RESEARCH

QTL Mapping of Ineffective Nodulation and Nitrogen Utilization-Related Traits in the IC-1 Mutant of Cowpea

Erik W. Ohlson, Sirando L. Seido, Suheb Mohammed, Carlos A. F. Santos, and Michael P. Timko*

ABSTRACT

Biological nitrogen fixation is a valuable component of sustainable agriculture. Improved understanding of nodulation pathways can facilitate enhancement of nitrogen utilization-related traits. Previously, a cowpea [Vigna unguiculata (L.) Walp.] mutant, 'IC-1', with ineffective nodules was identified. Heritability studies indicated that ineffective nodulation in IC-1 was controlled by a single gene designated cpi. To map cpi, F2 mapping populations were developed from crosses between IC-1 and two commercial cowpea cultivars. The F2 populations were evaluated for leaf and nodule color, nitrogen content, dry plant weight, nodule number, and nodule fresh weight. Nitrogen content, aboveground plant dry weight, and nodule fresh weight were each significantly lower in individuals with ineffective nodulation, whereas nodule number was significantly higher. Ninety individuals from one population were selected and genotyped with the Cowpea iSelect Consortium single-nucleotide polymorphism (SNP) array. In total, 6083 polymorphic SNPs were used for linkage map construction and quantitative trait loci (QTL) analysis. A major QTL accounting for nearly 50% of the phenotypic variance was identified on linkage group (LG) 4 associated with all traits except nodule fresh weight, whereas a minor QTL for nodule fresh weight was mapped to LG 6. The major QTL was validated in a second F2 population with several polymerase chain reaction markers. Identification of these QTL provides a foundation for future identification of the cpi gene and potential loci for targeted improvement of nitrogen-related traits in cowpea.

THE symbiotic relationship formed between legumes and rhizobia is a major contributor to the global nitrogen cycle. In exchange for biologically available nitrogen, legumes provide rhizobia with a microaerobic environment and carbon supply. Symbiotic nitrogen fixation is estimated to inject ~40 Tg of nitrogen into agricultural systems annually (Herridge et al., 2008). When used in crop rotations, legumes can alleviate the burden of artificial fertigation on farmers and the environment. Furthermore, legumes are a primary source of protein in many diets. Increased understanding of pathways involved in rhizobial colonization, nodule development, and nitrogen fixation is vital for improvement of symbiotic nitrogen fixation in legume crops (El Msehli et al., 2011; Udvardi and Poole, 2013).

Rhizobial establishment and development of functional nodules involves a complex series of steps beginning with host recognition, followed by nodule development, maintenance, and regulation. Plants attract rhizobia through secretion of flavonoids,

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activating the release of nodulation factors by rhizobia. Host recognition of nodulation factors induces infection thread formation. The bacteria travel through the infection thread, where they are ultimately deposited within the cytoplasm of cortical cells, forming the symbiosome. Finally, rhizobia in the symbiosome differentiate into their nitrogen-fixing form, where they are maintained and regulated by the host.

Natural and artificial mutants in model legumes Medicago truncatula Gaertn. and Lotus japonicas L. have been key tools for dissecting components of the nodulation pathway. A large proportion of reported nodulation genes were identified within these two species, from which at least 26 were cloned (Kouchi et al., 2010). These include genes facilitating nod signaling (Kouchi et al., 2010; Madsen et al., 2010; Murray, 2011; Donomkos et al., 2013; Oldroyd, 2013; Venkateshwaran et al., 2013), nodule number regulation (Krusell et al., 2002; Nishimura et al., 2002; Schnabel et al., 2005; Varma Penmetsa et al., 2008), bacterial infection (Arrighi et al., 2008; Yano et al., 2009; Kiss et al., 2009), nodule differentiation (Wang et al., 2010), nodule maintenance (Kumagai et al., 2007; Bourcy et al., 2013; Donomkos et al., 2013), and nutrient transport (Krusell et al., 2005; Bagchi et al., 2012). Additional nodulation genes and quantitative trait loci (QTL) have been reported in several crop species including pea (Pisum sativum L.) (Bourion et al., 2010), common bean (Phaseolus vulgaris L.) (Nodari et al., 1993; Tsai et al., 1998), and soybean [Glycine max (L.) Merr.] (Tanya et al., 2005; Nicolás et al., 2006; Santos et al., 2013; Hwang et al., 2014). More than 40 genes related to rhizobial symbiosis have also been reported in pea (Borisov et al., 2007), and QTL for root, nodule, and shoot variability were mapped (Bourion et al., 2010). In soybean, QTL governing nodule number, size, and weight were identified (Tanya et al., 2005; Nicolás et al., 2006; Santos et al., 2013; Hwang et al., 2014), as well as QTL regulating acetylene reduction and plant dry weight (Tanya et al., 2005). Several QTL for nodule number have also been mapped in common bean (Nodari et al., 1993; Tsai et al., 1998; Heilig et al., 2017).

