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NOTE

BREEDING AND GENETICS

Does Overexpression of Tomato Fructokinase (*LeFRK1*) in Cotton Enhance Yield?

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ABSTRACT

Efforts to identify and introduce single genes that could maintain or increase cotton production in water-limited production settings have resulted in extremely limited success. The primary objective of this investigation was to test whether overexpression of the tomato fructokinase gene, *LeFRK1*, in field-grown cotton could improve fiber yield under variable growth conditions and contrasting irrigation levels in warm semi-arid environments characteristic of the Southern High Plains. A secondary goal was to determine whether a larger field-scale experiment might be justified based upon the results of this exploratory work. Cotton overexpressing *LeFRK1* was field grown in small plots for three years under irrigation, contrasting irrigation levels, or without irrigation. Increased yield was found when comparing all *LeFRK1* lines relative to that of the control line, though seasonal and plant-to-plant variability limited confidence in the extension of results to production scale. We hypothesized that yield improvements resulted from a suite of responses arising from increased availability of photosynthate at the leaf to the whole-plant level to developing fruits. The results suggested that *LeFRK1* overexpression might be a viable approach to improving cotton yield in warm, semi-arid environments characteristic of the southwestern U.S. However, field trials under agronomically relevant systems and at agronomically relevant scales are needed to confirm these findings.

Cotton plants are important for their cellulosic seed-coat trichomes (fibers). Cellulose synthesis contributes a strong, irreversible, structural carbohydrate carbon sink notably in cotton fiber development (Haigler et al., 2001; Mukherjee et al., 2015). The initial source of carbohydrate for cellulose synthesis in a developing trichome is sucrose transported from the leaves via the phloem (Granot, 2007; Nguyen et al., 2016; Ruan, 2012; Tarczynski et al.,1992). The key enzyme that catabolizes sucrose for subsequent metabolic pathways in the cotton fiber is sucrose synthase (Sus) (Anderson-Gunnerås et al., 2006; Mukherjee et al., 2015; Sturm and Tang, 1999; Tarczynski et al., 1992; Weber et al., 1997; Xu et al., 2012). This enzyme catalyzes the reaction between uridine diphosphate (UDP) and sucrose, yielding UDP-glucose (UDP-G) and fructose (Granot, 2007). Because of the close spatial association of Sus and cellulose synthase (Ruan, 2007), the UDP-G generated is readily available for cellulose synthesis needed for cell expansion and secondary wall deposition during cotton fiber development (Anderson-Gunnerås et al., 2006; Mukherjee et al., 2015; Sturm and Tang, 1999; Weber et al., 1997; Xu et al., 2012). However, Sus is inhibited by its other product, fructose, through a feedback inhibition mechanism (Granot, 2007; Granot et al., 2013, 2014; Mukherjee et al., 2015; Schaffer and Petreikov, 1997). Therefore, keeping the fructose concentration low minimizes the inhibition of Sus, and maximizes UDP-G availability for fiber production.

The phosphorylation of fructose is the mechanism that reduces the concentration of fructose in the cell. Fructokinase (FRK) is the primary enzyme that phosphorylates fructose (Granot et al., 2013); produces fructose-6-phosphate (F6P), which can

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enter synthetic and catabolic processes; and reduces Sus inhibition (German et al., 2003; Mukherjee et al., 2015). Previous studies indicated that FRK is important for plant growth and development, notably cells undergoing secondary cell wall synthesis. For example, suppressing FRK activity in tomato (*Solanum lycopersicum* L.) and hybrid aspen (*Populus tremula* L.× *Populus tremuloides* Michx.) negatively affected cell wall and vascular tissue development. This was interpreted as an indication of a synergistic relationship between Sus and FRK during cell wall development (Damari-Weissler et al., 2009; German et al., 2003; Odanaka et al., 2002; Roach et al., 2012).

