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Extreme sugar accumulation in late fig ripening is accompanied by global changes in sugar metabolism and transporter gene expression

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Abstract

Female fig (*Ficus carica* L.) fruit are characterized by a major increase in volume and sugar content during the final week of development. A detailed developmental analysis of water and dry matter accumulation during these final days indicated a temporal separation between the increase in volume due to increasing water content and a subsequent sharp increase in sugar content during a few days. The results present fig as an extreme example of sugar import and accumulation, with calculated import rates that are one order of magnitude higher than those of other sugar-accumulating sweet fruit species. To shed light on the metabolic changes occurring during this period, we followed the expression pattern of 80 genes encoding sugar metabolism enzymes and sugar transporter proteins identified in fig fruit. A parallel comparison with male fig fruits, which do not accumulate sugar during ripening, highlighted the genes specifically related to sugar accumulation. Tissue-specific analysis indicated that the expression of genes involved in sugar metabolism and transport undergoes a global transition.

1 | INTRODUCTION

The fig (*Ficus carica* L., family Moraceae) tree bears fruit with an enclosed inflorescence structure, termed syconium. There are two major sex types in fig: the female fig, which is the edible type, and the caprifig (hermaphroditic, having male and female flowers) known as the male fig, which is generally nonedible. The female type is further classified into three groups: common, Smyrna and San Pedro, depending on pollination behavior and cropping characteristics (Flaishman et al., 2008). These three female-type figs have long-styled female flowers within the syconium, which can be pollinated but prevent oviposition of wasps.

The development of female fig fruit follows a typical double-sigmoid growth curve based on fruit diameter, including two rapid growth phases (I and III) separated by the slower growth phase II (Flaishman et al., 2008). Notably, the ripening process (phase III) of summer crop fig is extremely rapid, occurring within a week, and in as little as 3 days in the peak summer season. Accordingly, ripening-related parameters of size, flavor and texture undergo major changes within a short time. Compared with the seemingly uneventful phase II, fruit size may increase as much as two- to three-fold and softness increases drastically during phase III (Rosianski et al., 2016a). With respect to fruit sweetness, more than 70% of the total dry weight and 90% of the total sugar

content accumulate in the fruit during ripening (Flaishman et al., 2008).

Sweetness is perhaps the main indicator of fruit quality in fig and is determined by the soluble sugar concentration. Ripe fig fruit are very rich in sugars, and sugar accumulation is a developmental process. Sugar levels remain low during phase I and II of development, and concentrations increase considerably during the final stages of ripening, until harvest (Flaishman et al., 2008). Fig is a monosaccharide-accumulating fruit, the major soluble sugars being glucose (Glc) and fructose (Fru), the products of sucrose (Suc) hydrolysis; Suc is present as well, but at low concentrations (Trad et al., 2012; Vemmos et al., 2013).

In most non-Rosaceae plants, Suc is the major photoassimilate translocated from the source to heterotrophic sinks over long distances in the phloem (Ruan, 2014). Since Suc is the predominant sugar in fig leaves (Vemmos et al., 2013), sugar metabolism in fig fruit is likely Suc metabolism, which has been characterized in detail in Suc-metabolizing plant tissues, including fruit (Ruan, 2014; Wan et al., 2018). Once Suc is transported to sink cells, it may be stored as such without further metabolism, or metabolized via hydrolysis into Glc and Fru by invertases (EC 3.2.1.26), or conversion to UDP-Glc and Fru in the cytosol by Suc synthases (SUS; EC 2.4.1.13), which can also catalyze the reverse reaction of Suc synthesis. Invertases can be grouped into three types based on their optimum pH and subcellular locations: insoluble cell wall acid invertase (CWIN) located in the apoplasmic space, soluble vacuolar-localized acid invertase (VIN), and cytoplasmic neutral invertase (CIN). After a series of conversions by several enzymes, Suc may be resynthesized in the cytosol by SUC PHOSPHATE SYNTHASE (SPS; EC 2.4.1.14) and SUC PHOSPHATE PHOSPHATASE (SPP; EC 3.1.3.24). SPS is a key enzyme of Suc synthesis. For some Suc-accumulating fruit, upregulation of SPS is consistent with enhanced Suc accumulation at the late stage of fruit development, while VIN is downregulated, allowing for the Suc accumulation (Burger & Schaffer, 2007; Hubbard et al., 1990, 1991; Li et al., 2012). However, for the Suc-transporting, monosaccharide-accumulating fruit, maintaining fruit invertase activity is crucial for Glc and Fru accumulation during ripening (Boss & Davies, 2001; Miron et al., 2002). For example, in hexose-accumulating tomato, both VIN and CWIN are involved in Glc and Fru accumulation in the developing fruit (Fridman et al., 2004; Miron et al., 2002). CWIN and VIN activities may be further regulated by their respective inhibitory proteins (Ruan, 2014).

