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1 **Overexpression of Arabidopsis *FLOWERING LOCUS T***  
2 **(*FT*) gene improves floral development in cassava**  
3 **(*Manihot esculenta*, Crantz)**

4

5 **Short Title: Flowering in FT overexpression lines of cassava**

6

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25

## 26 **Abstract**

27 Cassava is a tropical storage-root crop that serves as a worldwide source of staple food for over 800  
28 million people. Flowering is one of the most important breeding challenges in cassava because in most  
29 lines flowering is late and non-synchronized, and flower production is sparse. The *FLOWERING LOCUS T*  
30 (*FT*) gene is pivotal for floral induction in all examined angiosperms. The objective of the current work  
31 was to determine the potential roles of the *FT* signaling system in cassava. The *Arabidopsis thaliana FT*  
32 gene (*atFT*) was transformed into the cassava cultivar TMS 60444 through Agrobacterium-mediated  
33 transformation and was found to be overexpressed constitutively. *FT* overexpression hastened flower  
34 initiation and associated fork-type branching, indicating that cassava has the necessary signaling factors  
35 to interact with and respond to the *atFT* gene product. In addition, overexpression stimulated lateral  
36 branching, increased the prolificacy of flower production and extended the longevity of flower  
37 development. While *FT* homologs in some plant species stimulate development of vegetative storage  
38 organs, *atFT* did not stimulate storage-root development in cassava. These findings collectively  
39 contribute to our understanding of flower development in cassava and have the potential for  
40 applications in breeding.

41

42 **Key Words:** Flower initiation, tropical crops, breeding

43

## 44 **Introduction**

45 In storage-root crops such as cassava (*Manihot esculenta*, Crantz), research on flowering has received  
46 relatively little attention. This is partially because floral, fruit and seed organs are not the harvested  
47 parts of the plant. However, in cassava breeding, delayed and non-synchronous flowering is a major  
48 impediment for crossing selected lines [1, 2]. Many elite lines with desirable agronomic traits including

49 high yield of storage-roots and erect non-branched shoot architecture, are difficult to use as parents  
50 because their flowering is late and sparse [2]. Understanding the factors that regulate flowering in  
51 cassava would be valuable to facilitate progress in breeding programs. Furthermore, if the regulatory  
52 system were better understood, it might be possible to develop methods for hastening floral initiation  
53 so that desirable alleles, which are otherwise “locked up” in parents with poor flowering, will become  
54 available. Controllable flower induction could help breeders make more rapid progress by enabling  
55 earlier crosses, thereby shortening the breeding cycle [3].

56  
57 *Flowering Locus T (FT)* in *Arabidopsis* (*atFT*) is now recognized as the key component whose expression  
58 is regulated by upstream signaling components that perceive photoperiod, vernalization (cool  
59 temperatures of winter), and other factors in leaves [4]. The translated protein of *atFT* is the flowering  
60 stimulus which interacts with signaling factors in the apical meristem [5-7]. The “florigenic” signal is the  
61 translated protein of the *FT* gene that is transported via phloem from leaves to the apical meristem  
62 where it causes the switch from vegetative to reproductive development [8].

63  
64 The role of the *FT* gene in flower induction has been established in many species of angiosperms,  
65 including all examined dicots and monocots [4-6, 9]. There is evidence that *FT* signaling plays a role in  
66 photoperiodic and developmental regulation in species closely related to cassava. In Barbados nut  
67 (*Jatropha curcas*), which like cassava is in the Euphorbiaceae family, an *FT* homolog is primarily  
68 expressed in the reproductive organs and is thought to play a role in flower induction [10, 11]. In leafy  
69 spurge (*Euphorbia esula*), long photoperiods (16 h light) stimulates accumulation of *FT* homologs in a  
70 diurnal manner consistent with flower induction. On the other hand, under long days and cooling  
71 temperatures, *FT* expression is down regulated, and *DAM* (*DORMANCY ASSOCIATED MADS BOX*) is up-  
72 regulated, a response associated with induction of overwintering bud dormancy [12]. Similarly,

73 Böhlenius et al. [13] demonstrated that in poplar (*Populus trichocarpa*), which is in the Salicaceae family,  
74 closely related to Euphorbiaceae, flowering is induced by long days and corresponding induction of  
75 diurnal expression of *PtFT1*, while shortening days induce growth cessation and vegetative bud set in  
76 advance of winter.

