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Identification of quantitative trait loci associated with iron deficiency chlorosis resistance in groundnut (*Arachis hypogaea*)

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Abstract

Iron deficiency chlorosis is an important abiotic stress affecting groundnut production worldwide in calcareous and alkaline soils with a pH of 7.5–8.5. To identify genomic regions controlling iron deficiency chlorosis resistance in groundnut, the recombinant inbred line population from the cross TAG 24 × ICGV 86031 was evaluated for associated traits like visual chlorosis rating and SPAD chlorophyll meter reading across three crop growth stages for two consecutive years. Thirty-two QTLs were identified for visual chlorosis rating (3.9%–31.8% phenotypic variance explained [PVE]) and SPAD chlorophyll meter reading [3.8%–11% PVE] across three stages over 2 years. This is the first report of identification of QTLs for iron deficiency chlorosis resistance-associated traits in groundnut. Three major QTLs (>10% PVE) were identified at severe stage, while majority of other QTLs were having small effects. Interestingly, two major QTLs for visual chlorosis rating at 60 days (2013) and 90 days (2014) were located at same position on LG AhXIII. The identified QTLs/markers after validation across diverse genetic material could be used in genomics-assisted breeding.

KEYWORDS

groundnut, iron deficiency chlorosis resistance, QTL mapping, SPAD chlorophyll meter reading, visual chlorosis rating

1 | INTRODUCTION

Groundnut or peanut (*Arachis hypogaea* L.) is an important food legume that is grown globally on 28,52 million ha area with an in-shell production of 45,95 million metric tons (FAOSTAT, 2018; http://www.fao.org/faostat/en/#data/QC). India stands first in groundnut area (4,94 m ha), while second in production (6,7 m t) after China (17,39 m t) due to very less productivity in India (13.553 hg/ha) compared to China (37.476 hg/ha) and world average (16.114 hg/ha). Iron (Fe) is an essential element for plants that plays an important role in photosynthesis, respiration, nitrogen fixation, DNA synthesis, hormone production, chlorophyll formation and also component of various redox and iron–sulphur enzymes in plants (Zheng, 2010). Plants adopt two types of mechanisms (Strategy–I and II) for iron acquisition from the soils. The strategy–I is found in

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dicots and monocots, except graminaceous species which adopts strategy–II. Groundnut adopts strategy–I and found susceptible to iron deficiency (Gholizadeh, Baghban, Kohnehrouz, & Hekmatshoar, 2007). Strategy–I involves steps like acidification of the rhizosphere through H(+)-ATPase-mediated extrusion of protons to increase the solubility of Fe^{3+} (Santi & Schmidt, 2009), reduction of Fe^{3+} to Fe^{2+} through the ferric-chelate reductase (e.g. *AhFRO1*; Ding et al., 2009), and transport of Fe^{2+} throughout the root plasma membrane through metal transporter (e.g. *AhIRT1*; Ding et al., 2010). Strategy–II involves production and release of phytosiderophores from roots, solubilization of iron by forming Fe^{3+}-phytosiderophore complex and uptake through high-affinity transport system in plasma membrane of root cells. The Fe chelated by microbial siderophores might also be acquired (Prasad & Djanaguiraman, 2017).

Although iron is abundant in nature, it is often unavailable because it forms insoluble ferric hydroxide complexes in the presence of oxygen at neutral or basic pH as in calcareous soils (Guerinot & Yi, 1994). Iron deficiency chlorosis (IDC) is common worldwide among crops grown in calcareous and alkaline soils due to lower levels of available Fe (Fe^{2+}) for uptake. Calcareous soils are widespread with an estimated 800 m ha worldwide, mainly concentrated in areas with arid or Mediterranean climates (Land, FAO, & Plant Nutrition Management, 2000). In India, more than one-third of the soils are calcareous and spread mostly in the low rainfall areas of the western and central parts of the country, where groundnut is a major crop. The IDC is more prevalent in the Saurashtra region of Gujarat, Marathwada region of Maharashtra, and parts of Rajasthan, Tamil Nadu and Karnataka states in India causing significant reduction in pod yield (15%–32%; Singh, 2001; Singh, Basu, & Singh, 2003; Singh, Chaudhari, Koradia, & Zala, 1995). IDC is also a common problem in groundnut-producing areas with calcareous soils in northern China (Li & Yan-Xi, 2007) and Pakistan (Akhtar, Shahzad, Arshad, & Fayyaz-Ul-Hassan, 2013; Imtiaz, Rashid, Khan, Memon, & Aslam, 2010) causing significant reduction in yield. Severity of IDC will be usually quite high after excessive rainfall and also for groundnuts grown under irrigation due to high bicarbonate ion concentration in the rhizosphere (Singh et al., 1995; Zuo, Ren, Zhang, & Jiang, 2007).

Iron deficiency in groundnut initially appears as chlorosis on young rapidly expanding leaves which is characterized by interveinal chlorosis. Iron deficiency has been found to decline net photosynthetic rate resulting from the reduction of photosynthetic pigment contents and inhibition of PSII photochemistry (Su et al., 2015). During severe deficiency, veins also become chlorotic, leaves become white and papery and later turn brown and necrotic, while the plants show stunted growth resulting in reduced yield, seed Fe content and fodder. Acute iron deficiency leads to death of plants and complete crop failure. Although application of Fe-containing fertilizers into soil or as foliar spray has been suggested (Frenkel, Hadar, & Yona, 2004; Irmak, Çıl, Yücel, & Kaya, 2012), it is often associated with problems like conversion into unavailable form (Fe^{3+}) or poor translocation within the plant (Hüve, Remus, Lüttschwager, & Merbach, 2003). Though foliar application of Fe chelates can overcome this problem, it is not economical as groundnut is predominantly grown as a rainfed subsistence crop by the resource-poor farmers in semi-arid tropics. Hence, the development of Fe-efficient genotypes can be a successive tool to overcome the Fe deficiency in soil and also for the improvement in human health (Imtiaz et al., 2010).