Cowpea [Vigna unguiculata (L.) Walp.] is one of the most important legumes grown in the semiarid tropics including Asia, Africa, Southern Europe, the Southern United States, and Central and South America (Timko and Singh, 2008). Cowpea is especially valued in sub-Saharan Africa, where it is a primary source of protein. Improving the efficiency of nitrogen fixation in cowpea is an important objective due to the impact of nitrogen on yield-related traits and cowpea’s ability to enhance and grow in marginal environments. Previously, a cowpea mutant designated ‘IC-1’ was identified with ineffective nodules (fix−) (Pemberton et al., 1990). Acetylene reduction assays indicated no nitrogenase activity, consistent with the fix− phenotype. Additionally, IC-1 plants exhibit lighter leaf and nodule color and reduced root and shoot weights. Inheritance studies suggested the presence of a single, recessive gene, which was designated cpi (Pemberton et al., 1990). Despite the dearth of nitrogenase activity, IC-1 nodules contain both infection threads and bacteroids. Northern blot analysis of four nodulin genes indicated that VuENOD2, an early nodulin, was initially reduced, whereas VuB mRNA levels declined more slowly in IC-1. Additionally, late nodulins leghemoglobin and uricase were expressed at reduced levels. Combined, these results suggest that cpi may play a role in synthesis or maintenance of the peribacteroid membrane (Purdom and Trese, 1995). However, further research is needed to determine the function of cpi.

The objectives of this study were to improve the characterization of the fix− cowpea mutant IC-1 and map QTL associated with the ineffective nodulation phenotype.

MATERIALS AND METHODS

Plant Material and Population Development
An $F_2$ mapping population ($F_{2, MA} = 174$) was derived from a cross between the effectively nodulating (fix+) commercial cowpea cultivar ‘BRS Marataoa’ and the fix− mutant IC-1. Quantitative trait loci validation was performed in a second $F_2$ population ($F_{2, MA} = 175$) developed from a cross between commercial cowpea cultivar ‘BRS Pujante’ (fix+) and IC-1. For each population, IC-1 was used as the male parent. Both populations were grown at Embrapa Semiárido, Petrolina, Brazil.

Phenotypic Analysis
The two populations were grown in a protected environment under 70% shade cloth in 3-L plastic pots. Plants were irrigated daily with no supplemental fertigation. To ensure uniform colonization, all plants were inoculated with a cocktail of nitrogen-fixing bacterial strains BR 3262, BR 3267, and BR 3299. At planting and 5 d after emergence, inoculum was prepared and adjusted to $\sim 10^7$ cells mL$^{-1}$ and 2 mL was added to each pot. Bacterial strains were provided by Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil.

Each $F_2$ population was evaluated 40 d after emergence for six traits: leaf color (green/yellow), nodule color (dark/white), nitrogen concentration, aboveground plant dry weight, nodule number, and fresh nodule weight. Nitrogen concentrations were determined using the Kjeldahl method (Mendonça and Matos, 2005). Green leaf and dark nodule color or yellow leaf and white nodule color were used to classify $F_2$ individuals as fix+ or fix−, respectively.

