients in staple food crops from a human nutrition perspective. J. Exp. Bot. 55, 353–364 (2004).
5. Thavarajah, P. Evaluation of chickpea (Cicer arietinum L.) micronutrient composition: Biofortification opportunities to combat global micronutrient malnutrition. Food Res. Int. 49, 99–104 (2012).
6. Fletcher, R. J., Bell, L. P. & Lambert, J. P. Public health aspects of food fortification: a question of balance. Proc. Nutr. Soc. 63, 605–614 (2004).
7. Welch, R. M. & Graham, R. D. A new paradigm for world agriculture: meeting human needs: productive, sustainable, nutritious. Field Crops Res. 60, 1–10 (1999).
8. Cichy, K. A., Caldas, G. V., Snapp, S. S. & Blair, M. W. QTL analysis of seed iron, zinc, and phosphorus levels in an Andean bean population. Crop Sci. 49, 1742–1750 (2009).
9. Blair, M. W. et al. QTL for seed iron and zinc concentration and content in a Mesoamerican common bean (Phaseolus vulgaris L.) population. Theor. Appl. Genet. 121, 1059–1070 (2010).
10. Brown, K. E., Peerson, J. M., Rivera, J. & Allen, L. H. Effect of supplemental zinc on the growth and serum zinc concentrations of prepubertal children: a meta-analysis of randomized controlled trials. Am. J. Clin. Nutr. 75, 1062–1071 (2002).
11. White, P. J. & Broadley, M. R. Biofortifying crops with essential mineral elements. Trends Plant Sci. 10, 586–593 (2005).
12. Mayer, J. E., Pfeiffer, W. H. & Beyer, P. Biofortified crops to alleviate micronutrient malnutrition. Curr. Opin. Plant Biol. 11, 166–170 (2008).
13. Blair, M. W., Astudillo, C., Grusak, M. A., Graham, R. & Beebe, S. E. Inheritance of seed iron and zinc concentrations in common bean (Phaseolus vulgaris L.) Mol. Breed. 23, 197–209 (2007).
14. Diapari, M. et al. Genetic diversity and association mapping of iron and zinc concentrations in chickpea (Cicer arietinum L.). Genome 57, 459–468 (2014).
15. Uauy, C., Distelfeld, A., Fahima, T., Blechl, A. & Dubcovsky, J. A. NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314, 1298–1301 (2006).
16. Lu, K. et al. Quantitative trait loci controlling Cu, Ca, Zn, Mn and Fe content in rice grains. J. Genet. 87, 305–310 (2008).
17. Tiwari, V. K. et al. Mapping of quantitative trait loci for grain iron and zinc concentration in diploid A genome wheat. J. Hered. 100, 771–776 (2009).
18. Anuradha, K. et al. Mapping QTLs and candidate genes for iron and zinc concentrations in unpolished rice of Madhukar × Swarna RILs. Gene 508, 233–240 (2012).
19. Jin, Y. et al. The genetic architecture of iron and zinc content in maize grains as revealed by QTL mapping and meta-analysis. Breed. Sci. 63, 317–324 (2013).
20. Sankaran, R. P., Huguet, T. & Grusak, M. A. Identification of QTL affecting seed mineral concentrations and content in the model legume Medicago truncatula. Theor. Appl. Genet. 119, 241–253 (2009).
21. Thavarajah, P., Thavarajah, D. & Vandenberg, A. Low phytic acid lentils (Lens culinaris L.): a potential solution for increased micronutrient bioavailability. J. Agric. Food Chem. 57, 9044–9049 (2009).
22. Blair, M. W. et al. Variation and inheritance of iron reductase activity in the roots of common bean (Phaseolus vulgaris L.) and association with seed iron accumulation QTL. BMC Plant Biol. 10, 215 (2010).
23. Blair, M. W., Astudillo, C., Rengifo, J., Beebe, S. E. & Graham, R. QTL analyses for seed iron and zinc concentrations in an intragenepool population of Andean common beans (Phaseolus vulgaris L.) Theor. Appl. Genet. 122, 511–521 (2011).
24. Shumugam, A. S. K. et al. Mapping seed phytic acid concentration and iron bioavailability in a pea recombinant inbred line population. Crop Sci. 55, 828–836 (2014).
25. Kumar, A., Choudhary, A. K., Solanki, R. K. & Pratap, A. Towards marker-assisted selection in pulses: a review. Plant Breed. 130, 297–313 (2011).