Our initial hypothesis was that increasing FRK activity in cotton fiber cells would increase Sus activity and cellulose synthesis, leading to enhanced fiber quality and yield. Mukherjee et al. (2015) found that under near optimal growing conditions in a greenhouse experiment, constitutively overexpressing the tomato (*S. lycopersicum* L.) FRK gene, *LeFRK1*, in cotton (*Gossypium hirsutum* L. cv. Coker 312) resulted in an increase in boll number and seed cotton and fiber yield per plant. However, fiber length and strength were not enhanced by the over-production of FRK activity in fiber cells, suggesting that Sus was not inhibited substantially by fructose in the developing fiber cells of wild-type cotton plants. They proposed that the improvement in yield was due to an enhanced leaf number per plant and individual leaf area, increasing the over-all carbon gain by the *LeFRK1* plants.

We subsequently hypothesized that improved whole-plant net CO_2 assimilation by overexpression of *LeFRK1* might lead to increased yield in field conditions where water availability varies, and frequent supra-optimal temperature excursions occur during the growing season. It is generally accepted that such stressors lead to considerable reductions in both fiber yield and quality (Pettigrew, 2004) through effects on fiber biochemistry and photosynthate production during fiber development (Loka et al., 2011; Salvucci and Crafts-Brandner, 2004). To determine whether large-scale field studies would be justified, we conducted a small-scale preliminary field study for three years. Our working hypothesis was that the *LeFRK1* plants would exhibit a similar or even greater improvement in yield under a more agronomically relevant field setting as compared to those previously obtained under controlled greenhouse conditions (Mukherjee et al., 2015). The purpose of this report is to communicate results of these experiments.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Transgenic cotton (*G. hirsutum* L., cv. Coker 312) plants overexpressing the *LeFRK1* gene from tomato were generated as described in Mukherjee et al. (2015). Over the course of 3 yrs, four *LeFRK1* genotypes resulting from separate *LeFRK1* insertion events (2-2a, 19-3a, 35-1a, and 95-5a) were used along with a non-transgenic, "null" Coker 312 control. Homozygous *LeFRK1* seed was not available the first year but was planted thereafter. The genotypes used and the numbers of individuals evaluated differed from year to year (Table 1). In every case, each plant was screened for the presence of the *LeFRK1* gene using DNA-based screening (DNeasy Plant Mini Kit, Qiagen, Valencia, CA; Go Taq Gree Master Mix, Promega, Madison, WI).

The plants were grown in rows within borders of non-transgenic cotton (cvar. DeRudder Red) at the USDA-ARS facility in Lubbock, TX (33° 35' 38.9" N, 101° 53' 52.1" W) between early June and the end of October in 2013, 2014, and 2015. Rows were spaced 1 m (40 in) apart, and the lines (8-12 plants/line) were randomly distributed within a row. After *LeFRK1* gene screening, the plants were thinned to allow 15 to 20 cm (6-8 in) spacing between plants. The individual plant, not the line, was the statistically replicated unit. The soil at the Lubbock USDA location is an Amarillo fine sandy loam (fine-loamy, mixed, superactive, thermic Aridic Paleustalfs). Precipitation, temperature, and other selected weather variables were measured by the onsite meteorological station located approximately 300 m west of the plots (Stout, 2018).

Irrigation treatments varied. In 2013, plants were grown under surface drip irrigation. In 2014, the plants were grown in two adjacent plots under differential irrigation treatments, one receiving "high irrigation" and the other "reduced irrigation". In 2015, the plants were rainfed, grown without irrigation. Irrigation decisions during development were subjective and experientially based. For presentation and summarization of the four resulting environments, and to allow a preliminary investigation into the relative responsiveness of the null plants and the *LeFRK1* overexpressors, potential evapotranspiration was calculated and compared to the water delivered during the estimated time of fruit setting.