Genotypic Analysis
DNA was extracted from parental replicates and $F_1$ individuals using a modified cetromonium bromide (CTAB) protocol (Doyle and Doyle, 1987). Ninety of the $F_{2, MA}$ individuals and three replicates of each parent were genotyped using the Cowpea iSelect Consortium Array, which consists of 51,128 single-nucleotide polymorphisms (SNPs, WG-401-1002, Illumina). Genotyping was performed on an iSCAN System bead chip reader at
the Center for Public Health Genomics (University of Virginia School of Medicine, Charlottesville, VA). The SNPs were called and filtered in GenomeStudio 2.0 (Illumina, 2016) based on guidelines outlined in the Illumina genotyping technical notes (Illumina, 2014) and a previously developed cowpea cluster file (Muñoz-Amatriain et al., 2017). Genotype data were exported from GenomeStudio and additional filtering was applied based on parental calls and expected \( F_{2} \) segregation. An IC-1 replicate was removed from consideration due to high heterozygosity (>20%), and markers were excluded if parental genotypes were heterozygous or polymorphic among replicates. Informatively SNPs were identified between IC-1 and BRS Marataoã and used for linkage map construction and QTL analysis. A single \( F_{2}^{MA} \) sample was excluded due to suspected sample contamination or outcrossing during population development.

Chi-square tests (\( \alpha = 0.01 \)) were performed to identify and remove markers for which \( F_{2} \) segregation deviated significantly from the expected ratio.

**Linkage Map Development**

A genetic linkage map was developed using MSTmap with Kosambi mapping function and grouping logarithm of odds (LOD) 7 (Wu et al., 2007, 2008). The map order for each \( F_{2} \) individual was inspected, and questionable SNP calls were excluded to reduce the effects of genotyping error and residual heterozygosity in parental lines on linkage and QTL analysis. Linkage group (LG) number and orientation were assigned according to the cowpea reference genome (Vigna unguiculata v1.0, NSF, UCR, USAID, DOE-JGI, http://phytozome.jgi.doe.gov/).

**QTL Validation**

After the detection of a major QTL on LG 4 for five of the six measured traits, validation of the QTL was performed using \( F_{2}^{MA} \). Five tetra-primer amplification-refractory mutation system (ARMS)-polymerase chain reaction (PCR) markers (Ye et al., 2001) were developed from SNPs tightly linked to the QTL and used for genotyping. The PCR was performed in 20-\( \mu \)L reactions containing 1× Taq buffer, 200 \( \mu \)M deoxynucleotides, 50 ng of genomic DNA, appropriate primer, and MgCl\(_{2}\).

**Table 1. Tetra-primer amplification-refractory mutation system (ARMS) markers on linkage group 4 used for validation of ineffective nodulation quantitative trait loci (QTL) in the ‘BRS Pujante’ × ‘IC-1’ \( F_{2} \) population.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Primer sequence (( 5' \rightarrow 3' ))</th>
<th>Primer concentration</th>
<th>( T_{a} )</th>
<th>( Mg^{2+} )</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>2_00349</td>
<td>Forward inner AACTAGATGATGATATACCCCTTCTTTCCGG</td>
<td>0.25</td>
<td>55</td>
<td>2.0</td>
<td>202</td>
</tr>
<tr>
<td></td>
<td>Reverse inner AGCAGTCGCTAAATTAGTGGACAACGTT</td>
<td>0.25</td>
<td>236</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward outer GTGTTCTTCTAGGTTTCTTTACCAT</td>
<td>1.50</td>
<td>380</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse outer GGTATGTTAGAGGGATGAACTTTTGG</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2_00352</td>
<td>Forward inner TGGCAAAAGTTCTTGGAACCATTTCC</td>
<td>0.25</td>
<td>55</td>
<td>3.0</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>Reverse inner AGTAATCTGTTGACCACCCCAACAT</td>
<td>0.10</td>
<td>235</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward outer TTTGCTTTCTGATTGAAAATCAGTT</td>
<td>1.00</td>
<td>387</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse outer AAAACTACCAACCCAGCCATACAGAC</td>
<td>2.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2_15618</td>
<td>Forward inner TTTAAGATGTTGTGTTATGACCTGTTA</td>
<td>0.25</td>
<td>60</td>
<td>2.5</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td>Reverse inner GCCAAATCTATGATAAAAATCAAATCCC</td>
<td>0.25</td>
<td>192</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward outer GTATTGTGTTGAAAACCGAGATGTATT</td>
<td>1.00</td>
<td>371</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse outer TTTCTCTTTGAGACCCTTCTAAC</td>
<td>1.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2_16203</td>
<td>Forward inner TTTGATGTTATTTATGAGAGCTGCA</td>
<td>0.25</td>
<td>55</td>
<td>3.0</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>Reverse inner ATGGCCTAAATAATAAAATAGACACCAAC</td>
<td>0.25</td>
<td>261</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward outer TTATACTGTTATGCTGTTTGG</td>
<td>1.50</td>
<td>442</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse outer AATTACAGATGTTGAAATGAGTTTCCC</td>
<td>2.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2_24723</td>
<td>Forward inner ATGCATCCACAGTGAAATTCTTGTGAT</td>
<td>0.10</td>
<td>55</td>
<td>2.0</td>
<td>246</td>
</tr>
<tr>
<td></td>
<td>Reverse inner TTGCACTAGATGAAATCAATGAGCAAC</td>
<td>0.10</td>
<td>223</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward outer TTTACACAAGCCTACGAGGACTAACTT</td>
<td>1.00</td>
<td>412</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse outer AGGTGTACAGATGAAATGACAAAG</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
concentrations (Table 1), 50 ng DNA, and 1 U Taq polymerase. The PCR conditions consisted of 2 min denaturation (95°C); 35 cycles of 1 min denaturation (95°C), 1 min annealing (55–60°C), and 1 min extension (72°C); and 2 min final extension (72°C). Amplicons were visualized on 2% agarose gels stained with ethidium bromide.