Nevertheless, studies of sugar metabolism at the global level, made possible by comprehensive transcriptomic analysis, generally indicate that accumulation is not the result of individual enzymological changes but is rather accompanied by more global changes in the complex pathways involved in sugar metabolism (Dai et al., 2011; Li et al., 2012; Zhang et al., 2016), as well as in the accompanying components of sugar accumulation, that is, sugar transport mechanisms (Afoufa-Bastien et al., 2010; Li et al., 2015; Reuscher et al., 2014; Shammai et al., 2018; Wei et al., 2014).

Long-distance transport of sugars from source to sink and the inter- and intracellular allocation of sugars involve sugar transporters. There are three families of transporters—Suc transporters (SUT/SUC), monosaccharide transporters (MST) and SWEET—mainly participating in the distribution of sugars within most plant cells (Chen et al., 2015). SUC family members have been characterized as Suc/H^+ symporters (Kuehn & Grof, 2010). For example, in grape berries, *VvSUC11* and *VvSUC12* mediate the loading of Suc from the apoplast into the parenchyma cells (Lecourieux et al., 2014). The MST are further distributed among seven subfamilies: sugar transporter family (STP, also called MST in rice or hexose transporter [HT] in grape), sugar facilitator family (SFP, also called early response to dehydration 6-like [ERD6-like]), tonoplast sugar transporter family (TST, previously named tonoplast monosaccharide transporter [TMT]) (Jung et al., 2015), plastidic glucose translocator family (pGlcT), vacuolar glucose transporter family (VGT), polyol/monosaccharide transporter family (PMT) and inositol transporter family (INT). Members of these families have been reported in a large variety of plant species, such as tomato, grape and rice (Afoufa-Bastien et al., 2010; Johnson & Thomas, 2007; Reuscher et al., 2014). In grape berries, a high accumulation of Glc and Fru in the vacuole is linked to the expression of two HT genes, *TMT* and *VGT* (Afoufa-Bastien et al., 2010). The coordination and cooperation between *MdTMT1/2* and *MdEDR6* correspond to the massive accumulation of Fru in apple fruit (Wei et al., 2014). The SWEET family members function as energy-independent uniporters that facilitate sugar influx and/or efflux (Chen et al., 2010). Seventeen SWEET have been identified in *Arabidopsis thaliana* and classified into four clades with different sugar transport preferences (Feng & Frommer, 2015). AtSWEET are localized to different cellular compartments, including the plasma membrane (e.g., AtSWEET1, 8, 9, 11, 12, and 15), the tonoplast (AtSWEET16 and 17), and the Golgi (AtSWEET9 and 15) (Feng & Frommer, 2015). Genetic variability in SWEET has been recently shown to account for modified sugar accumulation patterns in tomato (Shammai et al., 2018).

In the present study, we characterize the novel and sharp accumulation of sugars in ripening fig fruit and describe the global changes in sugar accumulation-related gene expression during the ripening phase of fig fruit. Sugar concentration and composition were analyzed in receptacle and inflorescence tissues of female and male fig during the final stages of ripening. Based on our previous sequencing results (Freiman et al., 2014; Rosianski et al., 2016a), we identified sugar metabolism and transporter genes and further analyzed their expression patterns in two different tissues of female and male fig. Comparisons between the different tissue types were performed to help understand the underlying mechanism of sugar accumulation and transport in fig fruit. In addition, candidate genes are proposed that might play crucial roles in fig sugar metabolism and transport.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Two common-type female fig (*Ficus carica* L.) cultivars, Brown Turkey and Figaro15, from an orchard located in Bet Dagan, Israel (32°00′07.1″ N, 34°49′45.8″ E) were employed in this study. All fruits used in our study were nonpollinated, by covering the fruit with transparent net bags (100 mesh, 15 × 10 cm) to prevent natural pollination by fig wasp. For the initial survey of sugar accumulation, we collected "Brown Turkey" fruits at weekly intervals, beginning at the mid-phase II

