1 # A multi-parent advanced generation inter-cross population for genetic
2 # analysis of multiple traits in cowpea (*Vigna unguiculata* L. Walp.)

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25 ## Abstract

26 Development and analysis of Multiparent Advanced Generation Inter-Cross (MAGIC) populations
27 have been conducted with several crop plants to harness the potential for dissecting the genetic
28 structure of traits and improving breeding populations. We developed a first MAGIC population
29 for cowpea (*Vigna unguiculata* L. Walp.) from eight founder parents which are genetically diverse
30 and carry many abiotic and biotic stress resistance, seed quality and agronomic traits relevant to
31 cowpea improvement in sub-Saharan Africa (SSA) where cowpea is vitally important in the human
32 diet and in local economies. The eight parents were inter-crossed using structured matings to
33 ensure the population would have balanced representation from each of the founder parents,
34 followed by single-seed descent, resulting in 365 F8 recombinant inbred lines (RILs) each carrying
35 a mosaic of genome blocks contributed from all founders. This was confirmed by SNP genotyping
36 with the cowpea Illumina 60K iSelect BeadArray. Following filtering to eliminate duplicates,
37 sister lines and accidental selfing events, a core set of 305 F8 RILs was chosen as the primary
38 population. The F8 lines were on average 99.74% homozygous while also diverse in agronomic
39 traits including flowering time, growth habit, maturity, yield potential and seed characteristics
40 across environments. Trait-associated SNPs were identified for most of the parental traits. Loci
41 with major effects on photoperiod sensitivity and seed size were also verified by genetic mapping
42 in biparental RIL populations. The distribution of recombination frequency varied considerably
43 between chromosomes, with recombination hotspots distributed mostly in the telomeric regions.
44 Due to its broad genetic base, this cowpea MAGIC population promises breakthroughs in genetic
45 gain and high-resolution genetic mapping for gene discovery, enhancement of breeding
46 populations and, for some lines, direct releases as new varieties.

Key words

Association Mapping; Multiparental Populations; MPP; Quantitative Trait Locus; Recombination; Photoperiod; Flowering Time; Seed Size; Legumes

Introduction

Cowpea (*Vigna unguiculata* L. Walp) is a highly nutritious warm-season grain legume crop vitally important for food security in Africa where it provides a primary source of protein that complements cereals in the diet (EHLERS and HALL 1997; KUDRE *et al.* 2013) and fodder for livestock. However, in the Sudano-Sahel region of West Africa typical smallholder farmer cowpea grain yields are only 10-20 % of known yield potential (WIDDERS 2012). Biotic stresses caused by insect pests, and diseases caused by pathogens, the parasitic weed *Striga gesnerioides* and nematodes, and abiotic stresses from heat, drought and low-fertility soils are primary constraints to cowpea grain production. Many of these problems also affect cowpea production in parts of southern Europe, Asia, Australia, Latin America, and southern United States (EHLERS and HALL 1997; HUYNH *et al.* 2013a). Development of cowpea cultivars that tolerate or resist these constraints will increase yield and reduce costly chemical based crop-protection inputs and promote human and environmental health, thus directly benefitting resource-poor farmers.

The greatest opportunity to increase cowpea grain yields lies in the rich genetic variation within this diploid (2n = 22) species as it has numerous resistance and tolerance traits to combat biotic and abiotic stresses (HUYNH *et al.* 2013b; MUCHERO *et al.* 2013). Many of these traits have been genetically mapped using QTL discovery and mapping approaches (OUÉDRAOGO *et al.* 2002b; MUCHERO *et al.* 2010; MUCHERO *et al.* 2011; LUCAS *et al.* 2012; OUÉDRAOGO *et al.* 2012; POTTORFF *et al.* 2012; POTTORFF *et al.* 2014; HUYNH *et al.* 2015; HUYNH *et al.* 2016). Cowpea has the capacity to produce grain under magnitudes of water stress that would render comparable crops unproductive (EWANSIHA and SINGH 2006), yet significant differences in drought tolerance exist among cowpea lines at different stages of growth (WATANABE *et al.* 1997; MAI-KODOMI *et al.* 1999a). For example, there are significant phenotypic differences in ability to survive vegetative-stage drought stress (MAI-KODOMI *et al.* 1999b; MUCHERO *et al.* 2013), providing opportunity for cowpea breeders to incorporate early-season drought tolerance into improved varieties. Among genotypes exhibiting seedling drought tolerance, two types of responses were observed by Mai-Kodomi et al. (1999a). Type 1 response plants ceased all growth and conserved moisture in all plant tissues, thereby allowing subsequent recovery of the entire shoot upon re-hydration. In contrast, type 2 response involved plants mobilizing moisture from lower leaves to sustain growth of new trifoliates, with rapid senescence of unifoliates at the onset of water-stress conditions. Mid- and late-season drought stresses have received considerable attention, given their negative effects on yield parameters (HALL *et al.* 2003; PADI 2004; DADSON *et al.* 2005). On a physiological level, osmotic adjustment, carbon isotope discrimination, transpiration, assimilation rates, and stomatal conductance in cowpea have been studied in detail (HUSSAIN *et al.* 1999; ANYIA and HERZOG 2004; ODOEMENA 2004). In many cases, however, results were inconclusive or no meaningful differentiation between genotypes was achieved. Morphological investigations have tended to focus on root-related parameters where genotypes were compared for rooting depth and relative root biomass (MATSUI and SINGH 2003; OGBONNAYA *et al.* 2003). Phenologically, flowering and maturation times have been investigated for drought escape-related strategies (GWATHMEY and HALL 1992). Early maturing varieties may be able to complete their reproductive cycle in time to escape late-season drought (GRANTZ and HALL 1982; EHLERS and HALL 1997), but these varieties

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92   were sensitive to mid-season drought (THIAW *et al.* 1993). Early flowering coupled with the
93   delayed leaf senescence trait, which later promotes survival during mid-and late-season drought,
94   allowing plants to produce a second flush of pods, offers the greatest potential for managing both
95   mid- and late-season drought conditions (GWATHMEY and HALL 1992). Association mapping
96   identified multiple loci with pleiotropic effects on drought-related traits in cowpea across
97   experiments in West Africa under limited water conditions (MUCHERO *et al.* 2013). Because
98   drought tolerance is a complex trait, its genetic improvement combined with selection for biotic
99   resistance would need a systematic breeding strategy involving multiple trait donors.

100   Development of multi-parent advanced generation intercross (MAGIC) populations, termed by
101   Cavanagh *et al.* (2008), provides a state-of-the-art approach to advancing plant population
102   resources for genetic analysis and breeding. It involves inter-mating multiple elite parents for
103   several cycles followed by single-seed descent, resulting in recombinant inbred lines (RILs) each
104   carrying a mosaic of genome blocks contributed from all founders. Development and analysis of
105   MAGIC populations have been undertaken in a few crops including wheat, rice and chickpea due
106   to the potential of this approach for dissecting genetic and genomic structure (HUANG *et al.* 2015).
107   The goal of the current work was to develop an 8-parent MAGIC population for cowpea using
108   founder parents that are highly diverse and carry many key traits relevant to cowpea production in
109   SSA. In this paper, we report on the development, genetic analysis and validation of this new
110   genetic resource using high-density marker genotyping platforms (MUCHERO *et al.* 2009a). Due to
111   its broad genetic base, the cowpea MAGIC population provides opportunities for increasing
112   genetic gain and QTL/gene discovery in cowpea and related species.

113

Materials and Methods

115   *Choice of parents*

116   The eight cowpea parents used in the original crosses were elite cultivars and breeding lines
117   selected based on their high genetic diversity characterized by genotyping with 1,536 genome-
118   wide gene-based SNP markers (MUCHERO *et al.* 2009a; MUÑOZ-AMATRIAÍN *et al.* 2017). In
119   addition, they were chosen because collectively they carry multiple biotic and abiotic stress
120   resistance and tolerance traits relevant to SSA (Table 1). SuVita 2, also known as 'Gorom', a local
121   landrace in Burkina Faso, is resistant to the parasitic weed Striga (OUÉDRAOGO *et al.* 2002b) and
122   the fungal pathogen *Macrophomina phaseolina* (MUCHERO *et al.* 2011). CB27, a California
123   blackeye cultivar bred by University of California–Riverside (UCR) (EHLERS *et al.* 2000), is heat
124   tolerant (LUCAS *et al.* 2013a) and highly resistant to root-knot nematodes (HUYNH *et al.* 2016),
125   Fusarium wilt disease (POTTORFF *et al.* 2012; POTTORFF *et al.* 2014), and foliar thrips (LUCAS *et*
126   *al.* 2012). IT93K-503-1, a breeding line from the International Institute of Tropical Agriculture
127   (IITA) breeding nursery in Nigeria, is drought tolerant (MUCHERO *et al.* 2009b), and resistant to
128   root-knot nematodes (HUYNH *et al.* 2016), *M. phaseolina* (MUCHERO *et al.* 2011), and Fusarium
129   wilt (POTTORFF *et al.* 2014). The other five parents (IT89KD-288, IT84S-2049, IT82E-18, IT00K-
130   1263, and IT84S-2246) are also breeding lines from IITA, Nigeria; they also carry combinations
131   of key traits including grain quality and resistance to root-knot nematode, Striga, Fusarium,
132   viruses, and bacterial blight (Table 1).