77

78 Overexpression of transgenic *atFT* has been shown to induce early flowering in woody plants with long  
79 juvenile phases such as blueberry (*Vaccinium corymbosum* L.) [14] and eucalyptus (*Eucalyptus grandis* x  
80 *Eucalyptus urophylla*) [15]. Also, overexpression of an FT homolog from *Jatropha curcas* was  
81 constitutively overexpressed with CaMV-35S in *J. curcas* to demonstrate enhanced flowering [10], and  
82 FT overexpression in various paired species has accelerated flowering in apple (*Malus* spp.) [16, 17], and  
83 poplar (*Populus trichocarpa*) [18]. Given the effectiveness of this approach, it has been suggested that  
84 *FT* overexpression could be used to hasten flowering in breeding programs [15, 18-20]. In cassava,  
85 breeding might benefit if genotypes with abundant production of the FT signal were used as understocks  
86 in grafting such that breeding lines would not be stably transformed [21].

87

88 The objective of the current study was to overexpress the Arabidopsis *FT* gene in cassava and determine  
89 whether the cassava signaling system interacts with and responds to the Arabidopsis *FT* with earlier  
90 flower induction. Our findings indicate that cassava responds to overexpression of Arabidopsis *FT* with  
91 extremely early flowering. *FT* overexpression also substantially increased the number of flowers  
92 produced and lengthened the duration of cassava flowering such that abundant mature flowers were  
93 obtained. These studies improve our understanding of flowering regulation in cassava and indicate the  
94 potential for application in breeding programs.

95

## 96 **Materials and Methods**

## 97 **Molecular cloning and plant transformation**

98 The ORF of *FT* (At1g65480) was amplified by PCR, using GATEWAY™ compatible primers (FTGWFV-  
99 GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGTCTATAAATATAAGAGACCCTC and FTGWRV-  
100 GGGGACCACTTTGTACAAGAAAGCTGGGTCTAAAGTCTTCTCCTCCGCAGCCA). The resultant attB-FT-PCR  
101 product was cloned into the pDONR207 vector (Thermo Fischer Scientific) using BP Clonase, and the  
102 sequence-validated insert from FT-pENTRY clone was subcloned into the pNew-Mik1-antisense  
103 GATEWAY-compatible vector (Destination vector; Bekir Ülker, MPIPZ), using LR Clonase (Gateway;  
104 Invitrogen). The plant expression vector created expresses *FT*-cDNA under the control of a CaMV35S  
105 promoter and an ethanol inducible system (Fig. 1). This plasmid was introduced into *Agrobacterium* ABI  
106 [22] by electroporation and transferred to friable embryogenic callus (FECs) of cassava genotype TMS  
107 60444 (henceforth referred to as 60444) by the *Agrobacterium*-mediated transfer method, as described  
108 by Gonzalez et al. [23], with modifications that promote transformation in several cassava varieties [24].  
109 For these studies transformants from independent transformation events, designated FT-02, FT-11, FT-  
110 13, FT-17 and a non-transformed control, 60444 are reported. To confirm that the transgene was  
111 incorporated into cassava according to expectations, we performed a PCR of genomic DNA that shows  
112 the amplified product of *atFT* gene in the four transformants, the untransformed cassava, and in  
113 *Arabidopsis* control DNA (Supporting Information S1).

114

115 **Fig. 1. Schematic representation of the transformation vector.**

116 *Arabidopsis FT* cDNA was inserted into the construct through Gateway  
117 cloning. pAnos, nopaline synthase polyadenylation signal; pat,  
118 phosphinothricin acetyltransferase; Tnos, terminator of nopaline  
119 synthase; pAlcA, promoter of alcohol dehydrogenase I (*Adh-I*) encoded  
120 by the *alcA* gene; *FT* cDNA, cDNA of Flowering Locus (*FT*) gene; pA35S,

121 polyadenylation sequence of Cauliflower mosaic virus 35S gene; nos,  
122 nopaline synthase terminator; ALCR, transcriptional factor which binds  
123 to *AlcA promoter*; p35S, Cauliflower Mosaic Virus 35S promoter; LB, left  
124 border; RB, right border.

125

## 126 **Plant Materials and Growth Conditions**

127 The *in vitro*-maintained putative transgenic cassava plantlets which are maintained at CIAT  
128 (<http://genebank.ciat.cgiar.org>) were grown from subcultured stem segments for about 4 weeks to  
129 about the 3-leaf stage [25]. The plantlets were carefully removed from test tubes, agar was washed off,  
130 and planted in sterile peat/vermiculite/pearlite rooting medium. The plantlets were covered to maintain  
131 a humid environment with inverted clear polystyrene cups. After about one week cups were replaced  
132 with polyethylene bags, which were progressively punctured more and more over about three weeks to  
133 gradually lower humidity and promote root growth. Plantlets were carefully watered, as needed. They  
134 were then transferred to the green house where they were maintained with temperature controlled at  
135 30°C (day)/25°C (night), under long days (14h light and 10h dark) with natural illumination  
136 supplemented with about 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  of photosynthetically active radiation (400 to 700 nm) from  
137 metal halide lamps. These plants were propagated into four batches of plants which were used for  
138 subsequent studies of their architecture and expression of the introduced *FT* gene. Three batches were  
139 grown directly from *in vitro* plantlets; ethanol treatments were initiated at 4 months after planting  
140 (MAP) (batch 1 and 2) or 3 MAP (batch 4). Batch 3 was established from stem cuttings taken from batch  
141 1, and ethanol treatments were initiated at 3 MAP. In the *FT*-transformed lines in batches 2, 3, and 4,  
142 branch shoots and developing flowers were pruned off as soon as they appeared to create a more  
143 uniform plant architecture consisting of a single central stem. When ethanol treatments were initiated