developmental period (around 4 weeks before ripening), that is, the stationary stage of fruit growth (between May and June), and continuing until they were ripe.

For the experiment detailing the sugar-accumulation phase, summer ripening "Brown Turkey" and "Figaro15" fruits were collected in July 2016 (day temperatures of 29–33°C and night temperatures of 19–25°C). Based on the percentage of color coverage on the outer fruit skin (Figure 1A), in combination with fruit size and fresh weight, ripening phase III fruits were classified into four distinct ripening stages: R1—beginning stage of ripening with 30% color coverage; R2—mid-ripening stage with 60% color coverage; R3—fully ripened fruit with 100% color coverage; R4—2 days after full ripening.

In addition, we selected one representative nonedible and unsweetened male fig cultivar, Figaro725, which was generally planted to provide pollen and was set as the "negative control" for comparison with the edible and sweet female figs. R1 to R4 stage samples (Figure 1A) were taken during the ripening process in May 2016 (day temperatures of 23–30°C and night temperatures of 16–22°C).

Three biological replicates consisting of three fruits each were collected for each variety on each date and the fruits were carefully dissected to separate female flowers and pulp. Samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

2.2 | Soluble sugar analysis by HPLC

Soluble sugars were extracted three times from 0.5–1.0 g fresh samples with 10 ml 80% ethanol in a water bath for 45 min at 70°C. The combined ethanolic solution was evaporated and redissolved in 2 ml HPLC-grade distilled deionized (DDI) water, then centrifuged at 20,800*g* for 10–15 min. The supernatant was filtered using a 0.22 μm nylon syringe filter. The content of soluble sugars was determined by HPLC as described previously (Petreikov et al., 2006) using an Alltech 700CH carbohydrate column (300 × 6.5 mm, catalog number 70057) installed in a Shimadzu HPLC system with refractive index detector. The mobile phase was HPLC-grade DDI water at a flow rate of

