A mutant allele of the *flowering promoting factor 1* gene at the tomato *BRACHYTIC* locus reduces plant height with high quality fruit

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Abstract

Reduced plant height due to shortened stems is beneficial for improving crop yield potential, better resilience to biotic/abiotic stresses, and rapid crop producer adoption of the agronomic and management practices. Breeding tomato plants with a reduced height, however, poses a particular challenge because this trait is often associated with a significant fruit size (weight) reduction. The tomato *BRACHYTIC* (*BR*) locus controls plant height. Genetic mapping and genome assembly revealed three *flowering promoting factor 1* (*FPF1*) genes located within the *BR* mapping interval, and a complete coding sequence deletion of the telomere proximal *FPF1* (*Solyc01g066980*) was found in the *br* allele but not in *BR*. The knock-out of *Solyc01g066980* in *BR* large-fruited fresh-market tomato reduced the height and fruit yield, but the ability to produce large size fruits was retained. However, concurrent yield evaluation of a pair of sister lines with or without the *br* allele revealed that artificial selection contributes to commercially acceptable yield potential in *br* tomatoes. A network analysis of gene-expression patterns across genotypes, tissues, and the gibberellic acid (GA) treatment revealed that member(s) of the *FPF1* family may play a role in the suppression of the GA biosynthesis in roots and provided a framework for identifying the responsible molecular signaling pathways in *br* mediated pheno-typic changes. Lastly, mutations of *br* homologs also resulted in reduced height. These results shed light on the genetic and physiological mechanisms by which the *br* allele alters tomato architecture.

Significance statement

Experiments involving knocking out the tomato *BRACHYTIC* allele in a commercialized large-fruited fresh-market tomato variety showed that *brachytic* mutant plants are short but produce heavy fruits, which demonstrates the strong agronomic relevance of the mutant allele. The *brachytic* plants need other gene(s) to achieve commercially acceptable yield (fruit weight × fruit number) potential as it is evidenced by artificial selection.
1 | INTRODUCTION

Tomato (Solanum lycopersicum) is the most valuable horticultural crop worldwide (Food and Agriculture Organization of the United Nations, 2016) and provides essential micronutrients (U.S. Department of Agriculture, 2016). Fresh-market and processing tomatoes are the two most widely consumed types of tomatoes and account for more than $2.6 billion in annual farm cash receipts in the United States alone, with fresh-market tomatoes accounting for more than $1.2 billion (U.S. Department of Agriculture, 2016).

In particular, large-fruited fresh-market tomatoes cultivated in the United States (also called round tomatoes or beefsteak tomatoes) represent a unique type (fruit class) of tomato that is bred for direct consumption (Bhandari & Lee, 2021). These tomatoes account for more than $600 million per year in farm cash receipts (Florida Tomato Exchange, 2022). In this type of tomato fruit class, fruits that are medium-sized (medium fruit size > 5.715 cm in diameter; U.S. Department of Agriculture, 2005) or larger and those with high exterior standards (i.e., flawless skin and perfectly symmetrical fruit shape) are generally classified as marketable and are desirable in the fresh-market tomato industry and sold in the consumer market.

Most fresh-market tomato varieties have determinate vines (driven by the homozygous sp allele at the SELF-PRUNING locus; Barton et al., 1955; Pnueli et al., 1998). However, their stems grow off of conventional raised plastic beds (typically 71.12 cm in width), which expose the fruit/vegetative tissue to the soil unless the stems are tied on vertical stakes (e.g., Figure 1d in Lee et al., 2018). The displacement of plants, especially fruit laying on the soil, significantly reduces fruit yield and quality via damages from human activity, machinery, and soil-borne pathogens (Adelana, 1980; University of Florida, Institute of Food and Agricultural Sciences [UF/IFAS], 2022). Thus, the majority of US large-fruited fresh-market tomatoes are grown with the support of stakes and ties on raised plastic beds in the open field cultivation system. However, the shift toward farm machinery to increase productivity and market value (Pardey et al., 2016; Zahara & Johnson, 1981) necessitates the development of a ground cultivation system for shortened plants, which does not require stakes and ties on raised plastic beds to enable mechanical harvesting (discussion in Gardner & Davis, 1991; Florida Tomato Committee, 2022; Frasca et al., 2014; Kemble et al., 1994a; Kemble et al., 1994b). Essentially, a reduced plant height enables a higher plant density, which can compensate for the loss in vertical space utilization, and has the potential to increase commercially acceptable yield potential (Ozminkowski et al., 1990; Scott et al., 2010). Therefore, it would be desirable to develop shortened fresh-market tomato varieties that maintain the marketable fruit size for commercially acceptable yield potential.

A shorter stem driven by shortened internodes is a detectable phenotypic property (phenotype) of plants typically deficient in endogenous gibberellin (GA) biosynthesis or defective in perception of GA (Binenbaum et al., 2018; Yamaguchi, 2008). In tomato, multiple genes essential for GA synthesis are functionally characterized (Carvalho et al., 2011; Illouz-Eilat et al., 2019; Koornneef et al., 1990; Li et al., 2012). Similarly, this phenotype can be observed in tomatoes with different hormonal mutations, such as mutations in genes involved in brassinosteroid (Bishop et al., 1999; Li et al., 2016; Marti et al., 2006; Nie et al., 2017) or indole-3-acetic acid synthases (Higashide et al., 2014; Leyser, 2018; Zhao, 2018). However, such mutation-derived phenotypes with reduced height have not been utilized commercially, particularly in large-fruited fresh-market tomato varieties, often due to extreme reductions in the fruit size (medium-sized or larger fruits) classified as typical marketable fruits in large-fruited fresh-market tomato in the United States; Florida Tomato Committee, 2022) and marketable yield, which were observed for tomato germplasm releases and varieties (Scott & Harbaugh, 1989; J.W. Scott and R.G. Gardner, personal communications; a comparison of fruit size shown in Figure S1), and experimental tomato lines (Higashide et al., 2014; Liu et al., 2018; Srivastava & Handa, 2005) that harbor mutant allele(s).