Table 1. MAGIC founder parents and their traits relevant to SSA and other production areas

Name	Source[a]	Agronomic trait	Resistance or tolerance trait
SuVita 2	INERA	High yielding under drought in Senegal, Burkina Faso and Mozambique; large dark-brown seed	Drought tolerant, resistant to Striga, foliar thrips and *Macrophomina* disease
CB27	UCR	High yielding under drought in Mozambique; large black-eye seed; photoperiod insensitive; erect growth habit; early maturing	Heat tolerant, resistant to root-knot nematode, Fusarium wilt, and foliar thrips
IT93K-503-1	IITA	High yielding under drought in Senegal; brown-eye seed; stay-green under drought	Drought tolerant, resistant to nematodes, Fusarium wilt, and *Macrophomina*
IT89KD-288	IITA	High yielding under drought in Burkina Faso and Nigeria; brown-eye seed; photoperiod sensitive	Root-knot nematode resistant
IT84S-2049	IITA	High yielding under drought in Burkina Faso; brown-eye seed; erect growth habit	Resistant to aphid, bacterial blight, viruses, root-knot nematode
IT82E-18	IITA	High yielding under drought in Mozambique; early maturing, light-brown seed; photoperiod insensitive	Broadly adapted, resistant to root-knot nematode
IT00K-1263	IITA	High yielding under drought in Mozambique and Nigeria; dark-brown seed; stay-green under drought	Resistant to Striga, aphid, fusarium wilt, root-knot nematode
IT84S-2246	IITA	High yielding under drought in Burkina Faso and Mozambique; dark-brown seed	Resistant to aphid, bacterial blight, viruses, root-knot nematode

[a] INERA: Institut de l'Environment et des Recherches Agricole, Burkina Faso; UCR: University of California − Riverside, United States; IITA: International Institute of Tropical Agriculture, Nigeria.

Population development

The eight parents were inter-mated using a mating strategy described in Cavanagh et al. (2008) with some modifications. In spring 2010, initial crosses were made between 4 pairs of founder parents (IT89KD-288 x IT84S-2049, CB27 x IT82E-18, SuVita 2 x IT00K-1263, and IT84S-2246 x IT93K-503-1, designated as A x B, C x D, E x F, and G x H, respectively) to produce 2-way F1s. In spring 2011, reciprocal 4-way crosses were made between 2 pairs of the 2-way F1s to produce 4-way F1s. In fall 2011, 330 pair crosses were made between 330 4-way F1 plants of the pedigrees ABCD and 330 4-way F1 plants of the pedigrees EFGH to produce 331 8-way F1s. Single seed descent (SSD) was then applied for each unique 8-way F1 until the F8 generation. For each F8 RIL, seeds from the F8 single plant were harvested and maintained as an original seed stock (F8:9). The F8:9 seeds were then increased in bulk to make F8:10 seeds for phenotyping.

149 *SNP genotyping*

150 The F1 progeny from 2-way crosses were verified by genotyping their F2 seeds (up to 21 seeds
151 per cross) with 89 parent-unique SNPs using the kompetitive allele-specific polymerase chain
152 reaction (KASP) cowpea assay (LGC Genomics Ltd., Hoddesdon, UK) (SEMAGN *et al.* 2014),
153 which was converted from the 1536 SNP Illumina GoldenGate Assay developed by Muchero et
154 al. (2009a). True F1 plants were confirmed when polymorphic markers were found segregating in
155 the corresponding F2 progeny.

156 The F8 single plants derived from 8-way crosses were genotyped with 51,128 SNPs using the
157 Illumina iSelect BeadArray (MUÑOZ-AMATRIAÍN *et al.* 2017). A core set of MAGIC RILs was
158 selected through the following consecutive steps: (1) Lines carrying non-parental alleles or excess
159 numbers of heterozygous and ambiguous genotypes were excluded; (2) Based on parent-unique
160 SNPs, lines that did not carry male-parent alleles (i.e., selfing) at the 4-way or 8-way crosses were
161 also excluded; (3) Among true 8-way RILs and eight parents, genetic similarities were measured
162 using the allele-sharing method (BOWCOCK *et al.* 1994) with the software GGT 2.0 (VAN BERLOO
163 2008), from which phylogenetic relationships were generated using the neighbor-joining method
164 (Saitou and Nei, 1987) and visualized using the software MEGA 5.05 (Tamura et al., 2011); and
165 (4) For each set of genetically identical RILs (similarity 0.99 or higher), the line with the lowest
166 number of ambiguous genotypes was retained in the core set for further analyses.

167

168 *MAGIC phenotyping*

169 The MAGIC RILs and parents were screened for photoperiod sensitivity under long-daylength
170 conditions during summer, from June (14.5 hours) to September (12.8 hours), at the UCR Citrus
171 Experiment Station, California (UCR-CES, 33.97°N, 117.34°W). In 2015, each MAGIC RIL and
172 parent was planted in one row of 0.76 m wide and 5.5 m long at a density of 12 seeds per meter
173 using a tractor-mounted planter. The field was watered to capacity before and after planting up to
174 100 days using furrow irrigation. The experiment was repeated in 2016 but under restricted
175 irrigation. The field was watered to capacity before planting, and then irrigation was withheld until
176 the end of trial. For each line in both trials, calendar days to flowering were determined when 50%
177 of plants in the plot flowered.

178 The population was also screened under short daylength conditions during autumn, from
179 September (12.8 hours) to December (9.9 hours), at the Coachella Valley Agricultural Research
180 Station, California (CVARS, 33.52°N, 116.15°W). In 2015, the population was planted in two
181 blocks receiving different watering regimes (full irrigation and restricted irrigation) and separated
182 by a 6-row buffer (5 m). In each block, each MAGIC RIL and parent was planted in one row of
183 0.76 m wide and 3.5 m long at a density of 12 seeds per meter using a tractor-mounted planter.
184 The field was watered to capacity before and after planting using subsurface drip irrigation. After
185 two weeks when the seedlings were well established, the irrigation was withheld in the restricted-
186 irrigation block until maturity, whereas in the full-irrigation block the rows were watered to
187 capacity up to 100 days after planting. In 2016, the two experiments (full irrigation and restricted
188 irrigation) were repeated on adjacent field blocks at CVARS. For each line in four experiments,
189 calendar days to flowering were determined when 50% of plants in the plot flowered. Plant growth
190 habit was measured 40 days after planting using a visual rating scale from 1 to 6 based on the
191 angles formed between primary branches and the main stem: (1) Acute erect, branches form angles
192 less than 45° with the main stem, (2) Erect, branching angles between 45° – 90° with the main
193 stem, (3) Semi-erect, branches perpendicular to the main stem but not touching the ground, (4)

194 Intermediate, lower branches touching the ground, (5) Semi-prostrate, lower branches flat on the
195 ground but the main stem standing upright, and (6) Prostrate, the entire plant flat and spreading on
196 the ground. Days to maturity were determined when 95% of pods in the plot had dried. At maturity,
197 the plants in each plot were cut at the lower stems and machine-threshed for measurement of plot
198 yield and 100-seed weight.

199 For each set of repeated trials at UCR-CES and CVARS, analysis of variance (ANOVA) was
200 performed with the software GenStat version 11 (PAYNE *et al.* 2008). Factors in the ANOVA
201 model were lines and block, with each field site receiving one watering treatment considered as a
202 block. Broad-sense heritability was estimated based on the variance component attributable to
203 variation among lines (VG) and residual variation (VE) (h^2 = VG/(VG + VE)). Nonparametric
204 Spearman's rank correlation analysis was also used to examine the consistency in genotypic
205 ranking of the same lines between environments.

206

207 *Association mapping*

208 Polymorphic SNPs (success rate > 90% and minor allele frequency > 0.05) with known positions
209 across 11 cowpea pseudomolecules (LONARDI *et al.* 2017) were used for genetic mapping, with a
210 new chromosome numbering convention based on synteny between cowpea and common bean.
211 Genome-wide association studies (GWAS) were performed with the software TASSEL 5.0
212 (BRADBURY *et al.* 2007) using the mixed linear model (MLM) function that incorporated
213 population principal components and kinship analyses. A LOD threshold of 4 was used to indicate
214 QTL significance.