**Statistical Analysis**

Statistical analyses were performed using IBM SPSS Statistics version 24 (IBM Corporation, 2016). Multiple comparisons of mean phenotypes were tested via Tukey’s honest significant difference test with $\alpha = 0.05$. Visual representations of LGs and LOD scores were developed in MapChart 2.3 (Voorrips, 2002).

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**Fig. 1.** Frequency distributions of nitrogen-related traits in two cowpea F2 populations derived from the crosses ‘BRS Marataoa’ × ‘IC-1’ ($n = 174$) and ‘BRS Pujante’ × ‘IC-1’ ($n = 175$). Phenotypic data were collected from F2 cowpea grown in pot trials at Embrapa Semiárido, Petrolina, Brazil.
RESULTS
Phenotypic Analysis
Nodule and leaf color cosegregated in all F₂ plants and were used to designate individuals from each population into fix+ and fix− phenotypic classes. The F₂⁴MA consisted of 128 fix+ and 46 fix− phenotypes and approximated a 3:1 distribution (χ² = 0.10). However, a higher-than-expected number of plants were fix+ (n = 149) in F₂⁴PJ, compared with just 26 fix− individuals. Similarly, genotypic segregation on LG 4 was skewed towards the BRS Pujante genotype in F₂⁴PJ. Genotyping of F₂⁴PJ using markers located near the top of LG 4 indicated that 23 plants were homozygous IC-1, 78 were heterozygous, and 74 were homozygous BRS Pujante.

Mean nitrogen content differed significantly between the three parental genotypes, averaging 6.2 g kg⁻¹ in IC-1 compared with 19.6 and 16.3 g kg⁻¹ in BRS Marataoa and BRS Pujante, respectively. No statistical differences were found between F₁ generations, fix+ F₂ individuals, or their respective fix+ parents. Contrastingly, the mean nitrogen concentrations of fix− individuals in the two mapping populations were significantly diminished and most similar to IC-1 (Fig. 1, Table 2).

Plant dry weight for each parent ranged from 1.1 g in IC-1 to 3.4 g in BRS Pujante. No statistical differences were observed in mean dry weight among the fix+ parents, F₁ generations, or fix+ F₂ individuals. Additionally, no statistical differences were identified between the F₂ fix− individuals and IC-1 (Fig. 1, Table 2).

Mean nodule number varied significantly among all three parental genotypes, averaging 36.2 g kg⁻¹ in IC-1 compared with 21.4% of the phenotypic variance. A single QTL for nodule number mapped between SNP markers 2_12850 and 2_54418 near the top of LG 4 and accounted for 42.6 to 49.3% of the phenotypic variance. A single QTL for nodule color detected the presence of a single recessive allele. The categorical trait interval mapping of leaf and nodule color detected the presence of a single recessive QTL. The QTL mapped to between 0.8 and 4.0 cM on LG 4 with a LOD score of 17.9 (Table 3, Fig. 2). The location corresponded with QTL for three of the four continuous traits detected via ICIM, as well as the joint-trait analysis. Major QTL for nitrogen content, dry plant weight, and nodule number mapped between SNP markers 2_12850 and 2_54418 near the top of LG 4 and accounted for 42.6 to 49.3% of the phenotypic variance. A single QTL for nodule fresh weight mapped between 33.5 and 34.5 cM on LG 6, flanked by markers 2_11936 and 2_49231, and accounted for 21.4% of the phenotypic variance. The joint-trait analysis mapped a single QTL to a similar interval on LG 4, as