Unlike typical dwarfism in which all organs are reduced in size, the plant BRACHYTIC (BR) gene typically influences stem growth, which was observed in both monocots, such as barley (Rossini et al., 2006) and maize (Balzan et al., 2018; Knöller et al., 2010; Multani et al., 2003; Xing et al., 2015), and dicots, such as chickpea (Gaur et al., 2008) and soybean (Cui et al., 2007). Mutations in an ATP binding cassette type B auxin transporter decreases internode length in br2 mutant maize (Multani et al., 2003; Xing et al., 2015). In peach, a GA receptor GID1c mutant causes the brachytic dwarfism trait, but does not impair fruit development (Hollender et al., 2016). Collectively, these studies suggest that different mechanisms/genetic loci affect organ development.

Tomato BR is a known genetic locus and defined by phenotype (Balint-Kurti et al., 1995; Barton et al., 1955; MacArthur, 1931), although its DNA sequence is not published yet. Plants with long (hereafter referred to as the wild-type phenotype), intermediate, and short (br phenotype) stems have homozygous BR allele (BR/BR), heterozygous alleles, and homozygous br allele (br/br), respectively (Lee et al., 2018). The br is the primary determinant of the shortened internode phenotype, which inevitably reduces stem length, and is a focus of fresh-market tomato breeding programs (Frasca et al., 2014; Scott et al., 2010; Tichelaar, 1986; UF/IFAS, 2022). Furthermore, the br has been used to generate a shorter architecture in indeterminate fresh-market tomato lines facilitating horticultural practices, as the production can be on shorter vertical stakes rather than the tall growing indeterminate tomatoes but still maintain indeterminate habit for its benefits (Panthee & Gardner, 2013; North Carolina State University [NCSU], 2022).

**KEYWORDS**

applied tomato genetics, plant breeding, plant genetics, plant physiology, Solanum lycopersicum
Notably, there is no evidence for a negative correlation between marketable fruit yields and the br (Frasca et al., 2014; Gardner & Davis, 1991). However, in the absence of a completely known pedigree for tomato materials, the effect of the genetic background on quantitative yield traits of br plants is unclear. Moreover, although mapping of the tomato BR locus was reported (Lee et al., 2018), the genetic and molecular characteristics have not been reported in a peer-reviewed forum. Therefore, knowing the genetic basis of br would contribute to knowledge-based breeding, by which an ideotypic plant architecture can be designed and the appropriate gene targets can be manipulated to create such improved varieties.

**FIGURE 1** DNA sequence variation in a flowering promoting factor 1 gene (FPF1, Solyc01g066980) at the BRACHYTIC (BR) locus causes a reduced tomato height. (a) Diagram of the BR locus. A scaffold (a sequence assembly carrying genomic DNA from the br genetic interval, which is 481-kb in size; scaffold ID 143086) spanning the BR locus (175-kb) was compared to the genome of Heinz 1706. Numbers and black marks indicate tomato genes (e.g., Solyc01g066915 according to the annotation ITAG4.0; Fernandez-Pozo et al., 2015). Three FPF1 genes are shown by black-filled marks. Red arrow indicates a complete coding sequence deletion in a FPF1 gene in the br allele. Black arrows indicate sequence polymorphisms in introns. The prefix brM indicates molecular markers used for fine mapping. brM10 and brM12 indicate the beginning and end of the mapping interval, respectively, supported by two different recombinant inbred line (RIL) populations and brM11 indicates a recombination breakpoint supported by a single RIL population. C indicates the direction toward the centromere. (b) Generation of Solyc01g066980 mutations by the CRISPR-Cas9 using two independent single-guide RNAs (sgRNA1 in red and sgRNA2 in green). Black horizontal lines and a block icon indicate untranslated regions and the coding sequence of Solyc01g066980 at the BR allele, respectively. A complete coding sequence deletion found in the br allele is indicated by a dashed rectangle. Nucleotide sequences of Solyc01g066980 mutant alleles br.8.1CR and br.8.2CR are shown with the corresponding amino acid sequences, and deletions are indicated by red or green dashes. The sequence gap length is shown in parentheses. (c) A mature transgene-free, homozygous mutant plant (br.8.2CR; right) was compared to its wild-type large-fruited fresh-market tomato Fla. 8059 (wild-type allele BR/BR; left). Fla. 8059 is one of the two parents of a commercial hybrid (F1), Tasti-Lee™ (Bejo Seeds, Oceano, CA), currently in the US market. The soil level in a 5-gallon soil bag is indicated by horizontal dashes. Each plant was supported by seven strings. White vertical bar represents 50 cm. (d) Representative picture of fresh-market tomato plants with the support of stakes and ties on raised plastic beds. Three plants of each genotype are shown. An approximation of the plant height is indicated by horizontal dashes. Each plant was supported by seven strings. White vertical bar represents 50 cm. (e) The stem lengths of 6-week-old plants were measured. WT, wild-type. Mutants are transgene-free, homozygous M2 generation. The n value represents the total number of plants evaluated. Statistical significance is indicated by ***P < 0.001, as determined by one-way ANOVA in conjunction with a two-tailed Tukey’s HSD multiple comparison test. Error bars indicate 95% confidence intervals. (f) Scanning electron microscope images of the first internode of WT (left image) and mutant (right) plants. White bar indicates 100 μm.
Therefore, br is a key target for the optimization of the fresh-market tomato architecture for various breeding goals. In this study, we mapped and identified sequence variants at the BR locus in large-fruited fresh-market tomatoes (Fla. 8044 [BR/BR], Fla. 8624 [BR/BR], Fla. 8834 [br/br], and Fla. 8916 [br/br]) and found that one of three flowering promoting factor (FPF) genes within the fine mapping interval is directly responsible for reduced plant height, as validated using CRISPR-Cas9-driven mutants. Using CRISPR-Cas9-driven br mutants and a pair of sister lines (one with and the other without the br allele, respectively) derived from conventional crossing/selection methods, we evaluated the effects of the br trait on fruit yields and horticultural traits of a large-fruited fresh-market tomato in two growing seasons in the greenhouse or open field in the Southeastern United States, where this tomato was originally bred. We compared gene expression patterns across tissues, genotypes, and GA treatments by a network analysis and evaluated the role of FPF genes in br-mediated traits. We created new sources of fresh-marketable tomato plants harboring mutated versions of FPF homologs adjacent to br. We discussed the implications of this gene for future breeding approaches.