Table 1. Selected yield parameters of null controls and *LeFRK1* genotypes in different environments (as years and irrigation treatments). Numbers of matured bolls per plant, seed cotton mass per plant (SCM/plant), fiber mass per plant (FM/plant), calculated seed cotton mass per boll (SCM/boll), and fiber mass per boll (FM/boll) of *LeFRK1* overexpressing lines and the nonexpressing, null line are presented. Values are the means ± s.e. Significance by Dunnet's t-test comparing *LeFRK1* genotypes against null controls at $p_t \leq 0.05$ are indicated by *Bold Italics*

Environment	Isoline	n	Bolls	SCM/plant (g)	FM/plant (g)	SCM/boll (g)	FM/boll (g)	n	Mainstem Nodes
2013 Irrigated	Null	4	11 ±1	45.1 ±9.3	11.9 ±3.5	4.1 ±0.6	1.1 ±0.2	4	11 ±0.9
	2-2a	6	13 ±1	64.7 ±6.1	20.2 ±2.0	5.5 ±0.7	1.7 ±0.3	4	13 ±0.6
	19-3a	4	***29 ±6***	***165.8 ±34.5***	***55.0 ±12.8***	5.7 ±0.1	1.9 ±0.1	4	14 ±2.8
	95-5a	4	21 ±3	90.7 ±12.9	26.2 ±4.8	4.5 ±0.2	1.3 ±0.1	5	11 ±1.0
2014 High Irrigation	Null	6	14 ±1	55.6 ±9.3	21.3 ±3.8	3.9 ±0.5	1.5 ±0.2	7	9 ±0.9
	2-2a	7	***8±1***	39.9 ±6.7	15.4 ±2.6	5.1 ±0.5	2.0 ±0.2	7	11 ±0.3
	19-3a	7	12 ±1	68.8 ±11.7	26.1 ±4.5	5.7 ±0.7	2.2 ±0.2	5	11 ±0.7
	95-5a	6	16 ±2	82.1 ±10.2	31.7 ±3.9	5.1 ±0.1	2.0 ±0.1	7	11 ±0.3
	35-1a	3	18 ±3	87.8 ±20.2	32.6 ±7.5	4.7 ±0.6	1.7 ±0.2	4	12 ±1.3
2014 Reduced Irrigation	Null	8	6 ±1	24.8 ±3.6	9.4 ±1.5	3.8 ±0.3	1.4 ±0.1	8	9 ±0.4
	2-2a	9	10 ±1	41.7 ±7.3	15.5 ±2.8	4.1 ±0.2	1.5 ±0.1	10	***13 ±0.6***
	19-3a	7	***12 ±1***	***60.4 ±8.5***	***22.5 ±3.3***	***5.1 ±0.4***	1.9 ±0.2	8	***13 ±0.7***
	95-5a	6	11±1	56.1 ±14.6	20.6 ±5.7	4.9 ±0.5	1.8 ±0.2	6	11 ±0.7
	35-1a	5	10 ±2	38.5 ±8.4	13.3 ±3.4	3.8 ±0.1	1.3 ±0.1	7	***12 ±0.6***
2015 Rainfed	Null	8	20 ±2	81.2 ±9.5	31.9 ±3.7	4.3 ±0.5	1.7 ±0.2	8	13 ±1.1
	2-2a	7	***29 ±4***	***144.8 ±14.6***	***54.8 ±5.9***	5.1 ±0.3	1.9 ±0.1	7	15 ±0.8

Thermal units were calculated as growing degree days using the averaged daily maximal and minimal air temperatures recorded at the onsite weather station (Stout, 2018), subtracting a 15.6 °C (60 °F) base temperature (Wanjura and Supak, 1985), and letting accumulated thermal units equal zero when average temperatures were below the base temperature (Hereafter we use the less accurate, but more commonly used term, heat units). The accumulated heat units were used to drive a model (Grimes and El-Zik, 1990; Young et al. 1980) to calculate the crop coefficient (K_c) of a cotton crop allowed to develop under the absence of water stress in daily time steps. The standardized reference crop evapotranspiration (ET_0) for short crops (Allen et al., 2005) was obtained from the Texas Tech Mesonet (Burget, 2018). Data for calculation of ET_0 were from a station (Lubbock 3WNW-TTU) located approximately 1 km NE of the experimental plots within the TTU Natural Resources Management Rangeland Research Station (33° 36' 14.7" N 101° 53' 57.1" W). The calculated potential evapotranspiration (PET_c) of the developing crop was expressed as the product of ET_0 and K_c in daily time steps (Grimes and El-Zik, 1990). Available water was then calculated as the difference between the cumulative PET_c and the cumulative water delivered to the crops (irrigation plus precipitation) between the estimated time of first bloom and first open boll (Supak, 1984). The conditions experienced by the plants and the estimated times of first bloom and open boll in each of the four environments are summarized in Fig. 1.