144 no further pruning was conducted. Plants in each batch were randomly assigned ethanol or water  
145 drench treatments. Each genotype by treatment combination, Batches 1, 2, 3, and 4 had 1, 1, 2, and 3  
146 within-batch replicate plants, respectively. Ethanol/Water treatments consisted of twice weekly  
147 drenching of the soil with 500 mL of 1% (v/v) of ethanol/water over five weeks. Leaf tissue was sampled  
148 from the second most recently matured leaf on each plant, 24 hours after the fourth treatment. Leaf  
149 tissue was immediately frozen in liquid N<sub>2</sub>, and transferred to -80°C for storage until RNA extraction.

150

## 151 **Gene expression studies**

152 Tissue was ground to powder with mortar and pestle under liquid N<sub>2</sub>. Total RNA was extracted using a  
153 modified CTAB protocol reported by Monger et al. [26] and quantified by absorption at 260 nm  
154 (NanoDrop ND-1000, Wilmington, DE, USA). Two µg of the total RNA was used for cDNA synthesis. Prior  
155 to the synthesis, RNA was treated with 10U/µl DNase I (Roche) with DNase 1 Buffer and incubated at  
156 37°C for 30 min to remove any residual genomic DNA. cDNA synthesis was performed by qScript cDNA  
157 Supermix (Quanta) and Superscript III First strand synthesis supermix (Invitrogen), following the  
158 manufacturer's instructions. Quantitative Real Time PCR was performed using PerfeCTa™ SYBR® Green  
159 FastMix™ (Quanta) in a Bio-Rad CFX96™ Real-Time System, C1000™ Thermal Cycler. Primers for  
160 cassava 18S RNA were 18SF- ATG ATA CGA CGG ATC GC and 18SR- CTT GGA TGT GGT AGC CGT TT and  
161 for ubiquitin (UBQ10F-GCA ACT TGA GGA TGG CCG AA and UBQ10R-CTC CCC TCA AAC GCA GAA CA);  
162 these genes were used as internal controls. The Real-time quantitative PCR was repeated with 7  
163 biological replicates (1 each from batch 1 and 2; 2 from batch 3; and 3 from batch 4), and each sample  
164 was assayed in duplicate using primers **AtFTL2**- AAG TCC TAG CAA CCC TCA CCT C and **AtFTR2**- CAC CCT  
165 GGT GCA TAC ACT GTT. Data for the number of PCR cycles to reach the threshold (Ct), were normalized  
166 for 18S Ct values in each specimen by subtraction (ΔCt). Values were also normalized for each  
167 specimen's UBQ Ct value, and the 18S and UBQ normalized ΔCt values were averaged. These ΔCt values

168 were further normalized against the 60444 water-treated controls in each batch ( $\Delta\Delta C_t$ ) and interpreted  
169 as normalized fold expression ( $\log_2$ ) assuming a PCR efficiency of 1.0. When the data were plotted on  
170 this  $\log_2$  scale they were normally distributed, a requirement for statistical analysis. These  $C_t$  values  
171 were subjected to analysis of variance (ANOVA), as described below.

172

## 173 **Flowering traits**

174 In cassava, flowering is associated with fork-type branching which occurs via outgrowth of axillary  
175 meristems subtending the shoot apical meristem [27]. After the first fork, two to four second-tier  
176 shoots develop and each of them initiates flowers at their shoot apices (second tier flowers). Third and  
177 subsequent tiers of flowering develop similarly. Flowering traits were recorded weekly in Batches 3 and  
178 4, which had 2 and 3 biological replicates each, respectively to determine: a) date of flower or  
179 inflorescence appearance, b) number of flowers that exceeded a 2-mm diameter threshold size, and c)  
180 initial date of flower (and/or inflorescence) senescence. From these weekly records, the total number of  
181 flowers at each forking tier were calculated.

182

## 183 **Plant Growth Traits**

184 At 4.5 months after plant establishment in soil, plant height was measured and plants from Batches 1 to  
185 4 were harvested. The number of shoot nodes between the soil surface and first forks, between the  
186 first-tier and second-tier forks, and between the second- and third-tier forks were counted. Lateral  
187 branches which formed in the axils of leaves on the main stem were counted and the presence/absence  
188 of flowering at their shoot apices was recorded. Storage-roots were excavated from soil and counted.  
189 Storage-roots and above-ground plant parts were dried at 70°C to a constant weight, and weighed.