2 | RESULTS AND DISCUSSION

2.1 | A DNA sequence polymorphism in a flowering promoting factor 1 gene is present in the BRACHYTIC fine-mapping interval

The tomato BR was mapped to a 763-kb interval on chromosome 1 (Lee et al., 2018). To narrow this interval, three recombinant inbred line (RIL) populations segregating for plant height were used for further mapping of the BR locus. Four newly identified RILs had crossing overs mapping to two sites within the previous BR interval (Table S1). The BR locus determined by two different populations corresponded to a 174.8-kb interval (spanning molecular markers brM10 and brM12) in the genome (SL4.0 version, Tomato Genome Consortium 2015) of the fully sequenced tomato Heinz 1706 variety (BR/BR) (Figure 1a). An additional recombination breakpoint was identified at brM11 in three RILs derived from a single population, which further narrowed down the interval (spanning molecular markers brM11 and brM12). However, no recombinant(s) were identified in an independently sequenced inbred line (Figure S2c). Interestingly, two additional FPF1s (Soly01g066950 and Soly01g066970) were present immediately adjacent to the FPF1 (Soly01g066980), and the DNA sequence of each gene of the two phenotypes was identical.

2.2 | Gene-edited deletion mutations in an FPF1 causes a reduced tomato height

The absence of DNA polymorphisms in annotated genes, except in a single FPF1, and the observed deletion of the coding sequence of the telomere-proximal FPF1 (Soly01g066980) in the mutant allele of the BR gene (br) with three intact FPF1 genes in the wild-type allele of BR suggest that the telomere-proximal FPF1 carrying a single exon is the only candidate for the br allele-mediated reduced plant height. Furthermore, expression polymorphism of one or more of FPF1s may possibly contribute to a similar or identical br-mediated phenotypic change.

To validate whether Soly01g066980 is the causative gene for stem length differences between br and wild-type plants, we used the CRISPR-Cas9 system to create mutation(s) in Soly01g066980 (Figure 1b). Expectedly, plants with mutations in Soly01g066980, which no longer produced the wild-type Soly01g066980 protein, were significantly shorter than wild-type plants (Figures 1c–e and S3a), indicating that Soly01g066980 is directly responsible for reduced plant height. Scanning electron microscopy (SEM) images of epidermal cells of the first internode showed approximately 30% increase in the number of cells within a 1-mm² area in the mutant compared to that of the wild-type (Figure 1f). Furthermore, knocking-out the same gene, Soly01g066980, in the processing tomato M82 also shortened its architecture (Figure S4).

The br allele in tomato lines used in this study was derived from the inbred tomato line 823125-1-3 with the br phenotype (Gardner, 2000). We further evaluated the genomic sequences of nine tomato accessions known to show br phenotype and maintained by the Tomato Genetics Resource Center (https://tgrc.ucdavis.edu) to verify if other accessions harbor similar genetic lesions based on the alignment of whole-genome sequencing (WGS) reads. Of the nine accessions, five showed the sequence deletion of Soly01g066980 unique to the br allele, whereas the remaining four accessions did not show such deletion (Figure S5, Table S2). All nine accessions were S. lycopersicum, which is a modern (domesticated) tomato species. In
four accessions without the sequence deletion of Solyc01g066980, other loci and/or expression polymorphism of one or more of FPF1s may confer a similar or identical br phenotype.

2.3 | brachytic reduces yield but does not negatively impact the average fruit and heaviest-fruit weights

Large fruit size is a particularly important trait for fresh-market tomato production, especially in the United States, based on market demand. Fruit weight and size are analogous to yield, because fresh-market tomato fruits can be sold in packages that meet net standard weight and fruit size requirements as per USDA market standards (Guan et al., 2015; Scott et al., 2013; U.S. Department of Agriculture, 2005). Thus, there is a demand for fruits classified as larger than medium in size including extra-large fruits (fruit size >6.985 cm in diameter), where size is defined in accordance with the shipping point and market inspection instructions for tomatoes (U.S. Department of Agriculture, 2005). The br-mediated reduced height phenotype has been used in tomato breeding programs because of the lack of significant negative impacts of the br on the marketable fruit size and yield, which has been observed in such programs. Therefore, combining high yields with this source of a reduced plant height may be a feasible objective for breeding. However, other factors may contribute to the observed phenotypes, especially linkage disequilibrium around br and/or genetic recombination (i.e., background genes) during artificial crossing. Accordingly, the horticultural traits, fruit weights, and yield of CRISPR-Cas9-driven br mutants were tested.

Plant FPF1 typically promotes the flowering time—for example, Arabidopsis (Kania et al., 1997; Melzer et al., 1990), cotton (Gossypium L.) (Wang et al., 2014), rice (Guo et al., 2020), tobacco (Nicotiana tabacum) (Smykal et al., 2004). In two separate greenhouse trials, we did not observe statistically significant differences in the days to first flower and leaves to first flower among the three genotypes (the wild-type and two mutants) (Figures 2a,b and 3b,c). Furthermore, there was no difference in the days to first fruit (Figures 2c and 3d,e). The differences in the fruit Brix and acidity among genotypes were not significant (Figure 2d).