215 To verify QTL detected for photoperiod sensitivity in the MAGIC population, a biparental
216 mapping population including 92 F8-derived F9 RILs from a cross between the non-photoperiod
217 sensitive parent CB27 and the photoperiod sensitive IT97K-556-6 was screened under long-
218 daylength conditions at UC Riverside in 2016. Each RIL and parent were planted in one row 0.76
219 m wide and 5.5 m long at a density of 12 seeds per meter using a tractor-mounted planter. The
220 planting time and experimental conditions were similar to the MAGIC phenotyping trial in 2015.
221 For each plot, days to flowering were determined when 50% of plants in the plot flowered. The
222 biparental RIL population was genotyped with the 51,128 SNP Illumina iSelect BeadArray that
223 was used to genotype the MAGIC population. Construction of genetic maps and QTL analysis
224 were performed with the software QTL IciMapping 4.0 (MENG *et al.* 2015) using the Inclusive
225 Composite Interval Mapping (ICIM) method (WANG 2009).

226

227 *Recombination analysis*

228 Based on physical positions of polymorphic SNPs on 11 cowpea pseudomolecules, pair-wise
229 linkage distances (in centimorgans) between every two adjacent SNPs were estimated as d =
230 [(*recombinants*/n) x 100], where *recombinants* are MAGIC lines that carry SNP genotypes that
231 are not present in any of the eight parents (Supplemental File S1), and n is the number of MAGIC
232 lines carrying valid SNP genotypes. Using a sliding window of 2 Mb with 1 Mb increments along
233 each chromosome, recombination rates (cM/Mb) were calculated as the linkage distance divided
234 by the physical distance between the first and the last SNP of each window. The recombination-
235 rate variation was visualized by plotting the estimated recombination rate for every 1-Mb
236 increment along the 11 chromosomes.

6

237 *Resource and data availability*

238 The MAGIC core set and their 8 founder parents are available on request at the IITA, Ibadan,
239 Nigeria and University of California Riverside, USA cowpea germplasm banks. QTL information
240 for photoperiod sensitivity and seed size is provided in Supplemental Files S2 and S3, respectively.
241 Genotypic and phenotypic data used in GWAS and biparental mapping are included in
242 Supplemental Files S4 and S5, respectively.

243

244

245 **Results**

246 *MAGIC development and genotyping*

247 In total 365 MAGIC F8 RILs were generated from 330 unique 8-way crosses. Among these, 29
248 crosses produced two or more F8 RILs, which were sister lines separated from earlier generations.
249 These lines were purposely created to maintain the population size. Reciprocal crosses were made
250 at the 4-way and 8-way cycles, resulting in RILs with different maternal parents, including CB27
251 (225 lines), IT89KD-288 (111 lines), Suvita 2 (9 lines), and IT84S-2246 (5 lines). There were 15
252 lines with illegible pedigrees on tags that were bleached by sunlight and moisture in the
253 greenhouse.

254 Genotyping with the 51,128-SNP Illumina iSelect BeadArray resulted in 36,346 SNPs that were
255 polymorphic between the 8 parents (68.26%). Among these, 11,848 SNPs were parent-unique,
256 each of which could distinguish one parent from the other 7 parents. Based on parent-unique SNPs,
257 15 RILs lacked male-parent alleles at the 2-way/4-way intercrosses and probably resulted from
258 accidental selfing during artificial pollination. Three RILs were found to carry non-parental alleles
259 in that they were heterozygous at SNP loci that were monomorphic between the 8 parents. Except
260 for five RILs with more than 10% of heterozygosity, the rest of the population had a low level of
261 heterozygosity, ranging from 0 to 3.33%. There were 8 RILs each showing very similar SNP
262 genotypes (more than 99%) to another RIL, and these were considered as redundant duplicates.
263 After excluding lines with duplicates, selfing errors, non-parental alleles and excess
264 heterozygosity, a core set of 305 MAGIC RILs derived from 305 unique 8-way crosses was
265 selected for further analysis. These RILs appeared highly diverse and clustered uniformly relative
266 to their eight parents, among which IT89KD-288, IT84S-2246 and IT97K-503-1 were closer to
267 each other than the other parent to parent relationships which were dispersed throughout the
268 population structure (Fig. 1).

7
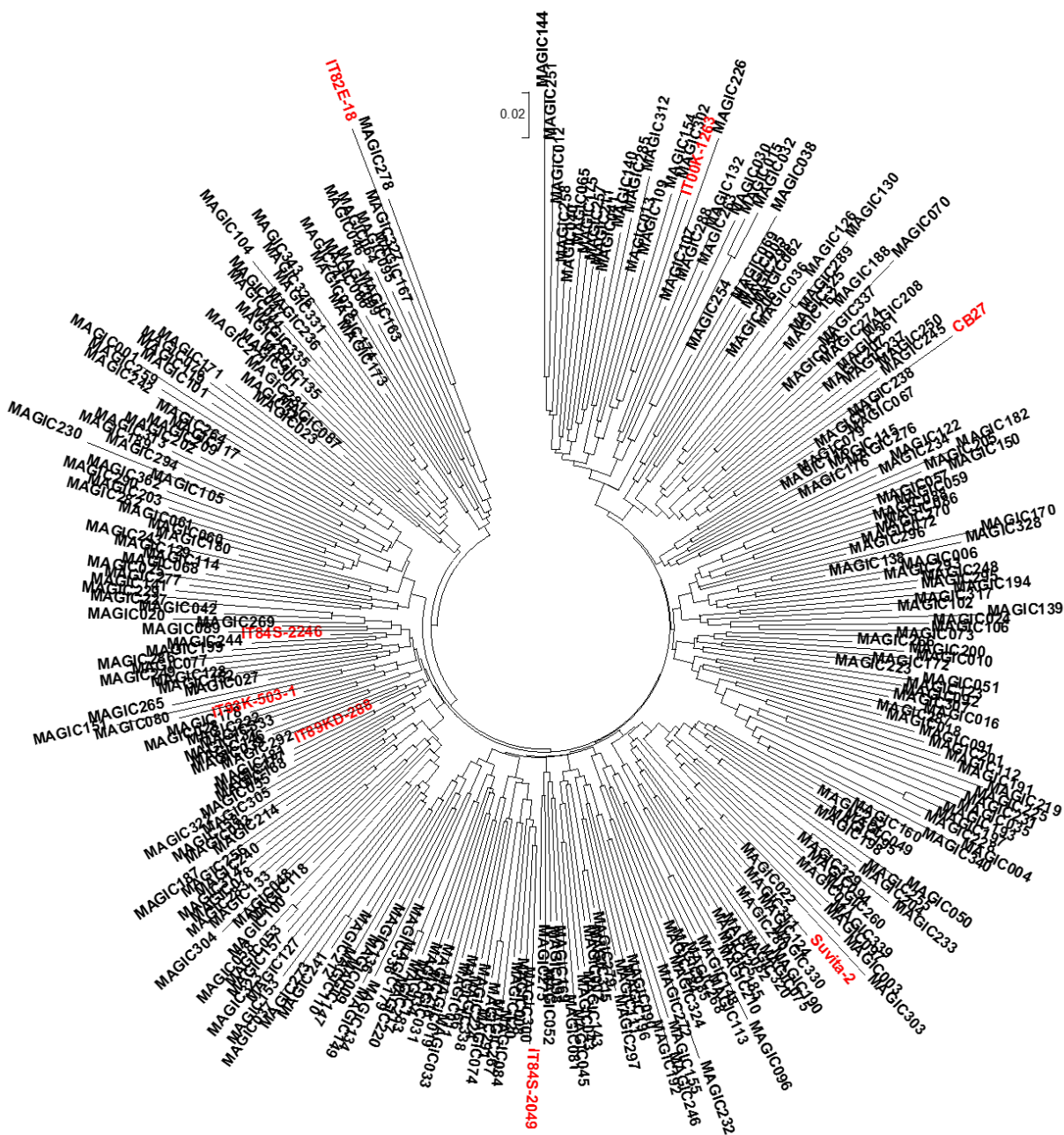
269

270 **Figure 1**. Phylogenetic relationships among the 305 F8 RILs of the cowpea MAGIC core set and
271 eight parents (in red) based on 11,848 parent-unique SNPs

272 *Phenotypic variation in the MAGIC population*

273 The F8 lines were highly diverse in morphological traits including flowering time, growth habit,
274 flower color, leaf shape, and seed characteristics (size, shape, color and texture) (Fig. 2). The
275 flowering time varied widely in the population under both long- and short-daylength conditions
276 (Fig. 3). The genotypic differences in flowering time were quite stable across contrasting watering
277 regimes in each daylength condition, with broad-sense heritability estimated as 0.77 and 0.71 at
278 UCR-CES (long daylength) and CVARS (short daylength), respectively. There was a significant
279 correlation ($r = 0.63$, $P < 0.001$) in phenotypic ranking between the long- and short-daylength
280 conditions, although the absolute flowering time varied considerably among lines. At UCR-CES
281 (long daylength), the population started flowering as early as 43 days after planting, but there were
282 many lines with delayed flowering beyond 60 days after planting (Fig. 3a and 4a). In contrast,
283 under short daylength at CVARS, the population started flowering as early as 34 days after planting
284 and the population completed flowering within another month (63 days) (Fig. 3b and 4b). Among
285 the parents, CB27 (36 and 44 days) was the earliest to flower while IT89KD-288 (46 and 88 days)
286 was the most delayed in both environments (CVARS and UCR-CES, respectively).