Measurements of Agronomic and Plant Traits. Seed cotton (fibers with seeds) was harvested by hand through October of each year. The small, immature, partially opened bolls that remained were less than 10% of the total number of bolls produced. After measuring the seed cotton mass per plant (SCM/plant), the cotton was ginned using a small research gin to determine the fiber mass per plant (FM/plant). The boll numbers, SCM/plant, and FM/plant were used to calculate values of average seed cotton mass per boll (SCM/boll), fiber mass per boll (FM/boll), and lint percentage (LP). Numbers of mainstem nodes were recorded at harvest.

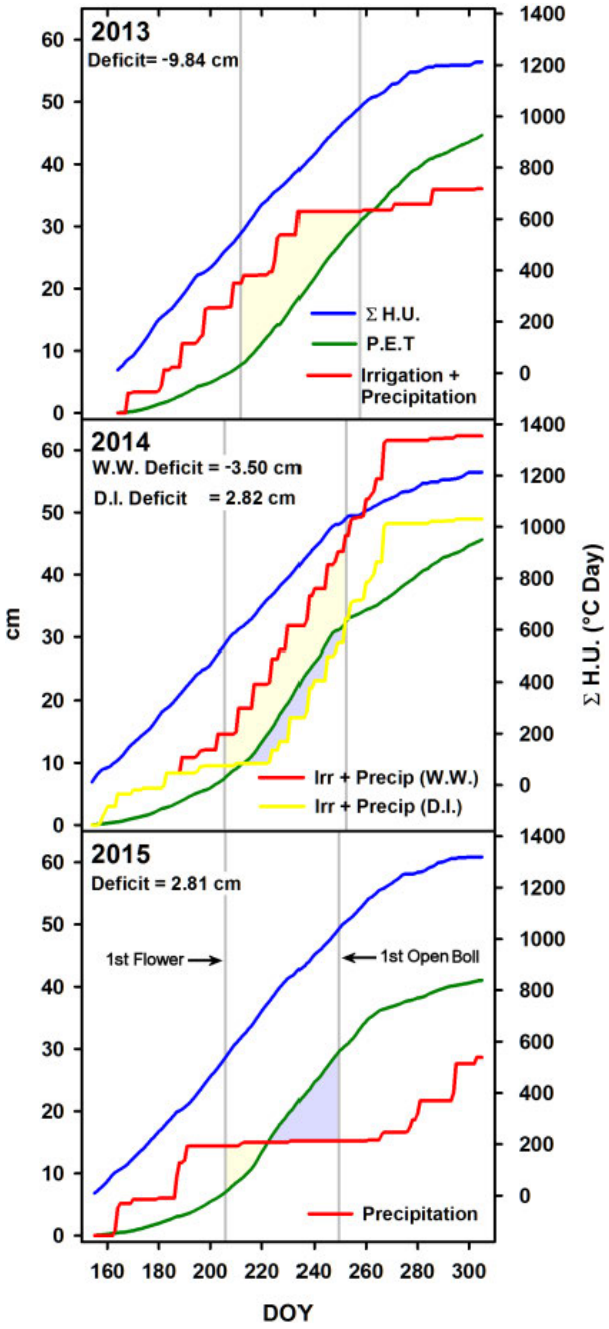

Figure 1. Estimation of water deficit (equal to negative surplus) based upon a simple heat unit. Water deficit is the difference between potential evapotranspiration (PET) and water input (precipitation + irrigation). Gray vertical lines are 1st flower and 1st open boll based on thermally driven model. Red and blue shaded areas are estimated deficit and surplus water, respectively.