There was wide distribution of fruit weights in the representative large-fruited fresh-market tomato variety at the time of harvest (Figure 3a). We did not find differences in the average weights of all fruits harvested or in the heaviest-fruit weight among the three genotypes (Figure 3b,c). Additionally, there were no differences in the average fruit weight stratified by fruit color among all the genotypes (Figure 3d,e).

The number of fruits tended to be lower in the two mutants than that in the wild-type plant in the two growing seasons in the greenhouse trials (Figure 3f). Both mutants showed lower total fruit yields per plant than that of the wild-type (Figure 3g). With respect to the yield of medium-, large-, or extra-large-sized fruits per plant, genotype effects were not consistently significant in the two growing seasons (Figure 3h). However, in greenhouse trial 1, both mutants showed a significant reduction in the medium-sized or larger fruit yields. A reduction in yield was observed in greenhouse trial 2 for both mutants (32% or 45% reduction compared to that in the wild-type). Similar results were observed in concurrent field evaluations (Figure 3i). Furthermore, a clear negative impact of the br mutation on the extra-large fruit yield was found in the field trials (Figure 3j). Interestingly, there were no differences in the dry weight of aboveground tissues, including stems and leaves (Figure 3k), which resulted in a reduction in the harvest index (HI; total fruit yield/dry weight) in mutants (Figure 3l). The lack of a difference in dry weight between the wild-type and mutants may be attributed to the denser vegetative tissues observed in br plants than that in the wild-type plant.

**Figure 2** The days to first flower or first fruit, and Brix/acidity levels of the brachytic mutant tomato. Days to first flower is defined as the number of days from sowing to the first full bloom (i.e., when petals create a 180° angle) (a), leaves to first flower the number of leaves produced before initiation of the primary inflorescence (b), days to first fruit is defined as days from sowing to the first fruit with 1 cm in diameter (c), and fruit Brix (%) and acid (%) (d). WT, wild-type. br.8.1C14 and br.8.2C14, transgene-free, homozygous M2 generation mutant lines. The n value represents the total number of plants for each genotype evaluated during each trial; ns indicates no significant difference (ANOVA at P > 0.05) was found between any genotypes, except for trial 2 in c; the Welch’s test (P > 0.05) was used for the comparison in trial 2 in c. Error bars indicate 95% confidence intervals. GH, greenhouse (a through d).
2.4 | Association between high extra-large-sized fruit yield of \textit{brachytic}-mediated shortened tomato and other genes

Marketable fruit yield losses due to the \textit{br} allele have not yet been reported. In contrast, we observed reductions in the extra-large and medium-sized or larger fruit yields of CRISPR-Cas9-driven \textit{br} mutants. This raises the intriguing possibility that background genes may have compensated for the negative impact of \textit{br} on marketable fruit yield by linkage disequilibrium and/or genetic recombination. We further evaluated variation in tomatoes with \textit{BR/BR} or \textit{br/br} alleles. We used two sister lines developed from Fla. 8653 (\textit{BR/BR}) × Fla. 8916 (\textit{br/b}), with a completely known pedigree to transfer the \textit{br} allele to a US-adapted large-fruited fresh-market cultivar (UF/IFAS, 2022) for yield evaluations. Unlike the CRISPR-Cas9-driven single gene mutant, a sister line can carry several to many different genes (traits) regardless of whether those can be easily observed by researchers when it compared to its mate (i.e., \textit{BR} sister line vs. \textit{br} sister line in Figure 4) other than the trait of interest. We focused on the extra-large and medium-sized or larger fruit yields, which are routinely evaluated by tomato

\begin{figure}
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\caption{\textit{brachytic} reduced fruit yields of the large-fruited fresh-market tomato, with no adverse effects on fruit weights. (a) Distribution of fruit weights in wild-type (WT) and mutant plants, \textit{br}.8.1\textsuperscript{CR} and \textit{br}.8.2\textsuperscript{CR}. The entire fruit harvested from each of the three different genotypes tested in a greenhouse trial are displayed. Medium-sized fruit was defined as described by U.S. Department of Agriculture, 2005. Number of fruits was calculated for a 1 g fruit weight window. (b) Average fruit weight (g) per plant. (c) Average heaviest fruit weight (g). An example of an extra-large fruit [as per the USDA standard size designations for tomatoes (U.S. Department of Agriculture, 2005)] harvested from \textit{br}.8.2\textsuperscript{CR} (right). White bar represents 2 cm. (d) Average green fruit weight (g) and (e) average red [breakers or later fruit color classification as per the equipment catalog for fresh and processed product inspections (U.S. Department of Agriculture, 2017)] fruit weight (g). (f) Total number of fruits per plant; (g) total fruit yield (kg) per plant; (h) yield of medium-, large-, or extra-large-sized fruits per plant evaluated in the greenhouse and field, respectively. (i) yield of extra-large fruit evaluated in the field. (j) Dry weight of above ground tissues per plant; (l) harvest index. Mutants are transgene-free, homozygous \textit{M}_{2} generation (a through l). The \textit{n} value represents the total number of plants for each genotype evaluated during each trial (a through l). Statistical significance (* \textit{P} < 0.05, ** \textit{P} < 0.01) was determined by one-way ANOVA in conjunction with a two-tailed Tukey’s HSD multiple comparison test (a through l). \textit{ns} indicates no statistical difference (ANOVA at \textit{P} > 0.05) among any of the genotypes (b through l). Error bars indicate 95% confidence intervals (b through l). Negative numbers (g, h, i, j, and l) indicate % decreases in the mutant compared to WT. GH and field trials indicate the evaluation of traits in the greenhouse and field, respectively (a through l).}
\end{figure}
breeders during germplasm/cultivar development (Hutton et al., 2015, 2017; Scott et al., 2008, 2009). Interestingly, during the yield evaluation, no negative impact of the br was detected compared to the line with the BR and control Fresh-market tomato ‘Sanibel’ (BR/BR) (Figure 4). The yield of extra-large fruit in the br sister line was significantly (P < 0.05) higher than that of the BR sister line in the two growing seasons (Figure 4b). Earlier observations by breeders are generally consistent with these results. This variation could explain the current widespread use of the br-derived phenotypes. While the br reduces the yield of marketable fruit (i.e., large size fruit) in large-fruited fresh-market tomatoes, our data clearly revealed that artificial selection contributes to commercially acceptable yield potential in br tomatoes.