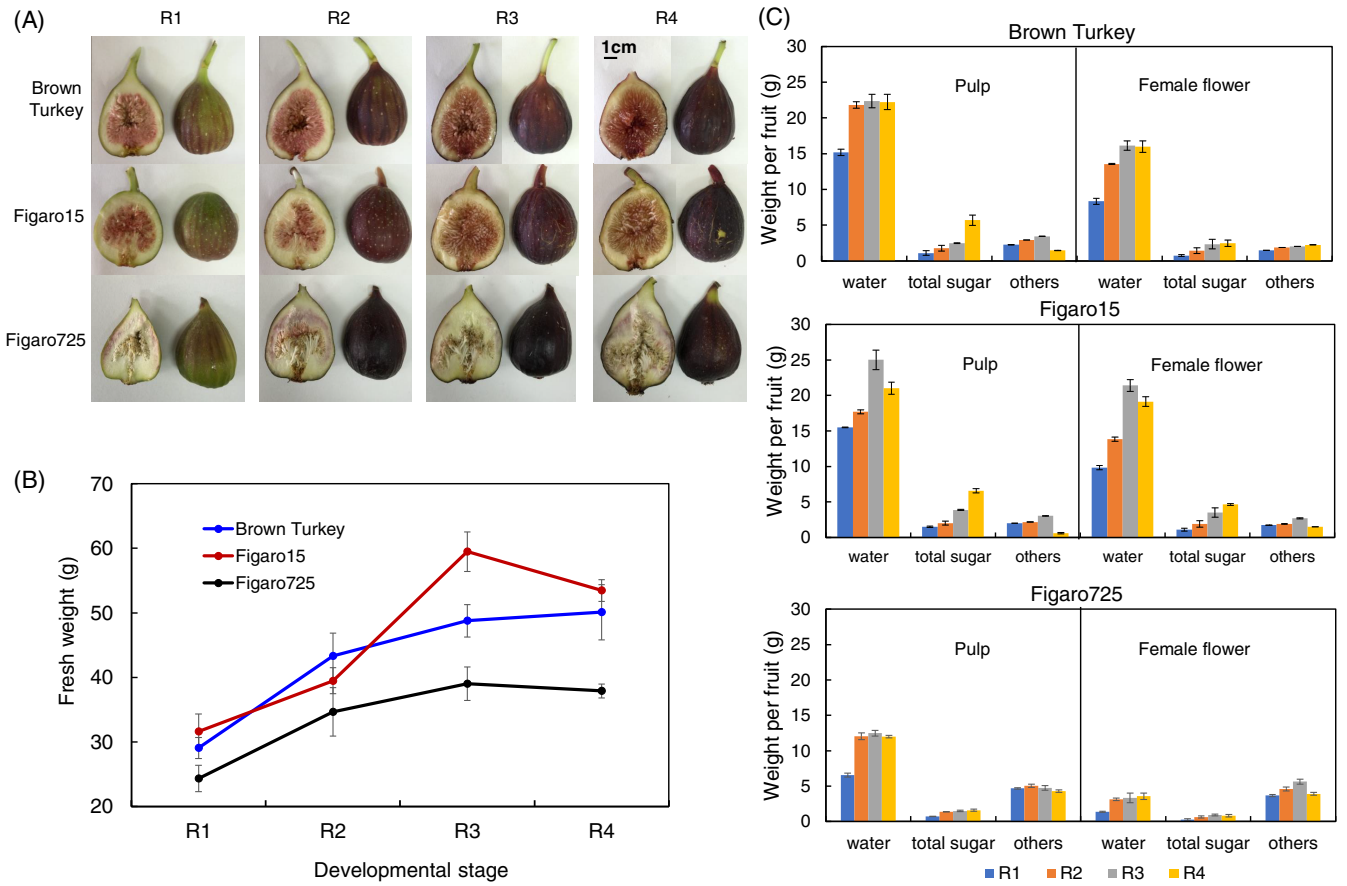

FIGURE 1 Water and sugar concentrations of pulp and female flowers per fresh fruit during ripening in the female figs "Brown Turkey" and "Figaro15", and the male fig "Figaro725." (A) Pictures of fruit and (B) changes in fresh weight during ripening. (C) Weights of the constituents in a single fruit. Total sugar refers to the sum of sucrose, glucose and fructose. R1, beginning stage of ripening with 30% color coverage; R2, mid-ripening stage with 60% color coverage; R3, fully ripened fruit with 100% color coverage; R4, 2 days after full ripening. Data in B, C are means ± SE of nine fruits

0.5 ml min^{-1}. Presented values are the means of three biological replicates.

2.3 | Water content analysis

Water content for the two selected female cultivars and one male fig cultivar was measured. After separating the fruit into pulp and female flowers as described above, samples were oven-dried at 60°C until the weight was stable. Dry matter and water content were calculated based on the mass difference before and after drying. Three biological replicates were measured for each sample.

2.4 | Identification of sugar metabolism and transporter genes in fig

Using amino acid sequences of known members of each recognized sugar metabolic enzyme and sugar transporter subfamily from *Arabidopsis thaliana*, *Solanum lycopersicum*, *Vitis vinifera*, *Malus domestica*, and/or *Morus notabilis* as queries, putative sugar metabolism-related genes and transporters were identified in fig by performing tBLASTn analysis via BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) under default parameters in our published transcriptome datasets (Freiman et al., 2014; Rosianski et al., 2016a). ORF (open reading frame) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used to verify the ORF of these genes, and then genes with complete coding sequences were used for further analysis. These genes were annotated by alignment against the nonredundant (NR) database from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