287 None of the MAGIC RILs or parents showed prostrate growth habit. Under full irrigation, the
288 majority of MAGIC RILs had a growth habit ranging from semi-erect to erect under both short-
289 and long-daylength conditions (Fig. 4). There was significant but moderate correlation in the
290 growth habit scores between the two daylength conditions ($r = 0.55$, $P < 0.001$), with about 55%
291 of the lines showing consistent growth habit between the two environments. Lines with semi-
292 prostrate growth habit under long-daylength became intermediate or semi-erect type when grown
293 under short-daylength condition. Among the parents, CB27 (acute erect) and IT84-2049 (erect),
294 IT89KD-288 (semi-erect) and Suvita-2 (semi-erect) maintained their growth habit in both short-
295 and long-daylength conditions under the full-irrigation regime. Under restricted irrigation, the
296 MAGIC RILs and parents mostly showed erect or acute erect growth.

297 Maturity varied considerably in the MAGIC population grown under different watering regimes
298 at CVARS in 2015 and 2016. Broad-sense heritability was estimated as 0.47, with significant but
299 moderate correlations ($r = 0.53$, $P < 0.001$) existing in the phenotypic ranking between the two
300 watering conditions (normal and restricted irrigation). Transgressive segregation was also
301 observed. Some lines were fully mature as early as 60 days after planting under both watering
302 regimes, while others were still green and kept producing pods after 120 days under restricted
303 irrigation in 2015, including two parents (IT00K-1263 and IT93K-503-1) and 66 MAGIC RILs
304 (21% of the population).

305 Grain yield and seed size also varied considerably under both water-restricted and full irrigation
306 conditions at CVARS (Fig. 5). The plants generally produced much higher yield and developed
307 larger seeds under full irrigation compared to water-stress conditions. Seed size appeared much
308 more stable in the genotypic ranking than grain yield, with broad-sense heritability estimated as
309 0.76 and 0.30, respectively. Transgressive segregation was observed for both traits. Approximately
310 11% of MAGIC RILs yielded higher than all parents under restricted irrigation conditions. Among
311 the parents, CB27 consistently had the highest yield and seed size across the two environments.
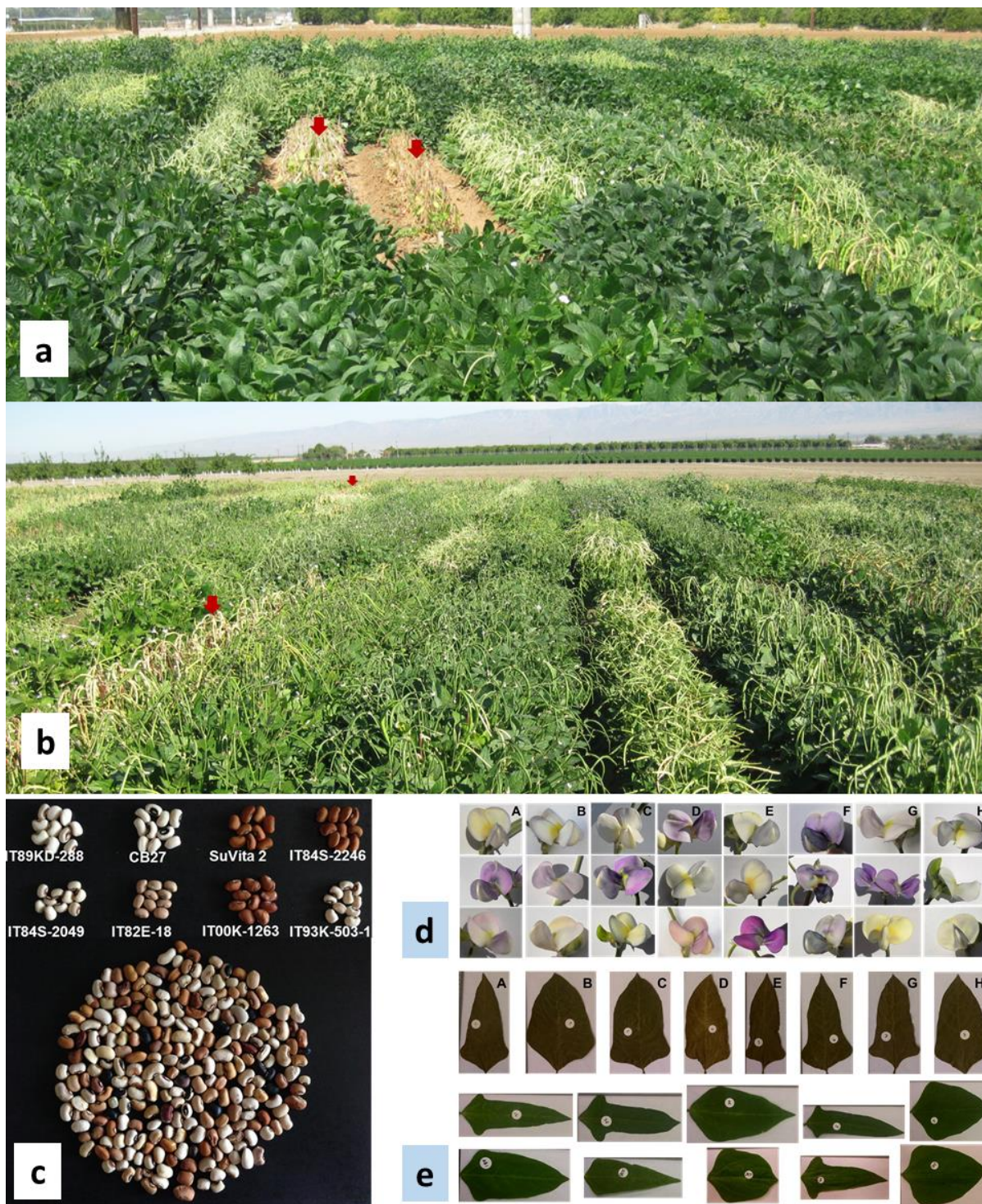
Figure 2. Morphological variation in the cowpea MAGIC population: Plant appearance at 65 days after planting under (a) long-daylength conditions at UCR-CES in 2015 and (b) short-daylength conditions at CVARS in 2016, both under full irrigation; (c) seed appearance, (d) flower color and (e) leaf shape of parents (top panel) and a representation of MAGIC F8 RILs (in lower part of 2c, each seed is from a different F8 RIL). In 2a and 2b, red arrows indicate examples of lines that matured earlier than other lines. In 2d and 2e, parent codes are: A, IT89KD-288; B, IT84S-2049; C, CB27; D, IT82E-18; E, Suvita-2; F, IT00K-1263; G, IT84S-2246; H, IT93K-503-1.