Statistical Analyses. The experiment was considered to have occurred under four discrete environments affected by time of planting and irrigation (Fig. 1). Two types of plants were used, nulls and *LeFRK1* overexpressors. Within the *LeFRK1* set, there were a total of four genotypes resulting from four separate insertion events. The replicated unit was the individual plant. The null control genotype was present in all seasons, but the *LeFRK1* genotypes planted and surviving varied from year to year (Table 2). All statistical analyses were done with SAS 9.2 (SAS Institute Inc., Cary, NC). Where reported, correlations are from the PROC CORR routine. Effects on yield were analyzed using a two-way ANOVA under General Linear Models (PROC GLM) controlling for errors that arose from the unbalanced nature of the experimental design. Within each environment, differences in absolute yield/plant were analyzed using Duncan's Multiple Range test using the nulls as control. Responses of yield to gene expression of all *LeFRK1* plant within all environments were also analyzed relative (%) to the nulls. Means of *LeFRK1* responses for all environments and genotypes were separated by t-test assuming both equal pooled error and by using Satterthwaite approximation to control for unequal standard errors. (The Satterthwaite method only increased the significance of means separation and did not add to the interpretation of the data, so only results of the more conservative pooled error is reported.)

RESULTS

Growth Conditions. The total amount of water (rainfall + irrigation), accumulated heat units, and the calculated PET_c received by the plots from planting through harvest are shown in Fig. 1. The amount of water available to the plots from planting to the first open boll (as total water delivered - PETc) varied from -2.82 cm to 9.84 cm. Though the amount of total water received in the 2013 environment was considered typical of fully irrigated commercial fields in the area, the total amount of water delivered to the plants was considerably less than that delivered to those under the deficit irrigation environment in 2014. The least water delivered was in the 2015 rainfed environment, which exhibited the highest yields across treatments.

Yield. Attempts to correlate plant yield response to water availability across environments was met with extremely limited success. The average estimated readily available water during thermally estimated fruit establishment was not correlated with any measured yield characteristics (r = -0.17-0.22, p = 0.02-0.22) except perhaps to bolls/plant and nodes/plant. The relationship between these yield parameters to water availability was weak: bolls/plant (r = 0.15, p = 0.15) or mainstem nodes (r = -0.17, p = 0.09).

Perhaps not surprisingly bolls/plant and mainstem nodes were the strongest predictors of yield on a per plant basis. SCM/plant was most strongly correlated with number of bolls (r = 0.94, $p < 0.0001$), whereas the number of mainstem nodes exhibited a much weaker though significant relationship (r = 0.44, $p = 0.0001$). Boll number was subsequently used as a developmental surrogate for yield in subsequent two-way ANOVA. Boll number was significantly affected by irrigation level ($p_r < 0.0001$), *LeFRK1* expression ($p_r = 0.0004$), and significant interaction between irrigation and *LeFRK1* expression was found ($p_r = 0.04$). No further attempt to partition yield data error across environments was made.

Fairly consistent improvements were found in yield as FM/plant in *LeFRK1* plants relative to null plants in 2013, in the reduced-irrigation plants in 2014, and for the rainfed plants in 2015, when examined by simple one-tailed t-tests, though these often were not significant by Dunnet's t-test, which controls for errors associated with multiple measurements. Only the more conservative results of the Dunnet's test, which controls for error associated with multiple measurements, are shown here. When a simple t-test was used to compare individual *LeFRK1* genotype responses to null controls, the significance of individual comparisons was increased (not shown). Greater yield values for the transgenic plants were associated with somewhat higher numbers of bolls per plant (r = 0.94, $p < 0.0001$) and higher SCM and FM per boll than for the null plants (Table 1). However, yield was variable across the four environments. Under high irrigation in 2014, increased yield with *LeFRK1* expression was less consistent across *LeFRK1* genotypes. Only in 2015 was there a slightly lower nonsignificant percentage of lint for the *LeFRK1* plants than for the null plants (not shown).