2.5 | Phylogenetic analysis

Prior to phylogenetic tree construction, full-length amino acid sequences of sugar metabolism genes and different sugar transporter subfamilies of *Arabidopsis thaliana*, *Solanum lycopersicum*, *Vitis vinifera*, *Malus domestica* and/or *Morus notabilis* were obtained from the NCBI database. The protein sequence alignment between fig and the other plants was carried out by MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/) under default parameters with a PHYLIP interleaved output format (Edgar, 2004). Phylogenetic trees were built using PhyML 3.0 software with 1000 bootstrap replicates and the blosum62 substitution model (Guindon et al., 2010) and visualized using Fig Tree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

2.6 | Quantitative expression analysis

Pulp and female flowers were thoroughly ground to a fine powder in liquid nitrogen. Total RNA was isolated as described previously (Jaakola et al., 2001). RNA quality and quantity were determined in a NanoDrop ND-1000 spectrophotometer (Wilmington) and electrophoresis in a 1% (w/v) agarose gel. To remove DNA, total RNA was treated with RQ-DNase (Promega). Total RNA (1 μg) was used for first-strand cDNA synthesis with the FastQuant RT Kit (KR106, Tiangen Biotech).

Forty-one putative sugar metabolism genes (Table S1) and 39 putative sugar transporter genes (Table S2) were obtained for further evaluation in female flower and pulp samples by high-throughput real-time quantitative PCR. Specific primers were designed using Primer3Plus software (Table S3) and synthesized by Hylabs (Rehovot, Israel). Primer specificity was determined by 1% agarose gel electrophoresis of the products and melting-curve analysis. High-throughput real-time quantitative PCR was performed on a BioMark 96.96 Dynamic Array (Fluidigm Corp.) with TaqMan Gene Expression Assays (Applied Biosystems) at the Weizmann Institute of Science (Rehovot, Israel). The relative expression level of target genes was normalized to the internal fig gene *FcACTIN* (Freiman et al., 2014) and calculated using a relative quantitative method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001).

3 | RESULTS

3.1 | Soluble sugar content in different tissues during early female fig fruit adevelopment

As part of our earlier study (Rosianski et al., 2016b), we characterized sugar levels during the earlier stages of fruit development (phase II), that is, before final ripening. During the second half of phase II, fruit sugar levels did not change significantly, remaining at a somewhat steady ~50 mg g fresh weight $(fw)^{-1}$ for both pulp and inflorescence tissue. The soluble sugars were comprised primarily of the Suc hydrolysis products Glc and Fru in nearly equimolar amounts (Figure S1). Based on that study, we followed in more detail the final days of ripening when most of the sugar accumulation takes place.

3.2 | Dynamic changes in soluble sugar accumulation and water content in different tissues of fig fruit during final stages of ripening

During the approximately 7 days from the R1 to R4 stage (Figure 1A), marked developmental changes occurred in the female fig fruit with respect to sugar and water accumulation. Perhaps the most striking observation was that the sharp increases in fruit expansion, fresh weight, and water content (Figure 1B, C), which occurred during the R1–R3 stages in both "Brown Turkey" and "Figaro15", were temporally disassociated from the sharp rise in sugar content. The latter occurred primarily after attainment of full size, during the final 2 days of ripening at the R3–R4 stage (Figure 1C). In the case of "Brown Turkey", sugar accumulation occurred mainly in the pulp tissue, whereas there was also a parallel, albeit lower increase, in the female flower tissue of "Figaro15."

The soluble sugars of all studied tissues and varieties were comprised primarily of the Suc hydrolysis products Glc and Fru in nearly

equimolar amounts, with small amounts of the disaccharide Suc accumulating in parallel with hexose accumulation (Figure 2). Out of the 25 g of pulp tissue, the sugar content increase from 2.7 to 5.7 g, and the soluble sugars reached 22.8% of the total 25 g biomass of "Brown Turkey" pulp tissue in only 2 days. Without taking into account any differential partitioning or compartmentalization in the pulp cells, this translated to a net increase of ~750 mM hexose over a relatively short period. "Figaro15" had slightly higher Suc accumulation, which was already higher at the R3 stage due to what seemed to be an earlier rise in the accumulation pattern.