### 2.5 | Five FPF1 homologs in tomato show tissue-specific expression

To identify the FPF gene family in Solanaceae, we performed a hidden Markov model (HMM) search using the PFAM FPF model against the 11 Solanaceae annotated protein datasets, which include three tomato species, one modern (domesticated) (S. lycopersicum) and two wild tomato species (Solanum pimpinellifolium and Solanum pennellii). We identified 57 protein sequences (including five modern tomato sequences) matching the model (Table S3). For each species, multiple sequences were identified in the datasets used in this study (ranging from three FPFs in Capsicum annum cv. CM334 to eight in Nicotiana N. benthamiana). A maximum likelihood phylogenetic analysis revealed that five modern tomato sequences can be clustered into two categories (Figures 5a and 5d). One contained all three FPFs on chromosome 1, and the other category clustered all three tomato species, including a single modern tomato gene, Solyc06g005530, close to a single terminal branch. Interestingly, both the wild tomatoes had five FPF1s, which is the same number as that found in the modern tomato. However, the modern tomato and its closest relative S. pimpinellifolium carried three FPF1s on chromosome 1, while S. pennellii carried four FPF1s on chromosome 1, implying molecular divergence in the FPF1 family in Solanum.

To obtain an overview of the expression profiles of the five tomato FPF1s, RNA-seq libraries were constructed from different tissue types, the first internode (stem), leaf, and root at the 6-week-old growth stage (the growth stage used in conventional brachytic pheno- typing; Lee et al., 2018) and small green fruit tissue in both phenotypes (wild-type and mutant [br.8.2<sup>cr</sup>]) for sequencing. Additionally, first internodes collected 3 h after the GA3 treatment at the 6-week-old stage were used for library construction. Comparing the expression profiles among homologs, both BR gene (Soly01g066980) and its immediately adjacent gene Soly01g066970 were expressed (Figure 5b). Notably, both genes were highly expressed in roots and their expression levels were not significantly affected by the GA3 treatment. The other three homologs had low expression levels in most or all tissue types.

### 2.6 | Identification of modules of coexpressed genes across tissues, genotypes, and the GA3 treatment by network analysis

A comprehensive catalog of expressed genes was identified from the same RNA-seq libraries outlined above. In fruits, 40 differentially expressed (DE) genes (up- or down-regulated combined; <0.10% of genes in the genome annotation ITAG4.0) at a false discovery rate (FDR) of <0.05 were found in a comparison between genotypes (Figure 5c, Table S4). The number of significant DE genes between GA3-treated and untreated plants was high in the stem tissues for both genotypes.

**Figure 4** Field evaluation of extra-large and medium-, large-, or extra-large-sized fruit yields of a pair of sister lines with or without the brachytic (br) allele during two growing seasons. (a) Yield of medium-, large-, or extra-large-sized fruits per plant; (b) yield of extra-large fruit per plant. A pair of sister lines (BR/BR or br/br) derived from conventional crossing/selection methods using parental lines homozygous for either BR or br alleles at the BR locus. Sanibel (BR/BR) is a commercial large-fruited fresh-market tomato cultivar currently in the U.S. market (Seminis, MO). The n value represents the total number of plants for each genotype evaluated during each trial; ns indicates no statistical difference (ANOVA at P > 0.05) among genotypes; * P < 0.05 determined by one-way ANOVA in conjunction with a two-tailed Tukey’s HSD multiple comparison test. Error bars indicate 95% confidence intervals.
To characterize the biological pathways related to expressed genes, we performed a weighted gene coexpression network analysis (WGCNA) and identified several genotype-specific (Figure 5d, Table S5) or the GA treatment-specific coexpression modules (Figure 5d). In an analysis of tissue-specific co-expression modules, module GTM46 included highly up-regulated genes in the mutant that was shared across the tissue types, while a comparison of fruit tissues showed less positive correlation. An analysis of tissue-adjusted data (i.e., identification of modules driven by the GA treatment effect in stem) revealed 30 modules (right panel in Figure 5d). In particular, modules GAM12 and GAM35 included highly up- and down-regulated genes, respectively, in the GA-treated stem for both genotypes, reflecting the effect of the GA treatment, respectively. Furthermore, those two modules were enriched for distinct Gene Ontology (GO) terms (bottom panel in Figure 5e, Table S6); module GAM12 was enriched for RNA modification and module GAM35 was enriched for integral component of the membrane. Interestingly, the module GAM14 included up-regulated genes in the GA-treated mutant compared to the GA-treated wild-type, while comparisons between with and without the GA treatment for any genotype showed a converse correlation. We found that 319 genes were assigned to the module GAM14, but enrichment of significantly DE genes in the module GAM14 did not pass a specified threshold ($P_{\text{Bonferroni}} = 0.05$) in this study. Highly down-regulated genes in a particular GA-treated...
Reduced plant height in plants harboring mutated brachytic homologs. (a) Diagram of two flowering promoting factor 1 (FPF1) genes (Solyc01g066950 and Solyc01g066970), the centromere-proximal homologs of brachytic. sgRNA3 in blue indicates a single-guide RNA used in the CRISPR-Cas9 system, which targeted a sequence region with only a single nucleotide difference between the two homologous FPF1s (i.e., “T” at 68,005,223 bp on Solyc01g066950 and “C” at 68,057,560 bp on Solyc01g066970). Black horizontal lines and block icons indicate untranslated regions and the coding sequences, respectively. The first nucleotide position of each start codon is given. Nucleotide sequences of WT, wild-type. The br.7CR, br.57.1CR, and br.57.2CR are shown with the corresponding amino acid sequences, and deletions and insertions are indicated by blue dashes and underlines, respectively. The sequence gap length between two genes is shown in parentheses. (b) The stem lengths of 6-week-old plants. Mutants are transgene-free, homozygous M2 generation. The n value represents the total number of plants for each genotype evaluated. **P < 0.01 based on one-way ANOVA in conjunction with a two-tailed Tukey’s HSD multiple comparison test. Error bars indicate 95% confidence intervals.
stage, we cannot rule out the possibility that changes in FPF expression are associated with developmental states. However, members of the tomato FPF1 family may play a role in the suppression of the GA biosynthesis in roots, which controls diverse aspects of growth and development. Furthermore, a gibberellin oxidase (Solyc01g093980) was up-regulated (FDR < 0.05) in the stem of the mutant compared to that of the wild-type stem (module \text{GTM14}), indicating that this gene could be responsible for the shortened stem length.