When yield data for all *LeFRK1* plants were expressed as increase (%) relative to null plants for each year, no significant differences between the responsiveness of individual genotypes to *LeFRK1* expression could be found, so the yield responses of the individual *LeFRK1* genotypes were combined across years. All yield components increased (Fig. 2) except for LP. Contrary to the results from the greenhouse study of the *LeFRK1* plants (Mukherjee et al., 2015), there were no significant genotypic differences in LP (Fig. 2). Mean FM and SCM across all four environments increased by approximately 80% with *LeFRK1* overexpression ($p_t < 0.001$).

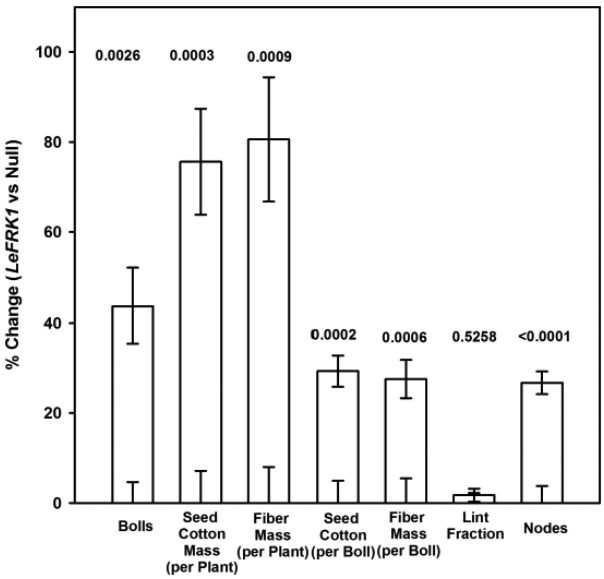

Figure 2. Response of selected yield and growth parameters to *LeFRK1* overexpression as compared to control (Null) plants (along x-axis, 0%). Significance (p_t) is shown above plots. Bars are s.e.

DISCUSSION

The previous greenhouse study demonstrated increased seed cotton and fiber yield of *LeFRK1* plants compared to the control genotype (null line) (Mukherjee et al., 2015). Because differences in potential crop plant performance observed in a controlled environment are often masked by variability in field settings, controlled environment results often cannot be replicated in the field. The aim of this study was to attempt to extend the initial greenhouse findings to a field setting. The scale of the experiment was small. Few individuals of several genotypes were grown, either because seeds were limited or due to the vagaries of the environment. In the first year, seeds were heterozygous and individual plants had to be enzymatically assayed to ensure *LeFRK1* was being overexpressed. The second year, few homozygous seeds were available. In the third year a single intense precipitation event destroyed much of the experiment leaving only one genotype in the field. The experiment was conducted across several environments (years and irrigation treatments) in small plots adjacent to a field research site. The results were used, in part, to determine whether a larger scale field trial of the *LeFRK1* technology could be justified. We found that *LeFRK1* overexpression significantly improved seed cotton

and fiber yield (Fig. 2). Although the results are consistent with the working hypotheses and are consistent with the earlier greenhouse study, the magnitude of the yield increases associated with *LeFRK1* overexpression were somewhat surprising. Because there are, to our knowledge, no reports of such large yield increases in response to overexpression of a single gene in cotton, this technology could represent an important technical advance.

Several aspects of the work should be borne in mind when evaluating the results presented. These include: not replicating the experiment with all *LeFRK1* genotypes and irrigation treatments across all environments; not controlling for the potential effects of field heterogeneity, ideally by planting several plots within the same field or in multiple locations; not consistently recording timing of plant developmental events; and using post hoc statistical comparisons. Post hoc statistical analyses are fraught with assumption. Even with these caveats, because the results are consistent with earlier greenhouse work (Mukherjee et al., 2015) and across multiple field environments (Fig. 2), it is difficult to attribute the results purely to experimental error.