190 Fibrous roots were not recovered. Harvest index (HI) was calculated as:  $HI = (\text{storage-root dry mass}) / [(\text{storage-root dry mass}) + (\text{above-ground dry mass})]$ .

192

## 193 **Statistical Analysis**

194 Gene expression, flowering, and growth traits were subjected to analysis of variance (ANOVA) using a  
195 model for determining effects due to ethanol drench treatment (T), effects due to *FT* overexpression  
196 genotype (G), effects due to batches (block) (B), and effects due to interaction of T×G. Each trait was  
197 analyzed using the linear model in R (version 3.1.1, R Foundation for Statistical Computing,  
198 <http://www.r-project.org/>).

199

## 200 **Results**

### 201 *Cassava transgenic lines over-express Arabidopsis FT*

202 The construct used for transformation of cassava line 60444 contained an ethanol-inducible promoter  
203 upstream of the Arabidopsis *FT* (*atFT*) gene (Fig. 1). The transgenic events generated from the  
204 agrobacterium-mediated transfer were numbered from 1 to 22. Of these initial independent  
205 transformation events, many of them were weak and slow growing with many flowers relative to leaves  
206 such that only four of them survived after several months in culture. For this manuscript, the four  
207 surviving transformants were used. The Arabidopsis-derived *FT* transcript, expressed on a logarithmic  
208 scale such that data are normally distributed, was abundant in all the transgenic cassava lines (FT-02, FT-  
209 11, FT-13 and FT-17), while it was not detected in the untransformed control (60444) (Fig. 2). Contrary  
210 to expectation, in most of the transformed lines (FT-02, FT-11 and FT-17), ethanol treatment did not  
211 further enhance expression in leaf tissue (Fig. 2). Only in the transgenic line FT-13 did ethanol  
212 significantly ( $P \leq 0.05$ ) increase expression of the *FT* transcript in comparison to its water treated

213 counterpart. The wild type, untransformed control, had no detectable atFT message with or without  
214 ethanol treatment.

215 **Fig. 2. Expression of Arabidopsis FT gene in Cassava.**

216 The qRT-PCR results were obtained from four biological replicates and  
217 two technical replicates for each sample. 60444 represents the non-  
218 transformed wildtype line and FT-02, FT-11, FT-13 and FT-17 represent  
219 the four independent transformants. The levels of detected  
220 amplification were normalized using 18S and Ubiquitin as reference  
221 genes. The expression cassette had an ethanol-inducible promoter. In  
222 each case, potted cassava transgenic plants were either watered  
223 normally (H<sub>2</sub>O), or the soil was drenched with 1% (v/v) ethanol for two  
224 weeks before leaves were harvested and analyzed.

225

226 *The Arabidopsis FT gene hastens flowering in Cassava*

227 Due to our interest in hastening reproductive timing, we evaluated the timing of flower appearance in  
228 the atFT transformed lines throughout their development. The untransformed line, 60444, displayed its  
229 first fork-type branching and corresponding floral stalks at 120 days after transplanting (Fig. 3). In  
230 contrast, the transformed lines first formed flowers while the plants were still at the seedling stage (Fig.  
231 4a-d), and had numerous branching events associated with flowering. Indeed, flowers were observed  
232 during *in vitro* growth before transplanting to soil (Fig. 4a).

233 **Fig. 3. Flowering traits in non-transformed wildtype line (60444) and**  
234 **in the four independent transformants.**

235 (a) Flowering time in days from establishment in soil to flowering at the  
236 1st, 2nd, and 3rd tier of flowering, as defined by fork-type branching at

237 the apical meristems. (b) Number of shoot nodes to forking events  
238 where inflorescences develop. The number of nodes between the soil  
239 surface and the first fork, between the first-tier and second-tier forks,  
240 and between the second- and third-tier forks. (c) Number of flowers  
241 per tier, per plant. (d) Time to start of floral and/or inflorescence  
242 senescence. Floral traits were recorded weekly to determine the date of  
243 inflorescence appearance, and initial date of floral senescence. The total  
244 number of days from flower appearance to start of inflorescence and/or  
245 flower senescence was calculated from these weekly records. Shown  
246 are the means  $\pm$  SEM.

247

248 **Fig. 4. Transformed and non-transformed plants at various stages of**  
249 **floral development.**

250 (a): FT-17 transgenic plant at 2 months *in vitro*. (b and c): FT-17  
251 transgenic plantlet at one month after transfer from *in vitro* to culture  
252 box and soil respectively. (d): Advanced stage transgenic plants  
253 flowering at 3 months. (e): Non-transformed (left) vs. transformed  
254 (right) plants at 5 months old. (f and g): Close up view of the apical  
255 region of 5-month old non-transformed (f) and transformed (g) plants,  
256 respectively. Arrows indicate flowers.