2.7 Mutated \text{br} homologs present new sources of a reduced plant height

Considering the observed sequence variation and expression patterns of the FPFs adjacent to the \text{br} on chromosome 1, we investigated phenotypes associated with mutated versions of the two \text{br} homologs, Solyc01g066950 and Solyc01g066970. Using a single-guide RNA targeting a sequence region only differentiated by a single nucleotide, three different mutants were obtained simultaneously (Figure 6a): \text{br.7CR}, with a 1-bp insertion in Solyc01g066970; \text{br.57.1CR}, with a 5-bp deletion in both Solyc01g066950 and Solyc01g066970; \text{br.57.2CR}, with a 1-bp insertion in Solyc01g066950 and a 5-bp deletion in Solyc01g066970. None of these mutants had DNA sequence variation in \text{BR} (Solyc01g066980). Expectedly, all three mutants showed significantly reduced height (Figure 6b). As the number of knocked out genes increased, the stem length reduced accordingly. Further experimental validation is needed to determine whether a reduced height can be obtained solely by mutation(s) in the centromere-proximal FPF (Solyc01g066950), which showed a relatively low expression level detected mainly in roots. However, our collective findings indicate that multiple \text{br} homologs confer a \text{br} plant-like shortened stem length. Targeting homologous sequence for simultaneous gene editing via one sgRNA using CRISPR-Cas9 system required considerably less time to generate double mutants in this study. However, this method does not necessarily allow the identification of sole gene contribution unlike single gene mutant.

High levels of genetic variation (e.g., copy number variation of DNA segments [CNV]) have been observed in plant genomes (Alonge et al., 2020; Cao et al., 2011; Hanikenne et al., 2013; Iovene et al., 2013; McHale et al., 2012; Swanson-Wagner et al., 2010; Zheng et al., 2011), and emerging evidence indicates that CNVs mediate a number of valuable crop traits—for example, CNV (1 to 11 copies)-mediated soybean cyst nematode (SCN) resistance (Cook et al., 2012; Cook et al., 2014; Lee et al., 2015; Lee et al., 2016), increased SCN resistance in high-copy-number (11 copies) individuals from a population of a single cultivar (Lee et al., 2016). Together with our results, this suggests further approaches to diversify the genetic resources of this large-fruited fresh-market tomato, such as the creation of tomato lines that carry diverse mutations in the FPF1 genes (e.g., knock-outs of all the three FPFs on chromosome 1 and/or homologs on other chromosomes). Expression polymorphism of these genes by either gene editing-driven knock-out(s) or recombination event(s) may result in considerably reduced plant architectures than those obtained by single or double mutants and should be further optimized for the ground cultivation system (e.g., compact growth habit tomatoes) and/or for other breeding goals (e.g., developing tomatoes for indoor agriculture). The impact(s) of such complex mutations on the fruit marketability should be evaluated.

Our work indicates that \text{br}-mediated reduced tomato height is attributable to a DNA polymorphism in the FPF1. Knock-out(s) of \text{br} homologs can confer a \text{br} plant-like short architecture. The \text{br} reduced the HI, while retaining the production of heavy fruits. Shared and divergent gene expression patterns associated with the genetic differences between genotypes (wild-type and \text{br} plants), and between \text{GA3}-treated and untreated tissues were observed in both genotypes. Given that the two FPF1s were highly expressed in roots and not in the stem, developmental and cell-to-cell signaling events from the roots to the stem are likely to drive phenotypic change(s) in \text{br} plants. The FPF1 homologs may have a wide range of functions. Genetic engineering of plant architecture is an effective approach for increasing marketable crop yield, and \text{br} plays a key role in reducing the plant height in tomato breeding programs (Frasca et al., 2014; NCSU, 2022; Scott et al., 2010; Tigchelaar, 1986; UF/IFAS, 2022). Clearly, future investigations of the genetic association between the \text{br}-mediated reduced plant height and the marketable fruit yield, especially the extra-large-sized fruit yield, are necessary to successfully develop shortened fresh-market tomato varieties.

3 EXPERIMENTAL PROCEDURES

3.1 Fine mapping of the BRACHYTIC (BR) locus

The \text{BR} locus was fine mapped following the same basic procedures used for the initial mapping of this locus described in our previous study (Lee et al., 2018). Further details are available in Supporting Information S1.

3.2 Genome assembly from brachytic haplotype

WGS of the tomato line 3040717, harboring the homozygous \text{br} allele, was conducted using Illumina technology. Detailed steps for genome assembly are available in Supporting Information S2. WGS of tomato accessions obtained from the Tomato Genetics Resource Center and Fla. lines was conducted using an Illumina HiSeq instrument as described in our previous study (Bhandari & Lee, 2021). The sequencing libraries were sequenced on average of 23 Gb for each tomato.