Identifying and developing IDC resistant genotypes is challenging due to high level of temporal and spatial variability of chlorosis expression in the field (King, 2011). Inconsistency in expression of iron deficiency symptoms could be due to various factors such as soil heterogeneity, bicarbonate ion concentration, soil moisture, temperature and relative humidity. The IDC response is usually assessed by visual chlorosis rating (VCR), chlorophyll content and SPAD chlorophyll meter reading (SCMR) in groundnut (Li & Yan-Xi, 2007; Mann et al., 2018; Samdur et al., 1999, 2000). Higher SCMR is an indicator of lesser incidence of leaf chlorosis. Higher VCR and lower SCMR indicate "susceptibility", while lower VCR and higher SCMR indicate "resistance" to IDC. Growing of IDC resistant groundnut cultivars under calcareous soils has shown significantly higher pod yield compared to susceptible cultivars (Li & Yan-Xi, 2007; Mann et al., 2018; Prasad, Satyanarayana, Potdar, & Craufurd, 2000; Samdur et al., 1999). For effective development of IDC resistant groundnut genotypes, it is necessary to understand the genetic basis and also identify the specific genomic regions associated with IDC resistance. Earlier, inheritance study by Gowda, Kulkarni, Nadaf, and Habib (1993) indicated recessive nature of IDC resistance in groundnut showing trigenic (21:43) and pentagenic (525:499) ratios in F_2 population. On the contrary, genetic investigations assessed by six generation mean analysis indicated dominant nature of IDC resistance and also presence of non-allelic interactions for related characters like chlorophyll and carotenoid content (Samdur, Manivel, & Mathur, 2005). Our recent investigations on inheritance of IDC resistance among four crosses of groundnut based on F_2 and F_3 behaviour indicated duplicate dominant genes governing this trait (Pattanashetti, Naidu, Prakyath Kumar, Singh, & Biradar, 2018). In groundnut, three genes involved in iron acquisition have been identified, that is *AhFRO1*, encoding an Fe(III)-chelate reductase involved in reduction of Fe^{3+} into Fe^{2+} (Ding et al., 2009), and two iron transporters, *AhIRT1* (Ding et al., 2010) and *AhNRAMP1* (Xiong et al., 2012). Recently, *AhIRT1* and *AhNRAMP1* expression have also been correlated with cadmium (Cd) uptake in groundnut under iron deficiency (Chen, Xia, Deng, Liu, & Shi, 2017).

The integration of available genomic resources together with modern genomics approaches, high throughput phenomics and simulation modelling will help in achieving higher genetic gains (Varshney et al., 2018). Breeding for IDC resistance using genomics-assisted breeding (GAB) can increase the selection efficiency and cost-effectiveness, reduce the duration of breeding cycle, but it requires identification of linked QTLs/markers (Pandey et al., 2012, 2016; Varshney et al., 2013, 2019). The deployment of linked markers in soybean has successfully demonstrated 2.6-fold increase in selection efficiency relative to phenotypic selection, wherein 73% of lines developed were with superior IDC resistance, and there was

70% reduction in cost of IDC evaluation compared to traditional breeding schemes (Charlson, Cianzio, & Shoemaker, 2003). Hence, the present study undertook the extensive phenotyping of IDC resistance-associated traits and analysed these data together with available genetic maps data for identification of QTLs and linked markers for IDC resistance in groundnut.

2 | MATERIALS AND METHODS

2.1 | Plant materials

The RIL population (318 lines) of the cross TAG 24 × ICGV 86031 was developed earlier at ICRISAT, Patancheru to map drought tolerance traits in groundnut (Faye et al., 2015; Gautami et al., 2012; Ravi et al., 2011; Varshney et al., 2009). As per field screening at College of Agriculture, Vijayapur, India during rainy season 2009, the parent TAG 24 was found IDC susceptible (VCR 4.0), while ICGV 86031 as IDC resistant (VCR 1.0; Figure 1a). Keeping the IDC response of parents in view, phenotyping of this RIL population for IDC resistance-associated traits like VCR and SCMR was undertaken towards identifying genomic regions associated with IDC resistance in groundnut.

2.2 | Phenotyping of RIL population

The field experiment was conducted for two consecutive years (2013 and 2014) during rainy season at College of Agriculture, Vijayapur, India (16°49'N, 75°43'E, 593 m above mean sea level, and 597 mm average annual rainfall) on calcareous vertisol soils that are alkaline (pH > 8) and deficient in available Fe (DTPA-extractable Fe < 4 mg/kg; Table S1). Field screening for IDC response of RIL population along with parents (320 lines) was done using randomized complete block design in two replications. Each genotype in a replication was planted as one row of 2.5 m length with an inter- and intrarow spacing of 30 and 10 cm, respectively. The recommended dose of nitrogen (25 kg/ha), phosphorus (75 kg/ha), potassium (25 kg/ha) and zinc sulphate (25 kg/ha) were added to the field at the time of planting. Iron-containing fertilizers were not applied. Recommended cultivation practices were followed to raise a good crop. The range of climatic factors during the crop period (June to October) at Vijayapur in 2013 and 2014, respectively, were as follows: maximum temperature (26.9–32.6°C; 26.6–34.2°C), minimum temperature (20.1–21.8°C; 14.7–21.9°C), average relative humidity (64.1%–85.5%; 57.2%–85.7%) and season rainfall (590 mm in 30 rainy days; 431 mm in 21 rainy days).

FIGURE 1 Phenotypic variability in parents and RIL population, and visual chlorosis rating (VCR) (1 to 5 scale) for IDC response. The figure indicates the (a) IDC response of parents, (b) variability for IDC response among RILs and (c) representation of VCR scale (1–5) used for assessment of RILs [Colour figure can be viewed at wileyonlinelibrary.com]

2.2.1 | Evaluation for IDC resistance

Iron deficiency chlorosis resistance-associated traits like VCR and SCMR were assessed across three stages, that is 30, 60 and 90 days after sowing (DAS) for two consecutive years. VCR scoring was done as per the scale proposed by Singh and Chaudhari (1993) (1 to 5 scale: 1—normal green leaves with no chlorosis, 2—green leaves but with slight chlorosis on some leaves, 3—moderate chlorosis on several leaves, 4—moderate chlorosis on most of the leaves, 5—severe chlorosis on all the leaves) (Figure 1c) on overall line basis. Higher VCR score indicates susceptibility, while lower VCR indicates resistance to IDC. Based on VCR score, lines can be considered as resistant (VCR 1 to 2), moderately resistant (>2 to 3) or susceptible (>3 to 5).

The chlorophyll meter SPAD 502 (Soil Plant Analysis Development meter, Konica Minolta, Japan) was used to measure the absorbance of the leaf in the red (at 650 nm) and near infrared region (at 940 nm). Using these two transmittances, it calculated a numerical SPAD value which is proportional to the chlorophyll present in the leaf and is negatively related to chlorosis of the plants. The SCMR (SPAD values) was recorded in the standard leaf (third leaf from the top on main stem, i.e., fully expanded) of five plants showing most severe symptoms per genotype or plot, and their mean was calculated. Higher SCMR indicates resistance, while lower SCMR indicates susceptibility to IDC. As the SCMR is a continuous variable, it is difficult to make classes for IDC response. However, for better understanding of the distribution for SCMR, we grouped the RILs into six categories with an interval of five, that is ≤20, >20–25, >25–30, >30–35, >35–40 and >40.