257

258 To create a set of atFT-transformed material that would be well matched in size and initial architecture  
259 so that the potential effects of ethanol-induced expression of atFT could be tested, we pruned away  
260 flowers and branches so that initially each plant would have just one main stem. These plants were then

261 allowed to form fork-type branches and flowers in the absence of ethanol treatment. The atFT plants  
262 treated in this way flowered at about 75 d after transplanting (Fig. 3). Drenching with ethanol to induce  
263 the expression of atFT did not significantly ( $P \leq 0.05$ ) hasten the second and subsequent forking and  
264 flowering events (Supporting Information S2). Given the absence of effect of ethanol treatment, the  
265 data on flowering phenology are presented as the overall average for treatments with and without  
266 ethanol treatment. Corresponding data for each of the ethanol and control treatments are shown in  
267 Supporting Information (S2-S8). Second fork-type branches and associated flowering occurred at only  
268 25 to 32 days after the first flush of flowers, and in two of the transformed lines (FT-11 and FT-17) a  
269 third tier of flowering occurred about 28 days after the second tier (Fig. 3). The four transformed lines  
270 did not differ significantly in the time interval between the first and second flowering events; however,  
271 FT-02 and FT-13 did not advance to a third tier of flowering during the observation period. Another  
272 indication of the timing of floral initiation events is the number of nodes between forking.

273 Overexpression of *FT* had similar effects on the number of nodes between fork-type branches (Fig. 3b).  
274 In atFT13, despite having an increased expression of *FT* in response to ethanol treatment, flowering was  
275 not further hastened between the first and second or subsequent forking and associated flowering  
276 events (Supporting Information S2).

277

278

279

### 280 *Overexpression of Arabidopsis FT in cassava results in profuse flowering*

281 While expression of atFT has been observed to hasten flowering time in many plant species, an  
282 additional effect in the current study was sustained flower development and greater longevity of  
283 flowers (Fig. 3d). We counted the number of flowers at each tier (fork) in each plant (Fig. 3c) and also  
284 observed the length of time they continued to develop in each tier before they began senescing (Fig.

285 3d). In the non-transformed controls, plants forked, and developed an inflorescence stalk with immature  
286 flower buds less than the 3-mm minimum for counting that wilted and senesced within 2-3 days (Fig.  
287 3d). In the transgenic lines, however, flower development at each tier was sustained such that more  
288 flowers were formed, and flowers continued development through anthesis rather than aborting  
289 development and senescing, as was observed in the untransformed 60444 control. Flower development  
290 traits differed in the four transformed lines corresponding to the earliness of floral initiation. The  
291 average number of flowers in FT-02, the latest to flower, was 33, followed by that of line FT-11 with 55  
292 flowers (summed over the first and second tier). FT-13 and FT-17, the earliest lines to flower, had 77  
293 and 60 flowers (summed over all tiers), respectively. Although third-tier flowering had commenced  
294 during the observation period in FT-11 and FT-17 (Fig. 3a and b), flowering at tier 3 was not advanced  
295 sufficiently to obtain flower counts in any of the genotypes (Fig. 3c). The longevity of the flowers  
296 produced by the over-expressing lines was also affected. Plants overexpressing atFT plants produced  
297 numerous female and male flowers, which developed fully and reached anthesis. Whereas  
298 nontransformed controls began senescing at 3 days after appearance, flower development in the  
299 transformed lines continued for almost a month and did not begin senescing until 25 to 27 days on the  
300 first tier, and 21 to 25 days on the second tier (Fig. 3d).

301 In addition to fork-type branching by outgrowth of axillary meristems subtending the shoot  
302 apical meristem, atFT overexpression stimulated the outgrowth of lateral branches in the axils of leaves  
303 (Fig. 5a), all of which forked at their apices and formed flowers during the observation period (Fig. 5b).  
304 Whereas the non-transformed control did not form lateral branches from axillary bud outgrowth, the  
305 transformed lines developed between seven (FT-02 and FT-17) and eleven (FT-11 and FT-13) lateral  
306 branches (Fig. 5).

307

308 **Fig. 5. Lateral branch development in the axils of leaves on the main**  
309 **stem.**

310 Lateral branches and flowers that formed in fork-type branches at the  
311 apex of these lateral branches were counted in the non-transformed  
312 wildtype line (60444) and in the four independent transformants. (a)  
313 Number of lateral branches per plant. (b) Total number of flowers on  
314 lateral branches. Shown are the means  $\pm$  SEM.