(a)

(b)
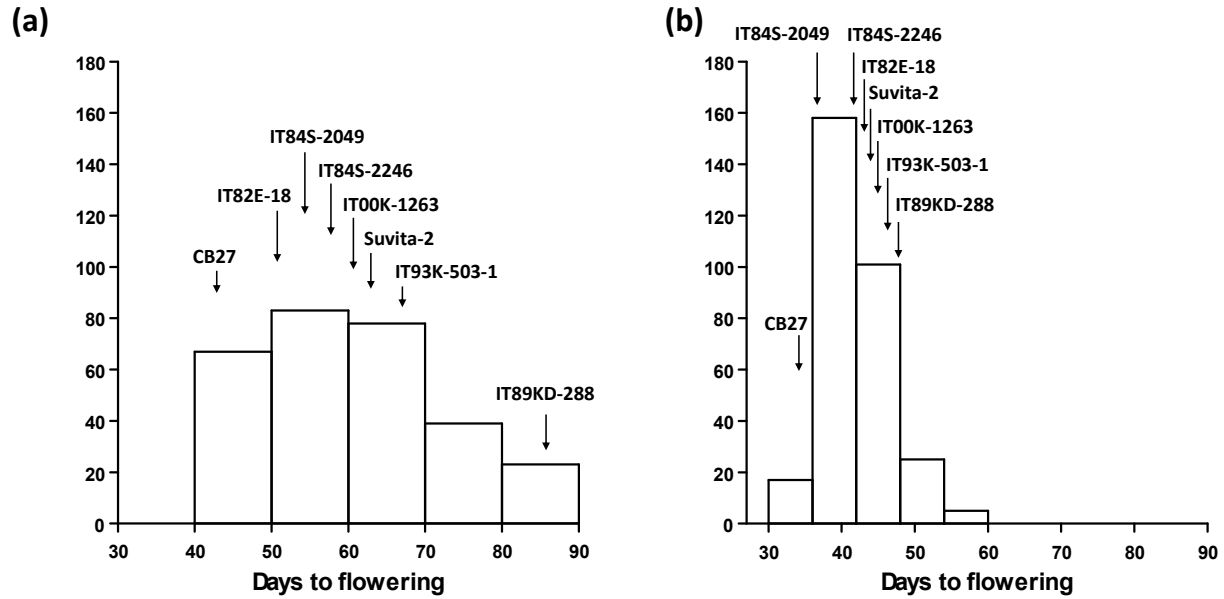
Figure 3. Variation in flowering time measured in the MAGIC core set and eight parents grown under (a) long-daylength conditions at UCR-CES and (b) short-daylength conditions at CVARS. Mean flowering time values for each line were derived from two experiments at UCR-CES and four experiments at CVARS during 2015-2016.

(a)

(b)
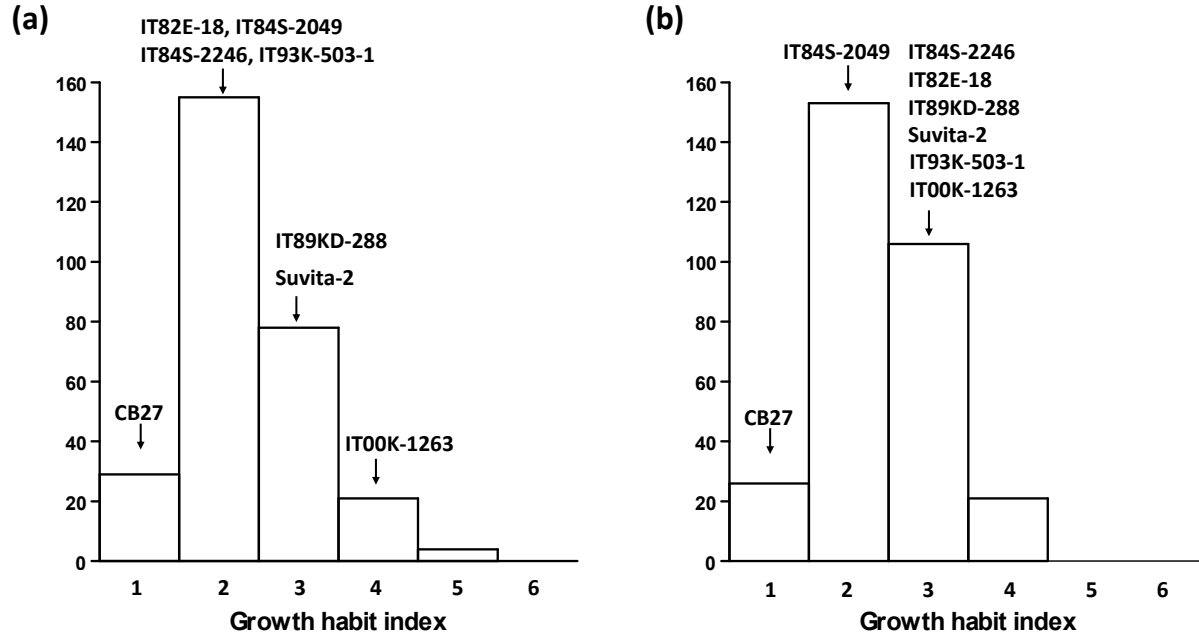
Figure 4. Variation in plant growth habit measured in the MAGIC core set and eight parents grown under full irrigation with different photoperiod conditions: (a) long-daylength at UCR-CES in 2015 and (b) short-daylength at CVARS in 2015 and 2016 (means of 2 years). Growth habit index: (1) acute erect, (2) erect, (3) semi-erect, (4) intermediate, (5) semi-prostrate, and (6) prostrate.

11
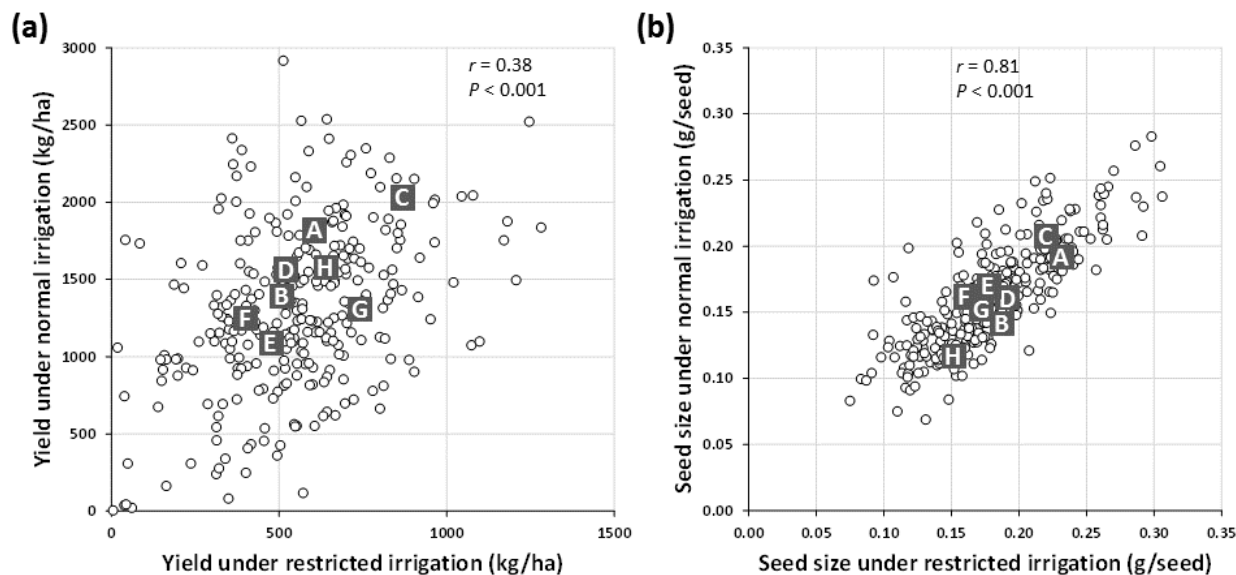
Figure 5. Variation in (a) dry grain yield and (b) seed size measured in the 305-RIL MAGIC core set and parents grown under restricted and normal irrigation regimes at CVARS in 2015 and 2016. Each dot represents a MAGIC RIL based on mean phenotypic values of two years of data. Labeled solid squares are MAGIC parents (A: IT89KD-288, B: IT84S-2049, C: CB27, D: IT82E-18, E: Suvita-2, F: IT00K-1263, G: IT84S-2246, H: IT93K-503-1).

Detection of marker-trait associations

From the 36,346 polymorphic SNPs, after removing those with MAF ≤ 0.05 and successful calling rate $\leq 90\%$, the remaining 33,768 SNPs with known positions on 11 cowpea pseudomolecules were used in GWAS. The MLM analysis consistently detected three clusters of SNPs on chromosomes 5, 9 and 11 significantly associated with photoperiod sensitivity expressed in the MAGIC population grown under long-daylength conditions at UCR-CES in 2015 (full irrigation, Fig. 6a) and 2016 (restricted irrigation, Fig. 6b). Markers with major effects were located on chromosome 9 (max LOD = 9.3), explaining up to 15% of total phenotypic variance, with favorable (earlier flowering time) alleles contributed from CB27 and IT82E-18. Markers with smaller effects were located on chromosomes 5 and 11 (max LOD = 5.8), explaining up to 9% of total phenotypic variance. Under the short-daylength condition, the significant peaks on chromosomes 5 and 9 remained, albeit with smaller effects (max LOD = 6.5, explaining 9% of phenotypic variance) while the peak on chromosome 11 was not significant (Fig. 6c and 6d).