Table 2. Means and standard deviations (mean ± SD) of nitrogen-related traits in parental lines and F₂ and F₃ generations derived from ‘BRS Marataoa’ × ‘IC-1’ and ‘BRS Pujante’ × ‘IC-1’. Populations were phenotyped 40 d after emergence. Nodulation effective (fix+) and ineffective (fix−) phenotypic classes were assigned according to leaf and nodule color where yellow leaves and white nodules were considered fix− and green leaves and dark nodules were considered fix+. Multiple comparisons of phenotypic classes were performed via Tukey’s honest significant difference and groups are indicated by the letters a through e.

<table>
<thead>
<tr>
<th>Genotype or population</th>
<th>No. of plants</th>
<th>Nitrogen</th>
<th>Shoot and leaf dry mass</th>
<th>Nodule no.</th>
<th>Nodule mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g kg⁻¹</td>
<td>g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC-1</td>
<td>45</td>
<td>6.2 ± 1.2a</td>
<td>1.10 ± 0.41a</td>
<td>125 ± 34a</td>
<td>0.40 ± 0.17a</td>
</tr>
<tr>
<td>BRS Marataoa</td>
<td>45</td>
<td>19.6 ± 3.5e</td>
<td>3.38 ± 0.82b</td>
<td>50 ± 16b</td>
<td>0.59 ± 0.24b</td>
</tr>
<tr>
<td>BRS Pujante</td>
<td>45</td>
<td>16.3 ± 3.6cd</td>
<td>3.41 ± 1.14b</td>
<td>72 ± 22c</td>
<td>0.54 ± 0.19b</td>
</tr>
<tr>
<td>BRS Marataoa × IC-1 F₁</td>
<td>60</td>
<td>18.5 ± 4.9de</td>
<td>3.41 ± 0.98b</td>
<td>51 ± 23b</td>
<td>0.59 ± 0.21b</td>
</tr>
<tr>
<td>BRS Pujante v IC-1 F₁</td>
<td>60</td>
<td>15.4 ± 3.6c</td>
<td>3.39 ± 0.83b</td>
<td>75 ± 26c</td>
<td>0.56 ± 0.18b</td>
</tr>
<tr>
<td>F₂ BRS Marataoa × IC-1 (fix+)</td>
<td>128</td>
<td>19.1 ± 5.6e</td>
<td>3.57 ± 1.42b</td>
<td>44 ± 17b</td>
<td>0.62 ± 0.25b</td>
</tr>
<tr>
<td>F₂ BRS Marataoa × IC-1 (fix−)</td>
<td>46</td>
<td>8.9 ± 3.7b</td>
<td>0.83 ± 0.30a</td>
<td>86 ± 30c</td>
<td>0.28 ± 0.17a</td>
</tr>
<tr>
<td>F₂ BRS Marataoa IC-1 (Total)</td>
<td>174</td>
<td>16.4 ± 6.9bc</td>
<td>2.84 ± 1.73b</td>
<td>55 ± 28b</td>
<td>0.53 ± 0.27b</td>
</tr>
<tr>
<td>F₂ BRS Pujante v IC-1 (fix+)</td>
<td>149</td>
<td>15.0 ± 4.1c</td>
<td>3.69 ± 1.42b</td>
<td>72 ± 28c</td>
<td>0.54 ± 0.23b</td>
</tr>
<tr>
<td>F₂ BRS Pujante v IC-1 (fix−)</td>
<td>26</td>
<td>7.0 ± 3.0ab</td>
<td>1.00 ± 0.28a</td>
<td>126 ± 36a</td>
<td>0.38 ± 0.20a</td>
</tr>
<tr>
<td>F₂ BRS Pujante × IC-1 (total)</td>
<td>175</td>
<td>13.8 ± 4.9b</td>
<td>3.29 ± 1.63b</td>
<td>80 ± 35c</td>
<td>0.52 ± 0.23b</td>
</tr>
</tbody>
</table>
previously identified by ICIM (Table 3, Fig. 2). However, no additional QTL were detected via this method.