315

316 *Yield Characters are hampered in cassava over-expressing FT gene*

317 Storage-root dry weight, total plant dry biomass, harvest index and root count of the transgenic plants  
318 as well as the control, were all measured as a function of crop yield and productivity. In general, the *FT*  
319 transformants were shorter (Supporting Information S8), had less storage-root production (Fig. 6a), less  
320 total plant dry biomass (Fig. 126b), a lower harvest index (Fig. 6c), and root count than in the non-  
321 transformed wildtype (Fig. 6d). The non-transformed line (60444) had the highest amount of storage-  
322 root production and harvest index, followed by FT-02, the intermediate line; and the three lines with the  
323 best flowering, FT-11, FT-13 and FT-17 had the lowest storage-root weights and harvest index (Fig. 6a  
324 and 6c).

325

326

327 **Fig. 6. Root and shoot production in non-transformed wildtype**  
328 **(60444) and the four independent transformants at harvest.**

329 (a) Storage-root dry weight; (b) total plant dry weight; (c) harvest index  
330 (HI), calculated as  $HI = (\text{storage-root dry mass}) / [(\text{storage-root dry mass})]$

331 + (above-ground dry mass)]; (d) number of storage-roots. Shown are  
332 the means  $\pm$  SEM.

333

334

335

## 336 **Discussion**

337 Delayed and scarce flowering in cassava has been a long-standing hurdle faced by conventional  
338 breeders, molecular biologists and geneticists in their attempts to cross desirable parents for  
339 improvement of cassava [1, 3]. The difficulties arising from the flowering biology of cassava have limited  
340 the development of inbred lines for use in cassava genetic enhancement and reduced the potential  
341 impact of genomic selection [1, 3]. In the current work, we overexpressed *Arabidopsis FT* in cassava  
342 cultivar 60444, which is an elite, high-yielding genotype that is normally late flowering [28]. Expression  
343 was driven with the ALCR/alcA promotor system, which is designed to be ethanol inducible [29] and has  
344 been used as such in several plant species [30-34]. We applied ethanol as a soil drench, which is  
345 expected to result in root uptake of ethanol and its delivery via the transpiration stream to leaves where  
346 expression is induced, as others have shown [34]. However, in this study, leaf expression of the *atFT*  
347 transcript was already high in the controls (water drench treatments) of all four independent  
348 transformation events, and was not increased further by ethanol treatment ( $P \leq 0.05$ ) except in the FT-13  
349 line (Fig. 2). In addition to expression in leaves, we also observed expression of a similar magnitude in  
350 flower buds and tissue of the apical region including unexpanded leaves and shoot meristem in  
351 transformed plants, whereas the untransformed cassava plants had insignificant *atFT* expression  
352 (Supporting Information S9). Furthermore, in the transgenic lines the plants given water versus ethanol  
353 treatment did not differ significantly for flower development traits (Supporting Information S2-S4).

354 Apparently the promoter gave constitutive overexpression in the absence of added ethanol. It is  
355 possible that cassava tissues produced sufficient ethanol to drive expression from the promoter.  
356 Studies have shown that hypoxia can develop in internal plant tissues such as vasculature [35], which  
357 might have elicited ethanol production in cells of internal tissue such as the phloem. A similar finding of  
358 constitutive expression was found with the ALCR/alcA promoter system in tobacco tissue cultures [33].

359         The current study showed that *Arabidopsis FT* (atFT) overexpression substantially reduced the  
360 time to flowering (Fig. 3) to the extent that flowering occurred in seedling plants grown *in vitro* (Fig. 4).  
361 This finding is in agreement with earlier work in other species where it has been established that the *FT*  
362 gene is a key signaling factor whose expression is regulated by photoperiod and other environmental  
363 factors, and its translated protein is the phloem-transported factor that initiates flower development in  
364 shoot meristems [4, 8, 16, 36, 37]. While flowering has been known to be sparse and delayed in  
365 cassava, it was not previously known whether this was due to deficiencies upstream or downstream of  
366 FT signal production. In another member of the Euphorbiaceae family, *Jatropha curcas*, an *FT* homolog  
367 was isolated, and when *Jatropha* plants were transformed with this gene under the control of the strong  
368 constitutive 35S-CaMV promotor, plants flowered extremely early [10], as expected for FT involvement.  
369 The current findings are also in agreement with studies in several species where overexpression of  
370 *Arabidopsis FT* induced earlier flowering. For example, in the late-flowering tree *Eucalyptus*, when atFT  
371 was driven by the 35S-CMV promotor plants flowered very early, within 1 to 5 months after  
372 transplanting [15]. Also, in apple trees, overexpression of *Arabidopsis FT* driven by 35S-CaMV promotor  
373 resulted in flower development directly from callus [16], and in poplar trees, atFT overexpression driven  
374 by a heat inducible promotor gave substantially earlier flowering [19]. Such studies, as well as the  
375 current investigation with cassava, indicate that the necessary components of the FT response system  
376 downstream of FT production are present and functional in the shoot apical meristems of these species,

377 and that they are capable of interacting with the Arabidopsis FT gene-product to induce flowers much  
378 earlier than normal.