2.2.2 | Estimation of chlorophyll and active Fe content

Chlorophyll (a, b and total) and active Fe (Fe^{2+}) content have been shown to be positively correlated with IDC resistance in several crop species. To confirm the IDC resistance/ susceptibility, chlorophyll and active Fe content were estimated at most severe stage (60 DAS) among both parents and selected five RILs each of IDC resistant and susceptible types during 2014. Sample from standard leaf of five plants per genotype was used to estimate the chlorophyll and active Fe content and mean was calculated. The chlorophyll content was estimated using method described by Shoaf and Lium (1976) and expressed as mg/g on fresh weight basis. Active Fe content was estimated using the method described by Katyal and Sharma (1980) using o-phenanthroline extractant and expressed as mg/kg on fresh weight basis.

2.3 | Statistical analyses

The phenotypic data of VCR and SCMR at three stages for individual years (2013, 2014) were analysed for analysis of variance technique by mixed model procedure of SAS version 9.3 (SAS Institute Inc., 2017) considering replication and genotype as random effect. Square root transformation has been applied on VCR before analysis. Best linear unbiased predictor (BLUP) values were estimated for VCR and SCMR at three stages during individual years. To assess genetic variability in the RIL population, components such as genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV) (Burton & Devane, 1953), broad sense heritability (H_{bs}) (Falconer, Mackay, & Frankham, 1996) and genetic advance as per cent mean (GAM) (Johnson, Robinson, & Comstock, 1955) were estimated using genotypic and phenotypic variances, and BLUP mean values. Association among traits was calculated using Pearson's correlation coefficients.

2.4 | Genetic mapping of RIL population

The genetic mapping data generated earlier for the TAG 24 × ICGV 86031 RIL population using 191 SSR marker loci were used for QTL analysis. The detailed information on identification of polymorphic markers on parental genotypes, parental polymorphism, genotyping of mapping population and construction of genetic map has been described in detail in Varshney et al. (2009) and Ravi et al. (2011).

2.5 | Quantitative trait locus (QTL) analysis

The QTL analysis for VCR and SCMR at three different stages (30, 60, 90 DAS) for two years (2013 and 2014) was performed by using BLUP values along with genotyping data for 191 SSR markers. The QTL analysis was performed by using Windows QTL Cartographer version 2.5. Composite interval mapping (CIM) approach was deployed for identification of location and effect of QTLs. This software uses a dynamic algorithm which considers various gene actions (additive and dominance), QTL-environment interactions and close linkage (Wang, Basten, & Zeng, 2007). Parameters such as model 6, scanning intervals of 1.0 cM between markers and putative QTLs with a window size of 10.0 cM were used for conducting the CIM analysis. In addition, forward–backward stepwise regression was selected for background control set by the number of marker cofactors along with 500 times permutations with 0.05 significance level and "Locate QTLs" option to locate QTLs. The QTL analysis was conducted on phenotyping data of individual years (2013, 2014) separately by using BLUPs.

3 | RESULTS

3.1 | Phenotypic variability for IDC resistance-associated traits

Large variation was observed among parents and RIL population for IDC resistance-associated traits like VCR and SCMR across three stages (30, 60 and 90 DAS) during both the years (2013, 2014) evident from highly significant differences for genotypes based on *F* test ($p < .0001$) (Table 1). For better clarity on variability for the

IDC associated traits among parents as well as RILs, mean values of the genotypes are presented in this section. In both the years, ICGV 86031 remained resistant to IDC across all three stages evident from lowest VCR scores (1.0) and higher SCMR values (39.1–45.3), while TAG 24 was found susceptible to IDC at all three stages evident from higher VCR scores (3.0–4.0) and lower SCMR values (10.95–23.05; Table 2). Wide variation was observed among the RILs for both VCR and SCMR across three stages for two years, wherein some values were beyond the parents (Table 2). Variability observed for IDC response among parents and RILs is depicted in Figure 1.

Frequency distribution of RILs (number) with VCR scores from 1 to 4 across three stages for two years is given in Figure 2a. None of the RILs showed VCR score of 5. During 2013, large number of RILs (277, 307, 286) were falling under resistant category (VCR 1 to 2) at 30, 60 and 90 DAS, respectively. However, during 2014, large number of RILs were falling under resistant category at 30 DAS (301), but it was drastically reduced at 60 DAS (170) showing IDC severity and further showed some recovery at 90 DAS (242). Frequency distribution of RILs based on SCMR into six categories across three stages for 2 years is given in Figure 2b. The distribution is skewed towards resistant/moderately resistant categories during 2013, while towards susceptible/ moderately resistant categories during 2014. During 2013, comparison of overall mean values across three stages for VCR (1.65, 1.51, 1.63) and SCMR (34.31, 36.21, 38.18) indicated almost similar IDC response across three stages. As evident from mean VCR and SCMR during 2014, there has been increased susceptibility to IDC from 30 DAS (1.49, 33.25) to 60 DAS (2.32, 24.76), and further some recovery at 90 DAS (1.89, 31.08), respectively. Comparison for IDC severity across the years indicated higher severity at 30 DAS during 2013, while much higher severity at 60 and 90 DAS during 2014. Majority of the RILs showing IDC resistance at all the three stages during both the years suggest dominance nature of IDC resistance compared to susceptibility in this RIL population.

Total chlorophyll and active Fe content estimations among parents and representative RILs during 2014 indicated their higher content among IDC resistant parent, ICGV 86031 (1.042 mg/g, 9.03 mg/kg) and resistant RILs (0.496 to 1.280 mg/g, 6.15 to 9.91 mg/kg) compared to IDC susceptible parent, TAG 24 (0.245 mg/g, 4.95 mg/kg) and susceptible RILs (0.231 to 0.475 mg/g, 4.19 to 5.86 mg/kg; Table 3). Highly significant

TABLE 1 Variance components for VCR and SCMR across three stages in RIL population during 2013 and 2014

		Visual chlorosis rating (VCR)			SPAD chlorophyll meter rating (SCMR)		
Year	Variance[a]	30 DAS	60 DAS	90 DAS	30 DAS	60 DAS	90 DAS
2013	σ_g^2	0.02886**	0.04125**	0.04243**	15.164**	14.847**	19.453**
	SE	0.00394	0.00385	0.00428	1.468	1.552	1.943
	Residual	0.03540	0.01390	0.02110	6.228	8.563	9.284
2014	σ_g^2	0.03057**	0.03727**	0.05080**	17.87**	29.95**	50.21**
	SE	0.00348	0.00377	0.00510	2.23	3.07	5.51
	Residual	0.02350	0.01870	0.02490	17.79	15.99	34.34

[a]σ_g^2—genotypic variance, SE, standard error for σ_g^2; DAS, days after sowing.
**Significant at $p < .0001$.