Leaf and nodule color QTL were validated by genotyping $F_2$ PJ with several PCR-based SNP markers. Genotyping of 175 $F_2$ individuals with five tetra-primer ARMS-PCR markers (Table 1) confirmed a strong association of the locus with the fix$^-$ phenotype. Nearly all $F_2$ PJ phenotypes matched the expected parental genotype (i.e., fix$^-$ genotyped homozygous IC-1). However, correspondence of phenotypes and genotypes for seven individuals (4%) deviated from expectations, suggesting incomplete linkage of the markers with the fix$^-$ gene.

DISCUSSION

Pemberton et al. (1990) previously reported a single recessive gene controlled the fix$^-$ phenotype in IC-1. This conclusion is supported by 3:1 segregation in $F_2^{MA}$ for leaf and nodule color and consistent with the majority of nodulation ineffective mutants previously identified (Bhatia et al., 2001). However, $F_2^{PJ}$ did not segregate in a 3:1 ratio. Despite abnormal segregation, there was near-complete
correspondence of the fix+ phenotype with the fix+ genotype and the fix− phenotype with the fix− genotype.

The fix− mutation in IC-1 influenced several nitrogen-related traits. Significant differences between fix+ and fix− individuals were found for all reported characters including lower nitrogen content, reduced plant and nodule weight, and increased nodule number. Reduced plant weight and nodule size in conjunction with increased nodule number were consistent with previous characterizations of IC-1 (Pemberton et al., 1990). Furthermore, several studies in soybean have reported strong correlation between greater nodule number and smaller nodule size (Purcell et al., 1997; King and Purcell, 2001; Santos et al., 2013; Hwang et al., 2014).

The F$_2$MA linkage map (Supplemental Table S1) corresponds well with the cowpea consensus map and previously reported cowpea maps (Lucas et al., 2011; Muñoz-Amatriain et al., 2017). Although the genome size in this study is shorter than the consensus map (735.6 vs. 837.1 cM), the result is consistent with potential effects of a smaller population size and fewer recombination events occurring in an F$_2$ population compared with the recombinant inbred line populations used in consensus map development (Ferreira et al., 2006; Muñoz-Amatriain et al., 2017).

We performed QTL mapping in F$_2$MA for six traits associated with ineffective nodulation. Mapping of leaf and nodule color identified a major QTL near the top of LG 4, suggesting that the fix− phenotype in IC-1 is controlled by a single gene, which is consistent with the 3:1 phenotypic segregation of leaf and nodule color (Table 3). Validation of the QTL in a second population indicated nearly 100% correspondence between fix− phenotype and genotype, providing a high level of confidence in the fidelity of the QTL. Furthermore, ICIM and multiple-trait composite interval mapping identified QTL for nitrogen content, dry weight, and nodule number at the same locus. However, the nodule fresh weight QTL did not colocalize with the other five traits. It is likely that the reduced individual nodule weights of fix− individuals were masked by the increased abundance of nodules produced by fix− individuals. However, colocalization for five of the six traits measured in this study suggests that a single, pleiotropic gene is most likely responsible for influencing several nodulation and nitrogen utilization traits in IC-1.

Examination of cowpea genome annotations within the LG 4 QTL indicates the presence of ~160 genes (Vigna unguiculata v1.0, NSF, UCR, USAID, DOE-JGI, http://phytozome.jgi.doe.gov/). Intriguing candidate genes include an aluminum-activated malate transporter gene, since malate is the primary source of carbon exported by legumes for rhizobial consumption (Udvardi and Poole, 2013), and a sulfate transporter gene (Krusell et al., 2005). Although large numbers of SNP markers were used in this study, no polymorphic SNPs were found between flanking markers corresponding to the fix− QTL. Consequently, additional markers may be needed to perform fine mapping and cloning of cpi.

The fix− cowpea variety IC-1 is a valuable model for studying ineffective nodules and their effects on nitrogen utilization. In addition to confirming reduced plant and nodule mass and increased nodule number in fix− individuals (Pemberton et al., 1990), this study identified significantly lower nitrogen content associated with the ineffective nodulation phenotype (Table 2). Furthermore, we report the identification of a major QTL associated with several nitrogen-related characteristics (Fig. 2, Table 3). The results of this study provide a strong foundation for future identification of the cpi gene.

**Supplemental Material Available**

Supplemental material for this article is available online.

**Acknowledgments**

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