To assist the reader in critically evaluating the results and the interpretation presented, the experimental conditions, methods, and the post hoc analytical approaches are reported explicitly. Lack of consistent replication of the isolines used during all three years made it difficult to discern whether there are consistent, reproducible performance differences between isolines. Differences in the yield increase between the different *LeFRK1* genotypes were not observed, or at least were not consistently observed; thus, the yield responses of all the *LeFRK1* overexpressors relative to that of the nulls were combined for analysis across years. It was also thought that problems associated with post hoc comparisons were balanced somewhat by the increased statistical power to detect differences afforded by increasing replicable units. The result was that *LeFRK1* overexpression increased all measured yield components, except for LP. All other yield components were significantly ($p < 0.001$) increased by *LeFRK1* expression (Fig. 2), and the fiber production per plant was nearly doubled (80% increase).

It might be important to note that in the previous greenhouse experiment (Mukherjee et al., 2015), LP was consistently lower in the *LeFRK1* overexpressors than in the null plants. In the greenhouse experiment plants were well-watered to eliminate confounding effects of drought stress. However, in the present study, no consistent reduction in LP was found. Only in 2015, when plants were grown under dryland conditions as opposed to the well-watered greenhouse conditions in the previous study, was a small reduction in LP observed. Moreover, in the present study there was no difference ($p_t = 0.52$) in LP when all *LeFRK1* overexpressors were compared to nulls across environments (Fig. 2). It was tentatively concluded that LP is unaffected, or at least less affected, by high FRK activity under field conditions. Whether and how water availability might have affected LP in *LeFRK1* plants remains unclear.

Several questions remain about the relative performance of the individual *LeFRK1* overexpressors. Plants containing *LeFRK1* generally out-performed the null controls, primarily when water availability was not excessive (2013, reduced irrigation in 2014, and 2015; see Table 1). Mean yield (SCM and FM per plant) for all but line 35-1a of the *LeFRK1* plants was consistently higher than the yield for the null line under these conditions. However, plant-to-plant variability resulted in an inability to demonstrate statistical significance with the conservative approach used. Simple t-tests against the nulls revealed significance but it was believed this was exaggerated and so not presented. This suggested a larger full-field scale study with various levels of water availability might be appropriate.

In the earlier study it was proposed that *LeFRK1* yield improvements resulted from increased photosynthate production and from alterations of fiber biochemistry during development (Mukherjee et al., 2015). This hypothesis was based in part on the observation that the greenhouse-grown *LeFRK1* plants had greater numbers of leaves and leaf areas as compared to the null plants. In the present work, we observed a significant increase in the number of mainstem nodes for the *LeFRK1* plants across environments (Fig. 2), though the genotypic response of the *LeFRK1* overexpressors was variable (Table 1). An increase in mainstem nodes increases the number of stem leaves, potentially increasing photosynthate production. Increasing mainstem nodes also increases the potential for more fruiting branches that will produce more flowers and bolls. The enhancement of SCM and FM per *LeFRK1* plant was strongly associated with enhanced boll numbers per plant, which was most evident in 2014 under reduced irrigation. Taken together, these results suggest that

a major contributor to the yield improvement for *LeFRK1* plants could be the improvement in branch number at cutout. Because water deficit can cause a reduction in the number of mainstem nodes and suppress flower development (Gerik et al., 1996; Stockton et al., 1961; Turner et al. 1986), *LeFRK1* overexpression might improve yield under such conditions. Whether mainstem node accretion rate of constitutive *LeFRK1* overexpressors is resistant to drought remains to be rigorously examined.

CONCLUSIONS

The results support the hypothesis that constitutive *LeFRK1* overexpression increases cotton yield in agronomically relevant environments. However, the magnitude of yield response to *LeFRK1* overexpression, an 80% yield increase per plant, was unexpected, so a larger scale experiment remains to confirm these results at the field scale. The responsiveness to *LeFRK1* overexpression might be affected by water availability and might be affected by the genotypes used. Further studies would ideally include differential or various preplanned irrigation water deficit levels and larger numbers of plants of each genotype to separate differences. But, from a practical agronomic point of view, a simple year-to-year, multilocation, or even mesoscale multiplot field trial using a single genotype or even simply bulking all *LeFRK1* overexpressors together might be appropriate.

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