3.3 CRISPR-Cas9 gene editing

Guide RNAs (gRNAs) targeting the FPF1 genes were designed using CRISPR-P (Lei et al., 2014) and CRISPR-PLANT (Xie et al., 2014), and each of the gRNA was cloned into a binary vector following the same
basic procedures described by Xie and Yang (2013) (Table S7). Duplex oligos carrying BsaI sites in binary vectors were synthesized (IDT, www.idtdna.com). The binary vector pHSN401 (www.addgene.org)-gRNA plasmid was introduced into Agrobacterium tumefaciens (A. tumefaciens) strain LBA4404 (Takara, www.takarabio.com) according to the manufacturer’s instructions. A. tumefaciens-mediated transformations of Fla. 8059 (a parental line of “Tasti-Lee F1” [Bejo, Seeds, Oceano, CA], which does not carry br; Scott et al., 2008; Tasti-Lee F1 is a fresh-market tomato cultivar currently in the US market [e.g., Publix Super Markets, Inc., www.publix.com]) were performed as described by Van Eck et al. (2019), with the following modifications in the preculture medium and selective regeneration medium steps: cotyledon explants from 7- to 9-day-old seedlings were precultured with 3 mg L\(^{-1}\) or 6 mg L\(^{-1}\) hygromycin was used.

Potential Cas9-gRNA-introduced mutations were examined by Sanger sequencing of PCR products and the T7 Endonuclease I assay (NEB, www.neb.com) (Figure S8a; Table S7). Details of the experimental procedures are available in Supporting Information S3. To identify homozygous transgene-free mutants, four primer pairs targeting the Cas9 gene in the binary vector or the Hyg gene were used (Figure S8b and S8c). Potential transgene-free mutants were further validated by WGS (Figures S8d–g). Potential off-target sites (i.e., up to four mismatches compared to each target region) were predicted using the Cas-OFFinder (Bae et al., 2014). A lack of off-target activity was verified (Figure S9, Table S8).

CRISPR brachytic mutants in the M82 background were developed by the Zach Lippman laboratory. Detailed steps are available in Supporting Information S4.

### 3.4 | Greenhouse trial

Greenhouse trials were conducted during two growing seasons at the University of Florida’s Gulf Coast Research and Education Center (UF GCREC, Wimauma, FL). Young plants were grown as previously described (“Phenotype analysis” in Lee et al., 2018). Each plant was transplanted into a 5-gallon soil bags 6 weeks after sowing. Soil bags were randomly placed at a distance 70 cm between the bags. Plants were managed as described by Hochmuth (2018). A total of 10 g of Nutri-Leaf® 20-20-20 (Miller Chemical, www.millerchemical.com) was applied weekly to each 5-gallon soil bag. Day and night temperatures were set to 26.6°C and 18.3°C, respectively. No artificial pollination or pruning was performed during the trials. No rotten fruits were observed at the time of harvest during the greenhouse trials. During the spring of 2020, seed sowing in the greenhouse (S) and fruit harvest (H) were performed on January 31 and July 9, respectively. For the second growing cycle, S and H were performed on September 3, 2020, and January 10, 2021.

The stem lengths of 6-week-old plants were measured, as described in our previous study (Lee et al., 2018). Days to first flower was defined as the number of days from sowing to the first full bloom (i.e., when petals create a 180° angle; Lee & Hutton, 2021). Leaves to first flower was defined as the number of leaves produced before initiation of the primary inflorescence. Days to first fruit was defined as the days from sowing to the first fruit (of 1 cm in diameter). Days to first flower and fruit color data were collected by the same individual throughout the seasons in this study. Ripe fruits were harvested and the tomato fruit Brix and acidity were measured immediately after harvest using digital refractometers PAL-1 (ATAGO; www.atago.net). The Brix or acidity values were measured for each individual fruit sample according to the manufacturer’s instructions, and the average value from replicates for each genotype was calculated.

All fruits (>0.5 cm in diameter) that developed in each seasonal trial were harvested on a single harvesting date, regardless of fruit weight, quality (e.g., irrespective of whether the fruits had defects such as cracks), or color. Fruit color (green vs. breakers; U.S. Department of Agriculture, 2017) was visually examined on the day of harvest (Figure S10). Fruits were sorted by size into four classes (i.e., any fruit smaller than medium size, medium, large, and extra-large) using the USDA Tomato Sizer (U.S. Department of Agriculture, 2017), and descriptions of medium-, large-, and extra-large-sizes were in accordance with the shipping point and market inspection instructions for tomatoes (U.S. Department of Agriculture, 2005). For each genotype and plot, the fruit weight and number were calculated from the average values for the weight and number of fruits from the plants in each trial, respectively.

### 3.5 | Field trial

Field trials of the CRISPR-Cas9-driven mutants were conducted during two growing seasons at the UF GCREC, where conditions are representative of typical field-grown fresh-market tomato production environments in the Southeastern United States. Plants were grown as described previously (“Phenotype analysis” section of Lee et al., 2018). A recommended fertilizer, pesticide, and irrigation program (Freeman et al., 2015) was followed throughout the growing season until harvest. In each season, the experiment was conducted using a randomized complete block design, as described in our previous study (Lee & Hutton, 2021). Six blocks were included in each season. Damaged or diseased plants were marked throughout the season, and data were not collected from such plants. During the spring of 2020, S and H were performed on January 22 and May 23, respectively, and For the second growing cycle during the fall of 2020, S and H were performed on August 3 and December 8, respectively.