Markers significantly associated with plant growth habit were identified on distal regions of chromosomes 1 and 9 based on data from short-daylength and full irrigation experiments at CVARS (Fig. 6e). Markers of highest significance (max LOD = 4.3) explained up to 9% of total phenotypic variance, with favorable alleles (more erect growth) contributed from IT89-KD288 and CB27. This region also coincided with markers affecting flowering time under the short-daylength condition (Fig. 6d), with the more erect-growth alleles associated with earlier flowering time. No significant association was found for growth habit under restricted irrigation.

Marker peaks on chromosomes 5, 8 and 9 with significant or marginal effects on maturity also coincided with marker peaks affecting flowering time under short-daylength conditions at CVARS

360 (Fig. 6g and 6h). For grain yield under restricted irrigation at CVARS, a significant peak was
361 detected, located on chromosome 8 (max LOD = 4.4, explaining 6% of phenotypic variance) (Fig.
362 6j) while no significant markers were identified for grain yield under normal irrigation (Fig. 6i).
363 In contrast, markers with major effects were identified for seed size on chromosome 8 based on
364 data from both water–restricted and full-irrigation conditions (Fig. 6k and 6l). Markers of highest
365 significance (max LOD = 7.3) explained up to 11% of total phenotypic variance, with favorable
366 (larger seed) alleles contributed by IT82E-18 and IT00K-1263. These markers also flanked the
367 region previously mapped for seed size using a biparental RIL population (CB27 x IT82E-18),
368 with the favorable allele contributed from IT82E-18 (LUCAS *et al.* 2013b) (Supplemental File S2).
369 The other region with minor effects on seed size under both water regimes was found located on
370 chromosome 6 (max LOD = 4.0), explaining 5% of total phenotypic variance, with favorable
371 alleles contributed from IT89KD-288, Suvita-2, IT84S-2246 and IT93K-503-1.

372 In general, markers significantly associated with agronomic trait determinants appeared to
373 distribute around distal regions of chromosomes. Neither maternal effect nor its interaction with
374 trait-associated SNPs for each agronomic trait were significant.

Full irrigation   **Restricted irrigation**

375
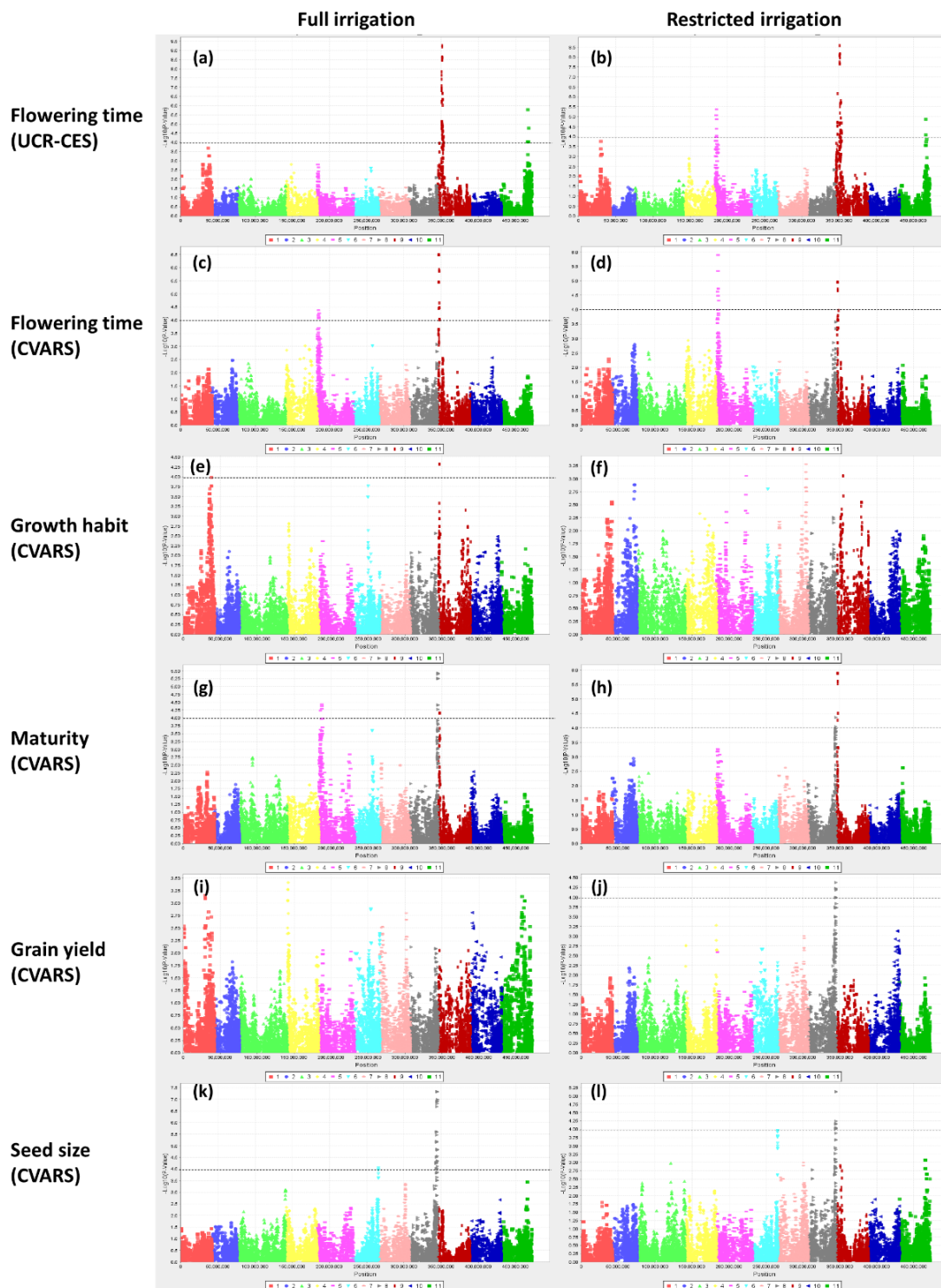
Figure 6. GWAS Manhattan plots depicting chromosomal regions associated with variation in agronomic traits measured in the MAGIC population grown under full irrigation (left) and restricted irrigation (right) with long-daylength conditions at UCR-CES (a-b) and short-daylength conditions at CVARS (c-l). Phenotypic values at CVARS were means of two years of data, except for growth habit which was not measured under restricted irrigation in 2016. The dashed lines correspond to the significance threshold $-\log_{10}(0.0001)$.