379           In cassava, branching occurs by outgrowth of axillary meristems subtending the shoot apical  
380 meristem (SAM), which results in two or more new shoot branches at the fork, occurs simultaneously  
381 with initiation of flower development at the original SAM [21, 27]. In the first tier of fork-type branching  
382 it is common in a large fraction of cassava genotypes for abortion of inflorescences and flowers such  
383 that these structures do not develop sufficiently to produce any mature flowers [27]. This was observed  
384 in the current study in the non-transformed genotype, 60444, which produced small flower stalks but  
385 did not produce any flower buds that exceeded the 2-mm diameter threshold for counting (Fig. 3c). In  
386 striking contrast, all four atFT over-expression lines produced abundant, fully developed flowers (Fig. 3c,  
387 4, and 5b). Furthermore, flower production on inflorescences continued over a longer time-frame such  
388 that more flowers were produced and flowers at each tier had greater longevity before senescence (Fig.  
389 3d). Previous studies of *FT* overexpression have not reported this effect on flower prolificacy and  
390 longevity. Apparently cassava, with its limited flower development on the first-tier inflorescences, has  
391 revealed another effect of FT on enhancing the continued development of flowers that goes beyond  
392 floral initiation.

393           An additional effect of *FT* overexpression was shoot architectural alterations in the cassava atFT  
394 overexpression lines. In contrast with the absence of lateral branches in the non-transformed 60444  
395 line, all lines overexpressing atFT produced abundant lateral branches, each of which forked and  
396 produced flowers (Fig. 5a and 5b). This finding agrees with studies in which the overexpression of *FT* in  
397 cotton increased the extent of branching, apparently by altering the balance between *FT* and the  
398 flowering inhibitor, TFL [38]. Increased branching has also been reported in transgenic plants  
399 overexpressing FT in tobacco (*Nicotiana* spp.) [11] and Eucalyptus [15]. In contrast to flower initiation,  
400 flower prolificacy, and branching, flower and leaf organogenesis was not apparently affected by *FT*

401 overexpression in cassava, as leaves and flowers were the same size and shape as in non-transformed  
402 plants (Fig. 4). This agrees with the outcome in most reported studies, but contrasts with findings in *FT*-  
403 overexpressing lines of apple, which had more numerous petals, fewer stamens, and no pistils [16], and  
404 in *FT* overexpression lines of tobacco where there was also altered leaf morphology, increased leaf  
405 chlorophyll content and photosynthetic rates, and flower abscission [11].

406 In some plant systems that have vegetative storage organs, one or more *FT* homologs have been  
407 associated with stimulating the initiation and growth of these organs. For example, in onion, bulb  
408 formation is regulated by two antagonistic *FT*-like genes. *AcFT1* promotes bulb formation, while *AcFT4*  
409 prevents *AcFT1* upregulation and inhibits bulbing in transgenic onions [39]. Another paralog, *AcFT2* plays  
410 direct role in floral induction. Also, in potatoes (*Solanum tuberosum*), floral and tuberization transitions  
411 are controlled by two different *FT*-like paralogues [40, 41]. In the storage-root crop sugar beet, one *FT*  
412 homolog acts as a stimulator of flowering while a second *FT* homolog functions in repression of  
413 flowering [42, 43]. In *Jatropha curcas* and *Populus spp* (poplar), which are species closely related to  
414 cassava, *JcFT* plays an inductive role in flowering while the *Populus* paralogs *PtFT1* and *PtFT2* both  
415 function to induce flowering but also perform other roles associated with growth cessation, promotion  
416 of vegetative growth and bud set [10, 13, 44].

417 In the present study, we observed that the transgenic lines overexpressing the Arabidopsis *FT* in  
418 cassava showed reduced storage-root development as indicated by less storage-root dry weight per  
419 plant (Fig.6a) and fewer number of storage-roots per plant (Fig.6d). The transformants also had a  
420 smaller total plant size (Fig. 6b), possibly because their increased development of flower primordia  
421 compromised the extent of new leaf production and hence restricted total plant growth. Alternatively,  
422 increased forking and axillary branch outgrowth and associated flowering in the *atFT* overexpression  
423 lines might have decreased production of leaves, which in turn affected whole-plant photosynthesis and  
424 growth. Studies have indicated that when branching is restricted, cassava storage-root yield is improved

425 [45]. Moreover, the cassava atFT overexpression lines had a lower harvest index (fraction of total dry  
426 matter in storage-roots) (Fig. 6c), indicating that rather than stimulating storage-root development, atFT  
427 might have had an inhibitory effect. Given that cassava is grown for storage-organ production, it is  
428 possible that domestication and breeding has led to genetic changes in FT that have the effect of  
429 increased storage-root production at the expense of flower development [46]. We hypothesize that  
430 cassava operates similarly to the species with vegetative storage organs discussed above, and may have  
431 regulatory pathways for floral development and storage-root development that are controlled by  
432 different *FT*-like genes.