One intriguing observation was that in practically all of the tissues studied, there was a decrease in the remaining "other" nonsugar components (NSC), concomitant with the increases in soluble sugars. This decrease was not accounted for by starch (not shown), which could otherwise indicate a starch-to-soluble sugar transition during this period. Accordingly, the surge in soluble sugar levels in the final stage of ripening could be attributed to either, or both, a net increase in the flux of carbohydrate import through the fruit pedicel, or accumulation of hexoses derived from the breakdown products of undetermined storage or structural components, occurring during the final stages of cell-wall softening.

To estimate the relative contribution of these two components, we followed the changes in soluble sugar content in fig fruit from the R2 stage to the R4 stage and compared fruit ripening on the tree to fruit harvested at R2 and R3, and ripened off the tree. The results indeed indicated a small increase in soluble sugars during the ripening off the tree, which accounted for ~15% of the total increase in soluble sugars in the fruit ripening on the tree (Figure 3). However, the remaining 85% of the increase in sugar content was due to the net import from source tissue to the fruit sink during this period.

Accordingly, considering that 85% of the accumulated soluble sugar was derived from translocation, and that sugar accumulation during the final 2 days of ripening at the R3–R4 stage amounted to a net increase of ~3 g per 25 g fruit, the import rate into a single fruit translated to approximately 1.25 g day^{-1}, or ~ 50 mg g fw^{-1} day^{-1}.

In parallel, we followed the fruit development of the male fig "Figaro725" (Figure 1), also separated into pulp and floral tissue. In stark contrast to the sharp sugar accumulation in the sweet female fig varieties, the male fig did not accumulate sugar during the R2 to R4 stages (Figure 2). This striking difference was observed for both the floral tissue and, to a larger degree, the pulp tissue. However, the modest increase in sugar accumulation observed between R1 and R2 was similar in both fruit types (Figure 2).