14

Validation of photoperiod QTL in biparental RILs

Flowering time varied widely in the CB27 x IT97K-556-6 RIL population grown under long-daylength condition at UCR-CES in 2016 (Fig. 7). CB27 began flowering 44 days after planting while IT97K-556-6 delayed flowering until after 70 days. A major QTL for flowering time was detected on linkage group 9 (LOD = 7.8, explaining 30% of phenotypic variance) (Fig. 8). The favorable (early flowering) allele was contributed from CB27. SNP markers flanking this QTL were 2_04691 and 2_00735 (and their co-segregating SNPs), which were found to be located in the same region as the major QTL detected in the MAGIC population grown under the same long-daylength condition in 2015 (Supplemental File S3).
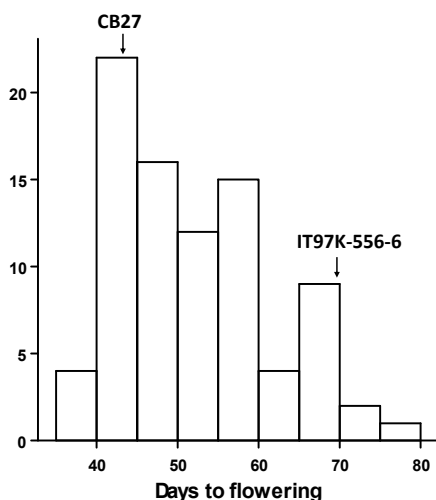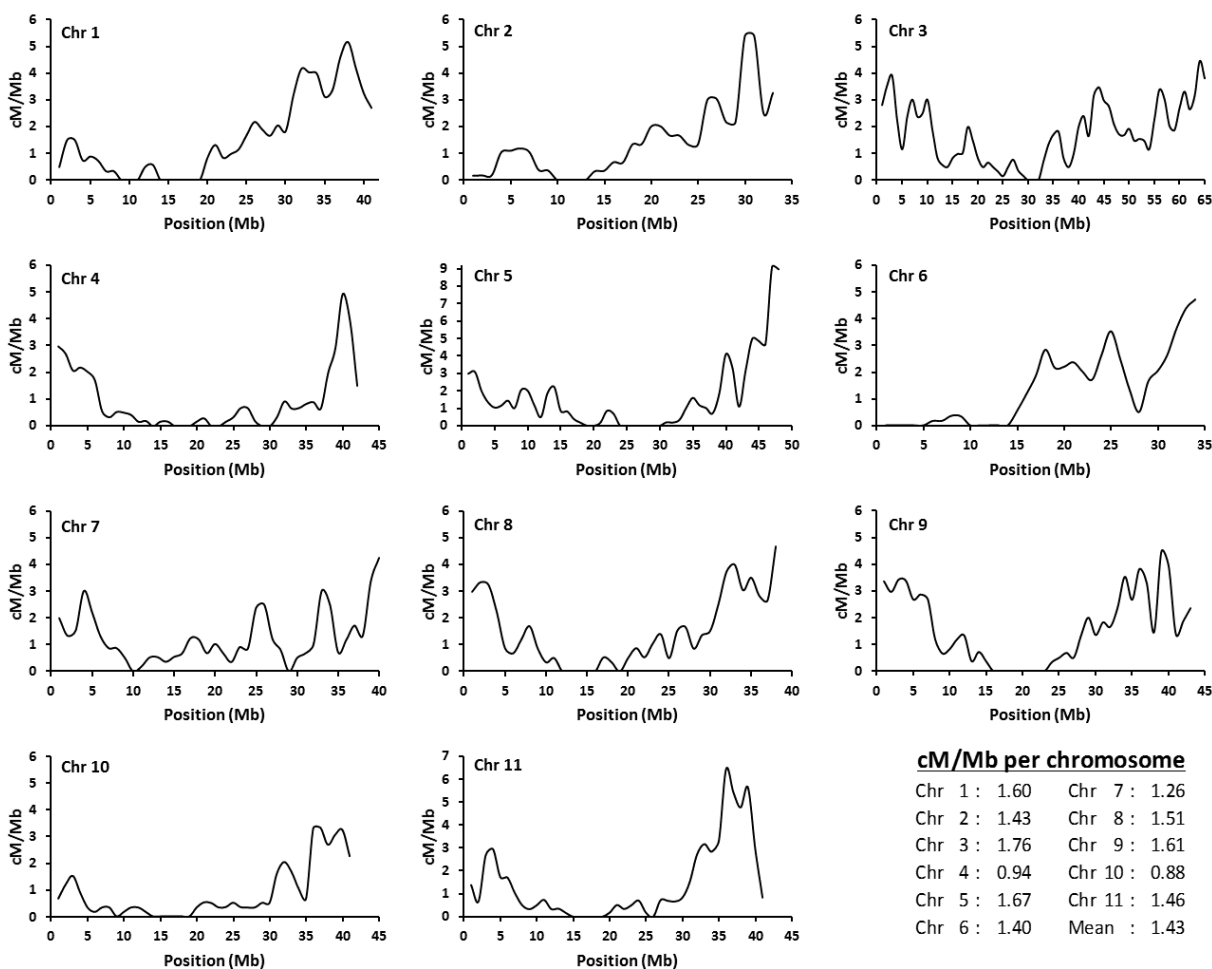

Figure 7. Variation in flowering time expressed in the CB27 x IT97K-556-6 biparental RIL population grown under long-daylength conditions at UCR-CES in 2016.
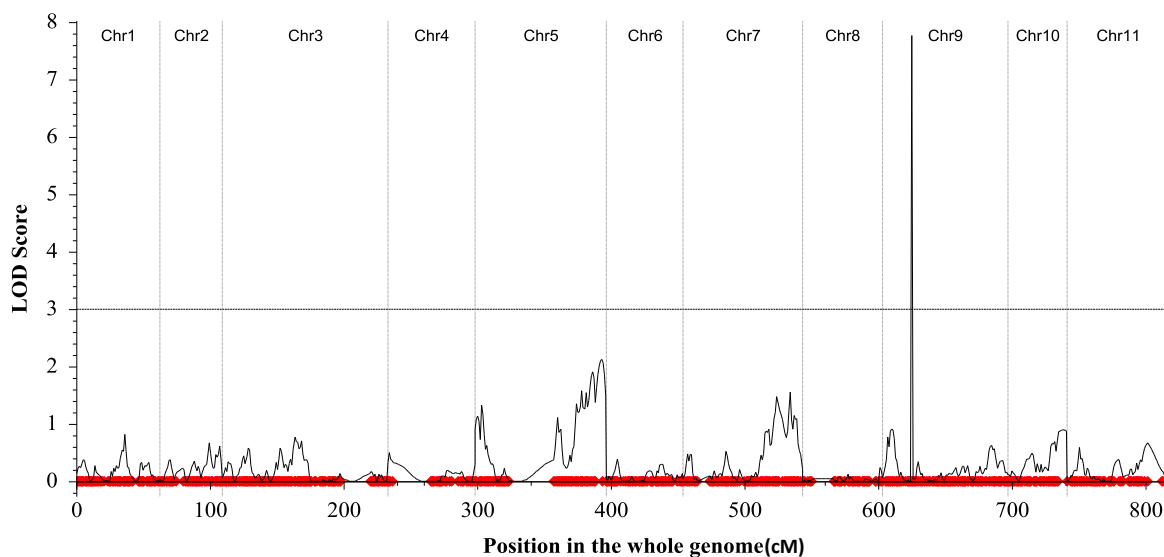
Figure 8. Chromosomal regions associated with variation in flowering time measured in the CB27 x IT97K-556-6 biparental RIL population under long-daylength conditions at UCR-CES in 2016. The LOD peak on linkage group 8 is flanked by SNP markers 2_10023 and 2_04691, which are in the same region of the major QTL detected in the MAGIC population (see Fig. 6a).

15

399 *Recombination rate variation*

400 Based on recombination analysis of 33,768 polymorphic SNPs, the crossovers appeared to
401 distribute throughout the MAGIC genome, at an average of 1.43 cM/Mb, and more frequently on
402 or near the telomeric distal regions of chromosomes (Fig. 9), where most trait-associated SNPs
403 were detected by GWAS (Fig. 6). Several recombination hot-spots with more than 5 cM/Mb were
404 detected on the distal long arms of chromosomes 1, 2, 5 and 11, while fairly large disequilibrium
405 blocks also were found on most chromosomes. On average, chromosome 3 had the highest
406 recombination rate (1.76 cM/Mb) while chromosome 10 had the lowest (0.88 cM/Mb).

cM/Mb per chromosome

Chr 1 : 1.60	Chr 7 : 1.26
Chr 2 : 1.43	Chr 8 : 1.51
Chr 3 : 1.76	Chr 9 : 1.61
Chr 4 : 0.94	Chr 10 : 0.88
Chr 5 : 1.67	Chr 11 : 1.46
Chr 6 : 1.40	Mean : 1.43

407

408 **Figure 9**. Recombination rate (cM/Mb) variation along 11 cowpea chromosomes measured in the
409 8-way cowpea MAGIC population using a sliding window of 2 Mb with 1 Mb increments.

16

410   **Discussion**

411   *MAGIC development*

412   The wide phenotypic variation with significant transgressive segregation observed in the cowpea
413   MAGIC population indicates that genome regions from parents were highly recombined in the
414   RILs. In fact, the population was developed in a way that maximized genetic variation. At the 2-
415   way crosses, plants in each F1 set were heterozygous and homogeneous because the 8 founder
416   parents were highly inbred lines. However, the F1s derived from the 4-way crosses segregated and
417   exhibited significant variation. To capture as much variation as possible, we performed more than
418   300 pair-wise crosses, also including reciprocal pairs, between different 4-way F1 individuals. In
419   addition, there was no intended selection for any trait during the SSD process. The plants were
420   grown in UCR greenhouses with optimal temperature, fertilizer, irrigation and pest and disease
421   management. In some cases, the plants were grown during long-daylength conditions in summer,
422   but the photoperiod sensitive lines which failed to become reproductive in the summer were
423   maintained and allowed to set flowers and pods later in the autumn when the daylength shortened,
424   to avoid selection against photoperiod sensitivity. There was also no selection for preferable seed
425   characteristics, plant type or yield components. This blind SSD process therefore helped create the
426   high diversity in morphological and agronomic traits that we observed in this MAGIC population
427   (Fig. 2-5).