TABLE 2 Genetic variability among parents and RIL population for VCR and SCMR across three stages during 2013 and 2014

Trait	Year	TAG 24	ICGV 86031	Range in RILs	Mean	GCV (%)	PCV (%)	H_{bs} (%)	GAM (%)
VCR 30 DAS	2013	3.00	1.00	1.00–4.00	1.65	13.47	17.11	62.02	21.85
	2014	3.00	1.00	1.00–4.00	1.49	14.61	17.18	72.34	25.60
VCR 60 DAS	2013	4.00	1.00	1.00–4.00	1.51	16.82	18.18	85.68	32.08
	2014	4.00	1.00	1.00–4.00	2.32	12.84	14.36	80.04	23.64
VCR 90 DAS	2013	4.00	1.00	1.00–4.00	1.63	16.47	18.40	80.00	30.36
	2014	4.00	1.00	1.00–4.00	1.89	16.74	18.68	80.25	30.91
SCMR 30 DAS	2013	23.05	39.50	14.70–43.60	34.31	11.35	12.46	82.96	21.30
	2014	21.30	41.40	17.25–43.65	33.25	12.71	15.56	66.77	21.40
SCMR 60 DAS	2013	16.80	39.10	12.75–44.85	36.21	10.64	12.08	77.62	19.31
	2014	10.95	40.25	8.15–40.25	24.76	22.10	24.88	78.93	40.45
SCMR 90 DAS	2013	17.70	41.65	17.70–45.10	38.18	11.55	12.86	80.73	21.38
	2014	11.60	45.30	9.55–46.55	31.08	22.80	26.41	74.52	40.54

Note: GAM, genetic advance as per cent of mean; GCV, genotypic coefficient of variation; H_{bs}, broad sense heritability; PCV—phenotypic coefficient of variation.

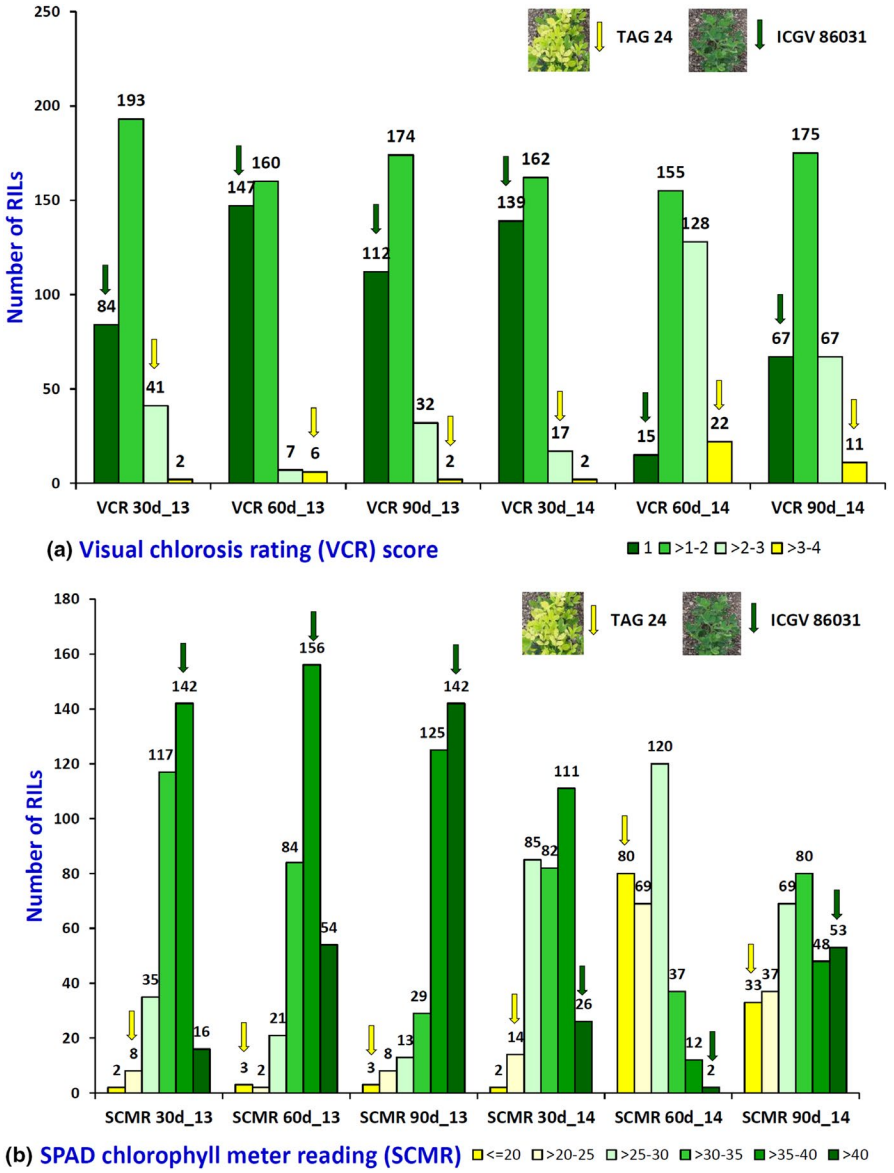

FIGURE 2 Frequency distributions of RILs and parents for VCR and SCMR at three stages during 2013 and 2014. The figure indicates the (a) frequency of RILs and parents (number) for VCR score (1 to 4) across three stages (30, 60 and 90 DAS) over two years (2013, 2014) and (b) frequency of RILs and parents (number) for SCMR groups (6) across three stages (30, 60 and 90 DAS) over two years (2013, 2014). For example, VCR 30d_13 indicates VCR at 30 DAS during 2013, while SCMR 30d_14 indicates SCMR at 30 DAS during 2014. Yellow and green arrows indicate the category in which the parents TAG 24 and ICGV 86031 are positioned [Colour figure can be viewed at wileyonlinelibrary.com]

correlations were observed between VCR at severe stage (60 DAS) with active Fe content (−0.872, $p < .001$), chlorophyll "a" (−0.819, $p < .01$) and total chlorophyll (−0.813, $p < .01$) suggesting them as good indicators for IDC response.