26. Varshney, R. K. et al. Achievements and prospects of genomics-assisted breeding in three legume crops of the semi-arid tropics. Biotechnol. Adv. 31, 1120–1134 (2013).
27. Jain, M. et al. A draft genome sequence of the pulse crop chickpea (Cicer arietinum L.). Plant J. 74, 715–729 (2013).
28. Varshney, R. K. et al. Draft genome sequence of chickpea (Cicer arietinum) provides a resource for trait improvement. Nat. Biotechnol. 31, 240–246 (2013).
29. Parween, S. et al. An advanced draft genome assembly of a desi type chickpea (Cicer arietinum L.). Sci. Rep. 5, 12806 (2015).
30. Fukami, A. K., Gaur, P. M., Gowda, C. L. L. & Chibbar, R. N. Nutritional quality and health benefits of chickpea (Cicer arietinum L.) a review. Brit. J. Nutr. 108, S11–S26 (2012).
31. Konishi, S. et al. SNP caused loss of seed shattering during rice domestication. Science 312, 1392–1396 (2006).
32. Tian, Z. et al. Allelic diversities in rice starch biosynthesis lead to a diverse array of rice eating and cooking qualities. Proc. Natl. Acad. Sci. USA 106, 21760–21765 (2009).
33. Kharabian-Masouleh, A., Waters, D. L. E., Reinke, R. F., Ward, R. & Henry, R. J. SNP in starch biosynthesis genes associated with nutritional and functional properties of rice. Sci. Rep. 2, 557 (2012).
34. Parida, S. K., Mukerji, M., Singh, A. K., Singh, N. K. & Mohapatra, T. SNPs in stress-responsive rice genes: validation, genotyping, functional relevance and population structure. BMC Genomics 13, 426 (2012).
35. Kujur, A. et al. Functionally relevant microsatellite markers from chickpea transcription factor genes for efficient genotyping applications and trait association mapping. DNA Res. 20, 355–374 (2013).
36. Kujur, A. et al. An efficient and cost-effective approach for genic microsatellite marker-based large-scale trait association mapping: identification of candidate genes for seed weight in chickpea. Mol. Breed. 34, 241–265 (2014).
37. Kujur, A. et al. Employing genome-wide SNP discovery and genotyping strategy to extrapolate the natural allelic diversity and domestication patterns in chickpea. Front. Plant Sci. 6, 162 (2015).
38. Kujur, A. et al. A genome-wide SNP scan accelerates trait-regulatory genomic loci identification in chickpea. Sci. Rep. 5, 11166 (2015).
39. Kujur, A. et al. Ultra-high density intra-specific genetic linkage maps accelerate identification of functionally relevant molecular tags governing important agronomic traits in chickpea. Sci. Rep. 5, 9468 (2015).
40. Negrao, S. New allelic variants found in key rice salt-14 tolerance genes: a association study. Plant Biotechnol. J. 11, 87–100 (2013).
41. Bajaj, D. et al. combinatorial approach of comprehensive genome mapping and transcript profiling identified a seed weight-regulating candidate gene in chickpea. Sci. Rep. 5, 9264 (2015).
42. Saxena, M. S. et al. An Integrated genomic approach for rapid delineation of candidate genes regulating agro-morphological traits in chickpea. DNA Res. 21, 695–710 (2014).
43. Zuo, J. & Li, J. Molecular genetic dissection of quantitative trait loci regulating rice grain size. Annu. Rev. Genet. 48, 99–118 (2014).
44. Deokar, A. A. et al. Genome wide SNP identification in chickpea for use in development of a high density genetic map and improvement of chickpea reference genome assembly. BMC Genomics 15, 708 (2014).
45. Jaganathan, D. et al. Genotyping-by-sequencing based intra-specific genetic map refines a “QTL-hotspot” region for drought tolerance in chickpea. Mol. Genet. Genomics 290, 559–571 (2015).
46. Curie, C. & Briat, J. F. Iron transport and signaling in plants. Annu. Rev. Plant Biol. 64, 183–206 (2003).
47. Kobayashi, T. & Nishizawa, N. K. Iron uptake, translocation, and regulation in higher plants. Annu. Rev. Plant Biol. 63, 131–152 (2012).
48. Bousain, N. et al. Phosphate and zinc transport and signalling in plants: toward a better understanding of their homeostasis interaction. J. Exp. Bot. 65, 5725–5741 (2014).