428   The genetic integrity of the cowpea MAGIC population was also confirmed by the results of high-
429   density SNP genotyping. We used 89 parent-unique SNP markers from the Illumina GoldenGate
430   Assay (MUCHERO *et al.* 2009a) to validate true 2-way F1 crosses to avoid possible mistakes from
431   the early stage of MAGIC development. We then used 11,848 parent-unique SNPs from the
432   recently developed Illumina iSelect 60K SNP assay (MUÑOZ-AMATRIAÍN *et al.* 2017) to confirm
433   true 8-way RILs and to eliminate those that appeared to be selfed at the 4-way or 8-way crosses.
434   The SNP genotyping also identified lines with non-parental alleles, identical SNP genotypes, or
435   excess heterozygosity. Fortunately, very few of these unexpected lines were found (16 out of 365
436   lines), some of which were replaced by sister lines that were purposely developed as backups. By
437   removing all erroneous lines and keeping one RIL from each unique 8-way cross, we created a
438   MAGIC core set of 305 RILs that are highly homozygous and genetically distinct from each other
439   and from the eight parents (Fig. 1). As such, they can serve as permanent genetic materials for use
440   in replicated phenotyping trials. Despite its compact size relative to MAGIC populations
441   developed for *indica* rice (2000 lines) (BANDILLO *et al.* 2013) and winter wheat (1091 lines)
442   (MACKAY *et al.* 2014), the cowpea 305 RIL MAGIC core set is less management-intensive and
443   easier to phenotype, especially under field conditions, while still capable of accurately detecting
444   trait-associated markers as discussed in the following section.

445

446   *MAGIC genetic analysis*

447   Based on SNP genotyping, the 36,346 markers segregating in the cowpea MAGIC population were
448   almost double those identified in every bi-parental RIL population genotyped with the same SNP
449   array (MUÑOZ-AMATRIAÍN *et al.* 2017). This is attributable to the 8 founder parents having been
450   chosen on the basis of phenotypic and genetic diversity found in earlier studies. The parents were
451   high yielding under drought in one or more countries, resistant to different biotic stress factors

17

452   (Table 1), and represented the two major gene pools of cowpea centered in West Africa and
453   southeastern Africa (HUYNH *et al.* 2013a). By applying multiple 2-way, 4-way and 8-way
454   intercrosses from those founders, plus 8 generations of single seed descent for over 300
455   independent 8-way pair crosses, one would expect more recombination events to occur in the
456   MAGIC than in bi-parental RILs. However, it is difficult to measure accurately the number of
457   crossovers between two SNP markers due to a lack of parent-specific alleles at each locus. At each
458   SNP marker, one allele represents one or more parents, and the alternative allele represents the
459   other parents, so in some cases it is impossible to identify the actual parent carrying the allele at
460   that locus if multiple parents bear a common allele. The recombination rate estimated in this study
461   was based only on the obvious recombinants between two SNPs (Supplemental File S1) and thus
462   may underestimate their true genetic distance. Despite this, the current estimated recombination
463   rate was still relatively high and varied considerably along 11 cowpea chromosomes (Fig. 9),
464   supporting a high level of crossing over as expected in the MAGIC population. The pattern of
465   variation along chromosomes appears consistent with information reported for *Drosophila* (HEY
466   and KLIMAN 2002) and human genomes (MCVEAN *et al.* 2004) where the recombination was often
467   elevated near telomeres and suppressed near centromeres.

468   The relatively high recombination rates near the telomeres imply that haplotype variation occurs
469   at a high level, thereby facilitating random association needed for QTL detection. This is in
470   accordance with our GWAS analysis showing that trait-associated SNPs were mostly distributed
471   near the telomeric regions (Fig. 6). In addition, markers with major effects detected in the cowpea
472   MAGIC population for photoperiod sensitivity and seed size were verified by bi-parental genetic
473   mapping, indicating that the MAGIC core set is effective for mapping genome regions harboring
474   major QTLs. This MAGIC core set comprised individuals which were carefully selected based on
475   genome-wide SNP diversity excluding sister lines and duplicates, so interference by kinship and
476   population structure would not be a problem during association analysis. The population size of
477   305 highly homozygous lines also provides sufficient internal replications of marker alleles needed
478   for marker-trait association; at a parent-unique locus, approximately one-eighth of the population
479   (~38 individuals or 12.5%) carry the parent-unique allele. The challenge for QTL detection in the
480   MAGIC population, however, is a lack of donor-specific markers flanking the QTL in question,
481   whose alleles cannot distinguish the QTL donor from other parents. In the case of the major
482   photoperiod QTL, it was fortunate that favorable alleles in the region were specific to the non-
483   photoperiod donors CB27 and IT82E-18 (Supplemental File S3). For other trait-associated SNPs
484   with lower LOD scores, their true effect might be diluted by 'hidden' unfavorable alleles during
485   GWAS analysis. Enriching such regions with more parent-unique markers would help improve the
486   efficiency of QTL mapping.

487

488   *Genetic improvement perspectives*

489   The strong transgressive segregation observed for agronomic traits provides opportunities for
490   selecting MAGIC lines that outperform the parents. Selecting for large seed size, which is
491   preferred by consumers in SSA, would be straightforward because the trait appeared highly
492   heritable (Fig. 5b). In contrast, selecting for higher yield will be more difficult given its relatively
493   low heritability (Fig. 5a); based on the pattern of variation in yield under restricted versus full
494   irrigation, it may be more effective to select for high yield under drought stress in which at least
495   11% of the RILs yielded better than the 8 MAGIC parents. These lines probably carry a

496 combination of different drought-tolerance genes contributed from multiple parents, because the
497 parents are known to yield well under drought conditions in different African countries (Table 1).
498 MAGIC lines that are not photoperiod sensitive could be grown widely across seasons and regions
499 with different latitudes. Lines that flower early may escape damage by flower/pod feeding insects
500 and abiotic stress such as heat and terminal drought in certain environments. Lines with
501 exceptionally early or delayed crop senescence are suitable for production systems requiring single
502 or double flushes of pods, respectively. Plants with acute erect growth could support heavy pod
503 load, allow more leaf area to capture sunlight for photosynthesis, and support high plant population
504 densities to increase yield under monocropping. Since the eight parents also vary in resistance to
505 many major insects and diseases (Table 1), the MAGIC population will segregate for many biotic
506 stress resistance traits and also contain lines with unique and novel combinations of defense genes.
507 Therefore, phenotypic screening of the MAGIC population for those traits will enable genetic
508 mapping and identification of lines carrying favorable trait combinations for selecting cultivars in
509 target environments.

510 For the longer term, the cowpea MAGIC population can also benefit breeding programs by
511 providing valuable pre-breeding resources. QTLs detected in the MAGIC population combined
512 with existing knowledge of QTL regions and haplotypes can be applied to develop novel
513 combinations of QTLs through intercrossing best MAGIC RILs, providing super trait-donor lines
514 for use in breeding programs. QTLs for many key traits were already mapped in bi-parent and
515 diversity populations where certain MAGIC parents were used in the crosses, such as seed size
516 (LUCAS *et al.* 2013b), heat tolerance (LUCAS *et al.* 2013a), drought tolerance (MUCHERO *et al.*
517 2013), resistance to foliar thrips (LUCAS *et al.* 2012), aphids (HUYNH *et al.* 2015), Fusarium wilt
518 disease (POTTORFF *et al.* 2014), root-knot nematodes (HUYNH *et al.* 2016), ashy stem blight or
519 charcoal rot disease caused by *Macrophomina phaseolina* (MUCHERO *et al.* 2011), viruses
520 (OUÉDRAOGO *et al.* 2002a), the parasitic weed *Striga gesnerioides* (OUÉDRAOGO *et al.* 2012), and
521 root architecture (BURRIDGE *et al.* 2017). It is therefore possible to track positive haplotypes
522 contributed by different MAGIC parents in each MAGIC line and then intercross best lines to
523 develop target ideotypes. The strategy would be similar to the multi-parent advanced generation
524 recurrent selection (MAGReS) approach proposed recently by Huang et al. (2015), except that (1)
525 prior knowledge of QTL information from cowpea bi-parental mapping will be utilized, (2) the
526 MAGIC RILs selected for intercrosses are more advanced (F8), and (3) the selection can be
527 targeted using both QTL haplotypes and predicted breeding values based on genome-background
528 diversity. The resulting MAGReS lines will be fixed for positive haplotypes at known QTLs and
529 carry additional recombinations in other unknown loci conferring high grain yield. They can thus
530 be a valuable resource both for genetic improvement (as super trait donors or new cultivars) and
531 for detecting novel QTLs when combined with the current MAGIC RIL set.

532

Supplementary Materials

534 Supplemental File S1: Illustration of recombinant identification
535 Supplemental File S2: Major QTL region associated with seed size
536 Supplemental File S3: Major QTL region associated with photoperiod sensitivity
537 Supplemental File S4: Genotypic and phenotypic data used in GWAS
538 Supplemental File S5: Genotypic and phenotypic data used in biparental mapping

539 **Acknowledgements**

551

552

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