433         We propose that this FT-expression system could be exploited to improve cassava breeding.  
434 Overexpression lines of cassava could be used as grafting partners, whereby the overexpression of atFT  
435 in understocks could provide a graft transmissible signal to scions of poor flowering lines. Graft-induced  
436 flowering with a profuse-flowering genotype as the understock has been used in other plant systems [7,  
437 19, 47, 48], including cassava [21]. *FT* overexpression might serve as a particularly effective means of  
438 producing and delivering the flower-inducing signal from understocks to scions.

439         In conclusion, we have demonstrated that atFT overexpression in cassava hastens flower  
440 initiation, and increases lateral branching, similar to reports in other species. In addition, our findings  
441 provide the first report that in cassava, atFT overexpression substantially improves the prolificacy of  
442 flower production and the longevity of flower development. We also show that while cassava has the  
443 necessary signaling factors to respond to atFT such that flower development was enhanced, atFT did not  
444 stimulate storage-root development. These findings have the potential for furthering our understanding  
445 of flower development and for use in stimulating flower production in breeding.

446

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450

## 451 **Author contributions**

- 452 • **Conceived and designed the experiments:** OSA, SJD, TLS
- 453 • **Performed construct formation, transformation and regeneration:** OSA, PC, SJD
- 454 • **Performed greenhouse experiments:** OSA, TLS
- 455 • **Contributed reagents/materials/lab tools:** JT, MF, SJD, TLS
- 456 • **Wrote the paper:** OSA, TLS
- 457 • **Obtained funding and provided supervision/mentoring:** JT, MF, SJD, TLS

458

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607  
608

## 609 **Supporting Information**

### 610 **S1 Figure. PCR of atFT in transgenic cassava and Arabidopsis genomic DNA.**

611 Lanes (left to right): cassava transgenic lines are labelled FT-02, FT-11, FT-13 and FT-17; No  
612 Template Control (NTC); non-transformed Arabidopsis Columbia ecotype (Col-0), and 60444 is

613 the untransformed cassava plant. The amplification product size of atFT is 189 bp in the cassava  
614 transformants. Lane Col-0 is Arabidopsis Col-0 DNA; the \* indicates the PCR product (1026 bp)  
615 of native FT including introns. Non-specific amplification products are labeled  $\Phi$ . Lane M  
616 contains a 1kb ladder (Thermo Scientific GeneRuler 1kb Plus DNA Ladder).

617 **S2 Figure. Number of nodes between forking events in non-transformed wildtype line (60444) and in**  
618 **four independent transformants.**

619 The number of shoot nodes between the soil surface and first forks, between the first-tier and  
620 second-tier forks, and between the second- and third-tier forks were counted at 5-6 months  
621 post planting in non-transformed wildtype line (60444) and in four independent transformants  
622 treated with water and 1% ethanol respectively. Shown are the means  $\pm$  SEM.

623 **S3 Figure. Total number of flowers per plant in water and ethanol treated control and transgenic**  
624 **plants.**

625 The number of flowers per plant were counted and recorded weekly, in non-transformed  
626 wildtype line (60444) and in the four independent transformants treated with water and 1%  
627 ethanol respectively. Shown are the means  $\pm$  SEM.

628 **S4 Figure. Time to start of flower senescence in water vs. ethanol treated transgenic plants and**  
629 **control.**

630 Flowering traits at each tier were recorded weekly to determine the time from flower  
631 appearance to initial date of flower senescence. Shown are the means  $\pm$  SEM.

632 **S5 Figure. Harvest Index in water vs. ethanol treated transgenic plants and control.**

633 Shown are the means  $\pm$  SEM.

634 **S6 Figure. Storage-root dry weight in water vs. ethanol treated transgenic plants and control. Shown**  
635 **are the means  $\pm$  SEM.**

636 **S7 Figure. Total plant dry weight in water vs. ethanol treated transgenic plants and control.** Shown are  
637 the means  $\pm$  SEM.

638 **S8 Figure. Harvest Index in water vs. ethanol treated transgenic plants and control.**

639 Shown are the means  $\pm$  SEM.

640 **S9 Figure. Total number of flowers per plant on lateral branches.** Data for plants treated with water  
641 and 1% ethanol were averaged. Shown are the means  $\pm$  SEM.