3.2 | Genetic components of variability and correlations

Genetic components were estimated for VCR and SCMR at three stages for individual years using variances and BLUP values to know the genetic variability in the RIL population. For VCR, moderate PCV and GCV (10%–20%) were observed during both years (2013, 2014) (Table 2). However, for SCMR, higher PCV and GCV (>20%) were observed during 2014 except at 30 DAS that was moderate, but they were moderate at all the three stages during 2013. Higher broad sense heritability (H_{bs}) (>60%) was observed for both VCR and SCMR across three stages during both the years. Genetic advance as per cent mean (GAM) was also higher (>20%) for VCR and SCMR across three stages during both the years.

Correlations between VCR and SCMR across three stages and their means were estimated using BLUP values to understand the associations among them. During both the years, highly significant positive correlations ($p < .00001$) were observed between different stages for VCR and SCMR (Table 4). However, highly significant negative correlations were observed between VCR and SCMR across three stages for both the years, wherein values were comparatively higher during 2014 compared to 2013. Highly significant negative correlation was observed between overall mean across three stages for both VCR and SCMR during 2013 (−0.860) and 2014 (−0.968).

3.3 | Identification of QTLS for IDC resistance

The QTL analysis was performed for all the traits using BLUPs for 2013 and 2014 independently. The QTL analysis for VCR in 2013

TABLE 3 Chlorophyll and active Fe content among parents and selected RILs during 2014

Parents/RIL #	VCR 60 DAS[a]	Active Fe (mg/kg)[b]	Chl. a (mg/g)[b]	Chl. b (mg/g)[b]	Total chl. (mg/g)[b]
Parents					
TAG 24	4.00	4.95	0.191	0.054	0.245
ICGV 86031	1.00	9.03	0.899	0.143	1.042
IDC Resistant RILs					
110	1.50	9.91	0.689	0.016	0.704
281	1.50	9.75	1.148	0.132	1.280
7	2.00	7.29	0.503	0.093	0.596
66	2.00	6.44	0.423	0.073	0.496
86	2.50	6.15	0.474	0.102	0.576
IDC Susceptible RILs					
4	3.50	5.04	0.293	0.054	0.347
213	3.00	4.19	0.199	0.052	0.251
228	4.00	5.86	0.396	0.079	0.475
95	4.00	4.32	0.285	0.047	0.332
186	4.00	4.41	0.192	0.039	0.231
Correlation with VCR 60 DAS	–	−0.872	−0.819	−0.542	−0.813
p-value	–	<0.001	<0.01	ns	<0.01

[a]VCR 60 DAS—visual chlorosis rating at 60 days after sowing.

[b]All biochemical contents were estimated at 60 days after sowing in standard leaf on fresh weight basis; Chl. a—Chlorophyll a; Chl. b—Chlorophyll b; Total chl.—Total chlorophyll; ns—non-significant.

TABLE 4 Pearson correlation coefficients (r) between VCR and SCMR values across three stages during 2013 and 2014

Year/Trait*	VCR 30 DAS	VCR 60 DAS	VCR 90 DAS	SCMR 30 DAS	SCMR 60 DAS	SCMR 90 DAS	Mean VCR
Year 2013							
VCR 60 DAS	0.563						
VCR 90 DAS	0.535	0.510					
SCMR 30 DAS	−0.681	−0.531	−0.433				
SCMR 60 DAS	−0.491	−0.791	−0.402	0.531			
SCMR 90 DAS	−0.580	−0.530	−0.809	0.493	0.484		
Mean VCR	0.808	0.843	0.838	−0.644	−0.681	−0.775	
Mean SCMR	−0.715	−0.748	−0.685	0.819	0.807	0.825	−0.860
Year 2014							
VCR 60 DAS	0.666						
VCR 90 DAS	0.633	0.775					
SCMR 30 DAS	−0.899	−0.665	−0.612				
SCMR 60 DAS	−0.642	−0.957	−0.782	0.663			
SCMR 90 DAS	−0.573	−0.739	−0.948	0.575	0.750		
Mean VCR	0.837	0.921	0.920	−0.788	−0.900	−0.866	
Mean SCMR	−0.760	−0.895	−0.915	0.796	0.916	0.917	−0.968

*Correlation values for all the traits were significant at $p < .00001$; DAS, days after sowing.

rainy season identified six QTLs across three stages (2 each for VCR 30 DAS, VCR 60 DAS, and VCR 90 DAS; Table 5, Figure 3). The LOD score for these QTLs ranged from 3.0 to 4.8, phenotypic variance explained (PVE) ranged from 3.9% to 22.4% and the additive effect from −0.29 to 0.16. Two QTLs each were identified on linkage group (LG) AhVIII and AhXIV, while one QTL each on AhXIII and AhIV. The QTL analysis for VCR in 2014 rainy season, identified 13 QTLs across three stages (3 each for VCR 30 DAS and

TABLE 5 QTLs identified for VCR and SCMR across three stages (30, 60 and 90 DAS) during 2013 and 2014