49. Nayak, S. N. et al. Integration of novel SSR and gene-based SNP marker loci in the chickpea genetic map and establishment of new anchor points with Medicago truncatula genome. Theor. Appl. Genet. 120, 1445–1451 (2010).
50. Gajaria, N. et al. Development and use of gene molecular markers (GMMs) for construction of a transcript map of chickpea (Cicer arietinum L.). Theor. Appl. Genet. 122, 1577–1589 (2011).
51. Hiremath, P. I. et al. Large-scale transcriptome analysis in chickpea (Cicer arietinum L.), an orphan legume crop of the semi-arid tropics of Asia and Africa. Plant Biotechnol. J. 9, 922–931 (2011).
52. Thudi, M. et al. Novel SSR markers from BAC-end sequences, DARt arrays and a comprehensive genetic map with 1,291 marker loci for chickpea (Cicer arietinum L.). PLoS One 6, e22725 (2011).
53. Gaur, R. et al. High-throughput SNP discovery and genotyping for constructing a saturated linkage map of chickpea (Cicer arietinum L.). DNA Res. 19, 357–373 (2012).
54. Sabbavarapu, M. M. et al. Molecular mapping of QTLs for resistance to Fusarium wilt (race 1) and Ascochyta blight in chickpea (Cicer arietinum L.). Euphytica 193, 121–133 (2013).
55. Varshney, R. K., Thudi, M. & Nayak, S. N. Genetic dissection of drought tolerance in chickpea (Cicer arietinum L.). Theor. Appl. Genet. 127, 445–462 (2014).
56. Neale, D. B. & Savolainen, O. Association genetics of complex traits in consifers. Trends Plant Sci. 9, 325–330 (2004).
57. Varshney, R. K. et al. Genome wide association analyses for drought tolerance related traits in barley (Hordeum vulgare L.). Field Crops Res. 126, 171–180 (2012).
58. Kim, S. A. & Guerinot, M. L. Mining iron: iron uptake and transport in plants. FEBS Letters 581, 2273–2280 (2007).
59. Liu, G., Xu, H., Zhang, L. & Zheng, Y. Fe binding properties of two soybean (Glycine max L.) LEA4 proteins associated with antioxidant activity. Plant Cell Physiol. 52, 994–1002 (2011).
60. Singh, S. et al. Comparative transcriptional profiling of two wheat genotypes, with contrasting levels of minerals in grains, shows expression differences during grain filling. PLoS One 9, e117118 (2014).
61. Santi, S. & Schmidt, W. Laser microdissection-assisted analysis of the functional fate of iron deficiency induced root hairs in cucumber. J. Exp. Bot. 59, 697–704 (2008).
62. Guerinot, M. L. The ZIP family of metal transporters. Biochim. Biophys. Acta 1465, 190–198 (2000).
63. Sahrawat, K. L., Ravi Kumar, G. & Rao, J. K. Evaluation of triacid and dry ashing procedures for determining potassium, calcium, magnesium, iron, zinc, manganese and copper in plant materials. Commun. Soil Sci. Plant Anal. 33, 95–102 (2002).
64. Kumar, V. et al. Genome-wide association mapping of salinity tolerance in rice (Oryza sativa). DNA Res. 22, 133–145 (2015).
65. Zhang, Z. et al. Mixed linear model approach adapted for genome-wide association studies. Nat. Genet. 42, 355–360 (2010).
66. Kang, H. M. et al. Variance component model to account for sample structure in genome-wide association studies. Nat. Genet. 42, 348–354 (2010).
67. Bajaj, D. et al. Genome-wide high-throughput SNP discovery and genotyping for understanding natural (functional) allelic diversity and domestication patterns in wild chickpea. Sci. Rep. 5, 11627 (2015).

Acknowledgements
The authors gratefully acknowledge the financial support for this study provided by a research grant from the Department of Biotechnology (DBT), Government of India (102/IFD/SAN/2161/2013-14). Shouvik Das acknowledges the DBT for Senior Research Fellowship award.

Author Contributions
H.D.U., D.B. and S.D. conducted experiments and drafted the manuscript. D.B., S.D. and V.K. involved in data analysis. H.D.U., C.L.L.G. and S.S. helped in constitution of association panel and performed phenotyping. S.K.P. 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
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Upadhyaya, H. D. et al. Genetic dissection of seed-iron and zinc concentrations in chickpea. Sci. Rep. 6, 24050; doi: 10.1038/srep24050 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/