Field trials of sister lines were conducted during two growing seasons at the UF GCREC. A pair of sister lines from a cross between Fla. 8653 (BR/BR × Fla. 8916 (br/br) and directionally selected (one with br and the other without) for the br allele were used. Both parental lines are large-fruited, determinate inbred lines. Three blocks were included in each season. Parental lines and the tomato cultivar “Sanibel” (BR/BR) (Seminis, www.seminis.com) were included as controls. F₆ and F₆:7 sister lines, with or without br, were used for yield evaluation in 2017 and 2018, respectively. The date of harvest was
determined as the day when at least 70% of the fruit in a plot had attained a mature color. During the fall of 2017, S and H were performed on July 31 and December 30, respectively. For the second growing cycle during the spring of 2018, S and H were performed on February 2 and May 26, respectively. Fruit collection and yield evaluations were performed, as described in our previous study (Lee & Hutton, 2021).

### 3.6 Statistical analysis

Two independent experiments (i.e., trial 1 and trial 2) were performed to collect phenotypic data. For each experiment, the statistical significance level \( P < 0.05 \) for comparisons between any two genotype mean values was determined by one-way analysis of variance (ANOVA) in conjunction with a two-tailed Tukey’s HSD multiple comparison test, or the Welch’s test. Error bars indicate the 95% confidence intervals.

### 3.7 Gibberellic acid treatment

Plants used for hormone treatments were grown simultaneously with those used in the greenhouse trial in the fall of 2020. At 14 days after sowing, plants were sprayed generously for 7 days with \( 10^{-4} \) M Gibberellic A3 (GA3; PhytoTech Labs, [https://phytotechlab.com](https://phytotechlab.com)) or \( 10^{-4} \) M GA4+7 (PhytoTech Labs) containing 0.02% Tween 20 (Fisher Scientific). Control plants were treated with a solution containing 0.02% Tween 20. The stem lengths of 6-week-old plants were measured, as described by Lee et al. (2018).

### 3.8 Identification of BR orthologs

Putative orthologs of Solyc01g066980 were identified as described in our previous study (‘Identification of C. sativa kelch-motif orthologs using hidden Markov models’ section in Zhang et al. [2020]). Detailed steps are available in Supporting Information S5.

### 3.9 RNAseq and expression analysis

In both genotypes, wild-type and mutant (M2 generation of br.8.2CR), tissue samples were collected from individual plants grown simultaneously with those used in the greenhouse trial in the fall of 2020. Five different tissue types were collected: stem without the GA3 treatment (specifically the 1st internode) at the 6-week-old stage, stem (specifically the 1st internode) collected 3 h after the GA3 treatment at the 6-week-old stage, leaf at the 6-week-old stage, root at the 6-week-old stage, and fruit at the time of harvest. The leaf, stem with or without the GA3 treatment, and root samples were collected from 6-week-old plants. Green fruits \( (5.1 \pm 1.0 \text{ g and } 5.2 \pm 1.1 \text{ g [mean \pm standard deviation] for the wild-type and mutant, respectively}) \) were collected at the time of harvest in the greenhouse trial. For each biological replication, the stem, leaf, and root were collected from the same individual plant, and four biological replications (four different plants) were collected for each genotype and tissue type. The samples were flash-frozen in liquid nitrogen immediately after excision.

Total RNA was extracted from a single plant per tissue using a Qiagen RNeasy Plant Mini Kit (Qiagen, [www.qiagen.com](http://www.qiagen.com)). The yield of mRNA was quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, [www.thermofisher.com](http://www.thermofisher.com)), and the quality was verified using an Agilent 2100 Bioanalyzer (Agilent, [www.agilent.com](http://www.agilent.com)). The libraries (250–300 bp insert cDNA library) were constructed and sequenced using Illumina HiSeq technology (PE 150) by the Novogene ([https://novogene.com](https://novogene.com)).

A total of 981.0 million Illumina reads were generated. After removing reads with adaptor contamination, uncertain nucleotides constitute \( >10\% \) of either read, or low quality nucleotides (base quality \( <5 \) and \( >50\% \) of reads), alignments of the raw reads to the tomato reference genome assembly SL4.0 (Fernandez-Pozo et al., 2015) were performed using STAR (Dobin et al., 2013). HTSeq v0.6.1 (Anders & Huber, 2010) was used to count reads mapped for each annotation (ITAG4.0; [https://solgenomics.net](https://solgenomics.net)). Then the expected number of Fragments Per Kilobase of transcript sequence per million base pairs sequenced (FPKM; Trapnell et al., 2010) for each gene was calculated. Furthermore, genes with counts per million aligned reads of at least 0.5 in at least four biological replications were retained, resulting in 23,869 genes for further analysis. A DE gene analysis was performed using DESeq2 (1.28.1; Love et al., 2014). DE was evaluated for the following groups of contrasts: (1) genotype (wild-type and mutant genotypes for the same tissue and treatment) and (2) treatment (GA3-treated and untreated for stem tissue). DE was evaluated based on the \( \log_2 \) fold change, Z-statistic \( (\log_2 \text{ fold change divided by its standard error}) \), \( p \) value, and FDR estimate. FDR was calculated independently for each test.

### 3.10 Gene coexpression network analysis

A gene coexpression network analysis was performed using the WGCNA R software package, as described by Langfelder and Horvath (2008). Two separate datasets were created: (1) one without any data adjustment and (2) one adjusted for tissue types (i.e., identification of modules driven by the GA3 treatment in stem). Enrichment of significantly DE genes in each module was identified at a threshold of Bonferroni correction \( <0.05 \) for 3778 GO terms for tomato (EnsemblPlants Biomart release 49; Kinsella et al., 2011; Howe et al., 2020).

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CONFLICT OF INTEREST
The Authors did not report any conflict of interest.

AUTHOR CONTRIBUTIONS
M.B.L. created CRISPR brachytic mutants in the Fla. 8059 background and performed some greenhouse evaluation. R.S. conducted the mapping. S.F.H. supplied seed, interpreted mapping data, and provided knowledge of fresh-market tomato breeding. T.G.L. designed the study, obtained funding, performed experiments, supervised and conducted data analysis, prepared figures, and wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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