Trait/QTL name_year[a]	Linkage group	Nearest marker	Marker interval	LOD value	PVE (%)	Additive effect[b]
VCR during 2013						
qVCR 30DAS AhIV_13	AhIV	Seq19D06	Seq19D06-GM723b	3.0	4.1	−0.12
qVCR 30DAS AhXIV_13	AhXIV	GM1996	RM14E11-GM1996	4.8	6.4	−0.15
qVCR 60DAS AhXIII_13	AhXIII	GM2259	Seq2C11-GM2259	3.1	22.4	−0.29
qVCR 60DAS AhXIV_13	AhXIV	Seq16C06	Seq16C06-RM14E11	3.2	3.9	−0.11
qVCR 90DAS AhVIII_13_1	AhVIII	IPAHM229, GM2746, GM2689	(IPAHM229, GM2746, GM2689) –IPAHM123	4.6	5.6	0.16
qVCR 90DAS AhVIII_13_2	AhVIII	TC9F10	IPAHM406-TC9F10	3.9	5.5	0.15
VCR during 2014						
qVCR 30DAS AhXIV_14_1	AhXIV	Seq16C06	GM2308-Seq16C06	3.2	4.5	−0.12
qVCR 30DAS AhXIV_14_2	AhXIV	GM1996	GM1996-GM 626	5.1	5.9	−0.13
qVCR 30DAS AhXV_14	AhXV	GM2602	GM641-GM2602	4.4	7.0	−0.14
qVCR 60DAS AhXIV_14_1	AhXIV	Seq16C06	Seq16C06-RM14E11	4.4	5.2	−0.15
qVCR 60DAS AhXIV_14_2	AhXIV	GM1996	GM1996-GM 626	5.4	7.2	−0.18
qVCR 60DAS AhXV_14	AhXV	GM2603	GM2603-Seq16G08	4.4	9.3	−0.20
qVCR 90DAS AhVIII_14	AhVIII	IPAHM606	IPAHM606-PM419	4.7	5.1	0.16
qVCR 90DAS AhXI_14	AhXI	GM672, GM1992a	GM672, GM1992a-TC2D06	4.2	4.6	−0.15
qVCR 90DAS AhXIII_14	AhXIII	GM2259	Seq2C11-GM2259	3.3	31.8	−0.43
qVCR 90DAS AhXIV_14_1	AhXIV	Seq16C06	GM2308-Seq16C06	3.7	4.9	−0.15
qVCR 90DAS AhXIV_14_2	AhXIV	GM1996	GM1996-GM 626	4.4	5.2	−0.16
qVCR 90DAS AhXV_14_1	AhXV	GM641	GM641-GM2602	3.4	5.3	−0.16
qVCR 90DAS AhXV_14_2	AhXV	GM2602	GM641-GM2602	3.4	5.0	−0.16
SCMR during 2013						
qSCMR 30DAS AhIV_13	AhIV	Seq19D06	Seq19D06-GM723b	4.1	6.0	1.05
qSCMR 30DAS AhVIII_13	AhVIII	IPAHM219	IPAHM219-IPAHM606	3.7	4.5	−0.91
qSCMR 60DAS AhXIV_13	AhXIV	RM14E11	RM14E11-GM1996	3.0	4.0	0.87
qSCMR 90DAS AhVIII_13_1	AhVIII	IPAHM229, GM2689, GM2746	(IPAHM229, GM2746, GM2689)-IPAHM123	6.2	7.3	−1.46
qSCMR 90DAS AhVIII_13_2	AhVIII	TC9F10	IPAHM406-TC9F10	4.0	5.5	−1.22
qSCMR 90DAS AhXIII_13	AhXIII	TC3B02	TC3B02-GM1609	3.3	3.8	−1.00
qSCMR 90DAS AhXIV_13	AhXIV	Seq16C06	Seq16C06-RM14E11	3.4	4.4	1.04
SCMR during 2014						
qSCMR 60DAS AhIX_14	AhIX	Seq7G02	gi1107-Seq7G02	3.0	5.0	1.39
qSCMR 60DAS AhXIV_14_1	AhXIV	Seq16C06	Seq16C06-RM14E11	4.1	5.3	1.49
qSCMR 60DAS AhXIV_14_2	AhXIV	GM1996	RM14E11-GM1996	4.5	6.4	1.64
qSCMR 60DAS AhXV_14	AhXV	GM2603	GM2603-Seq16G08	5.3	11.0	2.04
qSCMR 90DAS AhXII_14	AhXII	RM11H01	RM11H01-TC11B04	3.5	3.9	1.67
qSCMR 90DAS AhXV_14	AhXV	GM641	GM641-GM2602	3.6	5.7	1.95

[a]Year: 13 for 2013 and 14 for 2014; q, QTL; VCR, visual chlorosis rating; SCMR, SPAD chlorophyll meter reading; DAS, days after sowing.
[b]The additive effect of QTLs indicates that IDC resistance traits, that is, low VCR and high SCMR are contributed by the IDC resistant parent, ICGV 86031.

VCR 60 DAS, and 7 for VCR 90 DAS; Table 5, Figure 3). For these QTLs, the LOD score ranged between 3.2–5.4, PVE 4.5%–31.8% and the additive effect −0.43 to 0.16. Maximum QTLs identified were six on LG AhXIV, four on LG AhXV, while one each on LG AhVIII, AhXI and AhXIII. If the QTL for a trait was identified for two different years located in the same QTL region, it was considered as "Consistent QTL". For VCR at 60 DAS, one such consistent QTL was identified in the marker interval Seq16C06–RM14E11 on LG AhXIV. Interestingly, two major QTLs (>10% PVE) explaining 22.4% and 31.8% PVE were identified for VCR 60 DAS and 90 DAS during

2013 and 2014, respectively, at the same QTL region between the markers Seq2C11–GM2259 on AhXIII.

The QTL analysis for SCMR in 2013 rainy season revealed seven QTLs across three stages (2 for VCR 30 DAS, one for VCR 60 DAS and four for VCR 90 DAS; Table 5, Figure 3). The LOD score for these QTLs ranged from 3.0 to 6.2, PVE 3.8%–7.3% and the additive effect of individual QTLs from −1.46 to 1.05. Three QTLs were identified on LG AhVIII, two QTLs on AhXIV, while one QTL each on LG AhIV and AhXIII. The QTL analysis for SCMR in 2014 rainy season identified six QTLs (4 for SCMR 60 DAS, two for SCMR 90 DAS), while no QTL was detected for SCMR 30 DAS (Table 5, Figure 3). The LOD value for these QTLs ranged from 3.0 to 5.3, PVE 3.9%–11% and additive effect 1.39 to 2.04. Of the six QTLs identified, two QTLs each were located on LG AhXIV and AhXV, while one QTL each was on LG AhIX and AhXII. One major QTL was identified for SCMR 60 DAS located between marker interval GM2603–Seq16G08 on LG AhXV explaining 11% PVE.

Considering IDC resistance-associated traits like VCR and SCMR together across three stages, a total of 13 QTLs located on 4 LGs were identified for season 2013, while 19 QTLs located on 7 LGs for season 2014 (Table 5, Figure 3). Across years, four QTLs each were located between marker intervals Seq16C06–RM14E11 on LG AhXIV and GM641–GM2602 on LG AhXV; three QTLs each between marker interval RM14E11–GM1996 and GM1996–GM626 on LG AhXIV, and; two QTLs each between marker intervals Seq19D06–GM723b on LG AhIV, (IPAHM229, GM2746, GM2689)–IPAHM123 and IPAHM406–TC9F10 on LG AhVIII, Seq2C11–GM2259 on LG AhXIII, GM2308–Seq16C06 on LG AhXIV and GM2603–Seq16G08 on LG AhXV. The major region governing IDC resistance seems to be between marker intervals GM2308–GM626 on LG AhXIV, where 12 QTLs were identified.

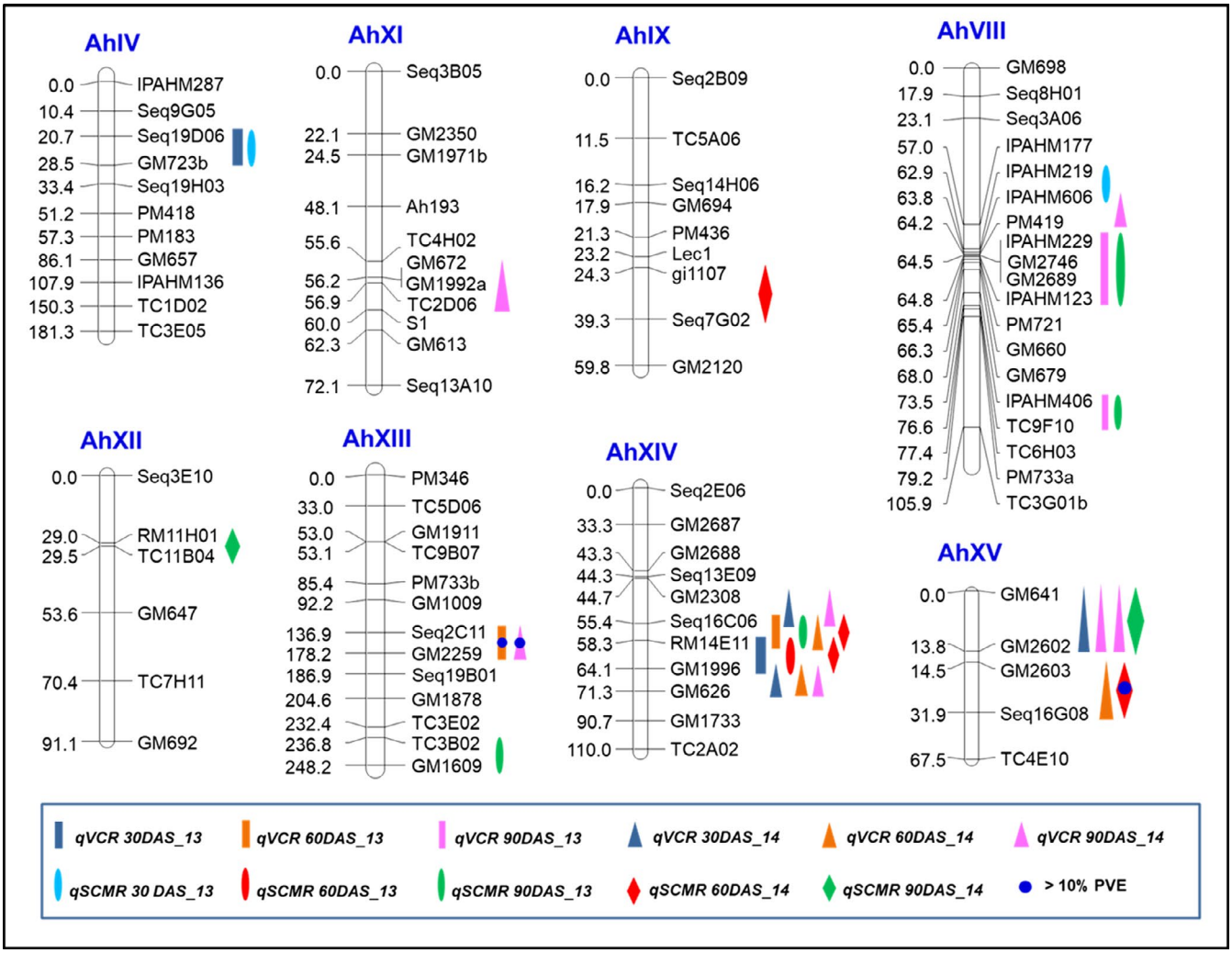

FIGURE 3 Genetic map showing location of identified QTLs in different linkage groups for VCR and SCMR across three stages. The QTLs identified for IDC resistance-associated traits, that is VCR and SCMR across three different stages (30, 60 and 90 DAS) are shown in the figure. The naming of QTL is as follows: *qVCR 30DAS AhIV_13* indicates QTL identified for VCR at 30 DAS on linkage group AhIV during 2013; *qSCMR 60DAS AhIX_14* indicates QTL identified for SCMR at 60 DAS on linkage group AhIX during 2014; *qVCR 90DAS AhVIII_13_1* and *qVCR 90DAS AhVIII_13_2* indicates first and second QTL identified on the same linkage group (AhVIII) for VCR at 90 DAS during same year, that is 2013

4 | DISCUSSION

Iron deficiency chlorosis is a major production constraint in groundnut growing areas with calcareous soils that are deficient in available Fe which causes significant yield loss in India (Singh, 2001), northern China (Li & Yan-Xi, 2007) and Pakistan (Akhtar et al., 2013; Imtiaz et al., 2010). In a recent study at Vijayapur (India), IDC response assessed among 26 released varieties, 13 advanced breeding lines and 4 germplasm lines indicated that majority of the released cultivars were found either susceptible or moderately resistant to IDC, while one each of released cultivar (ICGV 86031), advanced breeding line (ICGV 06146) and germplasm (A30b, an interspecific derivative) were found IDC resistant (Boodi, 2014). This shows the low frequency of IDC resistant cultivars in the farmer's fields and also advanced breeding lines in the pipeline. Hence, targeted development of high yielding groundnut cultivars with IDC resistance is essential to address the problem of IDC in calcareous and alkaline soils. Since IDC is a complex with high environmental influence, selection and development of improved varieties for IDC resistance based on visual scoring is difficult. Hence, identification of QTLs/markers linked to IDC resistance will be more useful towards the development of IDC resistant high yielding groundnut cultivars through marker-assisted selection (MAS). The present study reports identification of several QTLs for IDC resistance in groundnut.

Phenotyping for IDC resistance-associated traits like VCR and SCMR across three stages during both years revealed significant differences among parents and large variation in the RIL population (Tables 1 and 2). Earlier, huge variation has been noted among parents and RIL populations while mapping for IDC resistance-related traits like visual scoring and SPAD values (SCMR) in soybean (Butenhoff, 2015; Lin, Cianzio, & Shoemaker, 1997) and mungbean (Prathet, Somta, & Srinives, 2012), and also for zinc efficiency score in wheat (Genc et al., 2009). Though visual scoring is a fast and convenient method to evaluate for IDC in groundnut, due to the complexity associated with field screening, it is essential to confirm IDC resistance through biochemical parameters like chlorophyll and active Fe content. Since significant correlations have been established between SCMR, chlorophyll and active Fe content for parents and selected RILs in the present study (Table 3) and also by earlier researchers in groundnut (Akhtar et al., 2013; Li & Yan-Xi, 2007; Samdur et al., 2000), SCMR is found ideal for confirmation of IDC resistance since it is reliable, faster and convenient. Further, higher values of genetic components such as GCV, PCV, H_{bs} and GAM and correlations recorded for VCR and SCMR (Tables 2 and 4) also indicate their utility as important traits in breeding for IDC resistance in groundnut.

Iron deficiency chlorosis severity across two years indicated higher severity at 30 DAS during 2013, while much higher severity at 60 and 90 DAS during 2014. Earlier reports in groundnut indicate beginning of iron deficiency at 10–15 days after emergence, while attaining of maximum intensity at 30–70 days (Singh & Chaudhari, 1993) or 50–65 days after emergence (Li, Yan-Xi, & Jian-min, 2009). In this study also the severity of IDC was found increasing from the 30 d to 60 d, while slight recovery at 90 d as evident from VCR scores and SCMR values. Over three stages during both the years, majority of the RILs were showing IDC resistance rather than susceptibility which suggests dominance nature of IDC resistance compared to susceptibility in this RIL population. IDC resistance has been reported to be under the control of one or few dominant genes among several legumes including groundnut (Pattanashetti et al., 2018; Samdur et al., 2005), while some reports suggest polygenic inheritance with additive effect in soybean (Cianzio & Fehr, 1982) and tomato (Dasgan, Abak, Cakmak, Romheld, & Sensoy, 2004).

The BLUP values are more robust and better indicators of the phenotypic performance; hence, the QTLs identified based on BLUPs can be more precise and reliable. The QTL analysis using genotyping and phenotyping data identified a total of 32 QTLs located on eight LGs with PVE ranging from 3.9% to 31.8% (Table 5, Figure 3). Interestingly, majority of the identified QTLs were of minor effects, while three were of major effects. Maximum number of QTLs identified during 2014 (19 QTLs) compared to 2013 (13 QTLs) indicate more congenial environmental conditions for IDC severity in 2014. Considering QTLs for VCR and SCMR together across three stages over two years, the genomic regions between GM2308–GM626 on LG AhXIV harboured 12 QTLs, GM641–GM2602 on LG AhXV harboured 4 QTLs, while between Seq2C11–GM2259 harboured 2 major QTLs, hence they seem to be the major regions governing IDC resistance in groundnut. Several QTLs for VCR and SCMR identified in this study have been located to same position in comparison to previously identified QTLs for drought tolerance related traits on AhIV [Seq19D06-GM723b], AhVIII [IPAHM219-IPAHM606; (IPAHM229, GM2746, GM2689)-IPAHM123], AhIX [gi1107-Seq7G02], AhXII [RM11H01-TC11B04] and AhXIV [Seq16C06-RM14E11] using the same mapping population (TAG 24 × ICGV 86031] (Ravi et al., 2011). It was interesting to note that ICGV 86031 contributed favourable alleles for IDC resistance, that is, low VCR and high SCMR as evident from additive effects in the present study (Table 5).

Before this study, no literature was available on QTLs for IDC resistance in groundnut, so their role in controlling IDC has not been elucidated, and hence, no comparisons could be drawn. However, there has been preponderance of achievement documented in other legume/ staple food crops for IDC resistance. Genetic analyses have led to identification of SSR/AFLP markers associated with IDC resistance in crops like soybean (Butenhoff, 2015; Charlson, Bailey, Cianzio, & Shoemaker, 2005; Charlson et al., 2003) and mungbean (Sommanus, 2000; Srinives, Kitsanachandee, Chalee, Sommanas, & Chanprame, 2010). Mapping studies could detect QTLs associated with IDC resistance/ iron efficiency in soybean (Butenhoff, 2015; Lin et al., 1997) and mungbean (Prathet et al., 2012). According to "soybase website" (https://soybase.org), there are 40 QTLs identified for Fe efficiency. Some QTLs associated with seed mineral content for Fe and/or Zn, B, Mn and Cu have been reported in soybean (Bellaloui et al., 2015; King et al., 2013), Andean common beans (Blair, Astudillo, Rengifo, Beebe, & Graham, 2011), lentil (Aldemir

et al., 2014) and wheat (Genc et al., 2009). In Andean common bean (*Phaseolus vulgaris*), genes conditioning iron reductase activity in iron-sufficient plants appear to be associated with genes contributing to seed iron accumulation (Blair et al., 2011). Similarly in soybean, seeds with higher iron content were found to show higher degree of IDC resistance (King et al., 2013). Hence, seed Fe content and iron efficiency go together as significant correlations have been noted between them.

Several candidate genes associated with IDC resistance have been identified in soybean (Mamidi et al., 2011; Peiffer et al., 2012). For instance, a 12-bp deletion within the predicted dimerization domain in *Glyma03g28610* (bHLH gene) was found to hinder the FIT/bHLH heterodimer that induces iron acquisition genes like *FRO2* and *IRT1* thereby resulting in susceptibility to IDC in soybean (Peiffer et al., 2012). QTL analysis using high-density SNP genetic map in soybean identified seven major effect QTLs on seven chromosomes, wherein 12 candidate genes associated with iron metabolism were mapped near these QTLs supporting polygenic nature of IDC (Mamidi, Lee, Goos, & McClean, 2014). Early iron-efficiency response based on RNAseq in soybean roots and leaves could detect key changes for genes involved in hormone signalling, regulation of DNA replication and iron uptake utilization (Moran Lauter et al., 2014). Role of miRNA genes under Fe deficiency was assessed in *Arabidopsis*, wherein eight conserved miRNA genes were upregulated and 24 miRNA genes were found to contain cis-regulatory elements like IDE1/IDE2 (iron deficiency-responsive *cis*-Element 1 and 2; Kong & Yang, 2010). Hence, detailed studies on candidate genes and expression studies with diverse genetic material are essential in groundnut for in-depth understanding of the trait and in turn its utilization in GAB. The availability of reference genomes for diploid progenitors (Bertioli et al., 2016; Chen et al., 2016) and cultivated tetraploid (Bertioli et al., 2019; Chen et al., 2019; Zhuang et al., 2019) together with high-density genotyping SNP array "Axiom_Arachis" (Pandey et al., 2017) with 58K highly informative genome-wide SNPs have provided now further opportunities for fine mapping and candidate gene discovery for IDC resistance in groundnut.

5 | CONCLUSIONS

This study reports 32 QTLs for IDC resistance-associated traits VCR and SCMR across three stages over two years. Three major QTLs explaining 11%–31.8% PVE were identified on AhXIII and AhXV, while other QTLs of small effects having less than 10% PVE were identified on eight LGs. The LG AhXIV alone harboured 12 QTLs in the genomic region GM2308–GM626 and seems to be important for IDC resistance in groundnut. Further, the saturated linkage maps could be used to fine map these QTLs and also to find candidate genes controlling IDC resistance in groundnut. The identified markers associated with IDC resistance can be deployed in molecular breeding after validation across diverse genotypes for improving IDC resistance in groundnut.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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