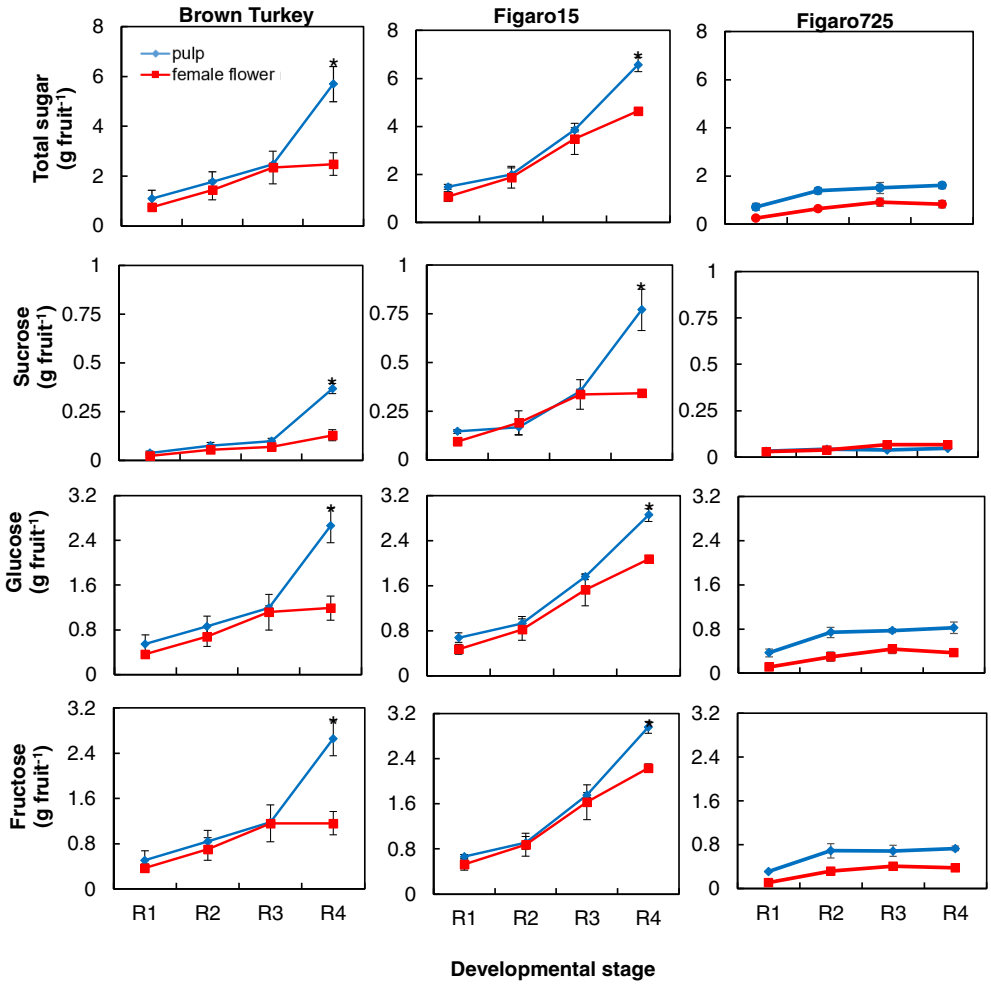

FIGURE 2 Total sugar (sucrose, glucose, and fructose) distribution in pulp and female flower of female figs "Brown Turkey" and "Figaro15", and male fig "Figaro725" during different ripening stages (see legend of Figure 1). Each value is the mean ± SE of nine fruits. Asterisk denotes significantly different at $p < 0.05$ by Student's *t*-test

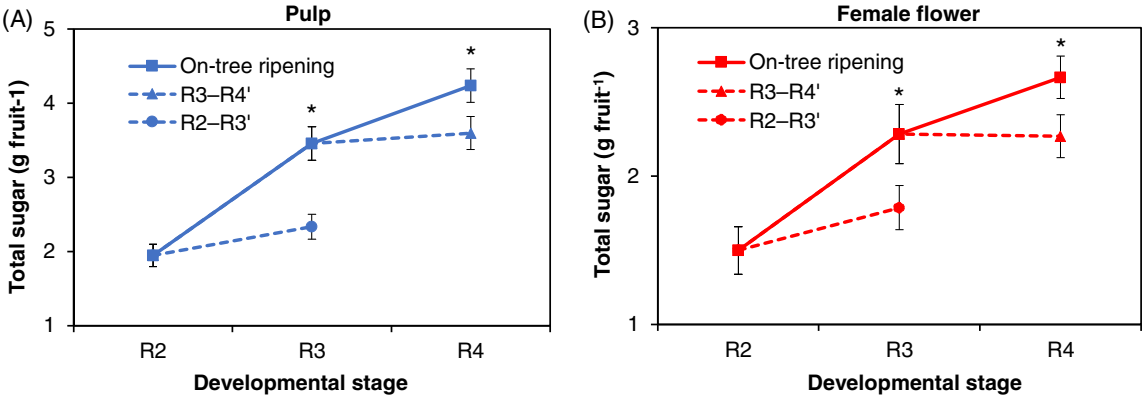

FIGURE 3 Comparison of total sugar accumulation in (A) pulp and (B) female flower of on-tree and off-tree ripened "Brown Turkey" fig. For developmental stages, see legend to Figure 1. R3', R3 ripening stage at room temperature (20°C) from R2; R4', R4 ripening stage at room temperature (20°C) from R3. Each value is the mean ± SE of 15 fruits. Asterisk denotes significantly different at $p < 0.05$ by Student's *t*-test

3.3 | Identification of putative sugar metabolism and transporter genes

To better understand the underlying processes of rapid sugar accumulation during female fig ripening, a comprehensive inventory of putative sugar-related genes was prepared based on the publicly available transcript data (Freiman et al., 2014; Rosianski et al., 2016a). Eighty transcripts, representing 41 sugar metabolism enzymes and 39 sugar transporter proteins, were identified in the database (Tables S1 and S2).

Most of the enzymes in the sugar metabolism pathway were encoded by multiple genes, including four *SPS*, five *SUS*, four *CIN*, two *VIN*, three *CWIN* and five invertase inhibitor (*C/VIF*) genes. As for phosphoglucomutase (PGM) and phosphoglucose isomerase, single copies of both cytosolic and plastidic forms were identified, as has been shown for other plants as well (Figure S2). In addition, three hexokinase (*HK*) genes and four fructokinase (*FK*) genes were identified.

The sugar transporters can be divided into three main families: SUT/SUC, SWEET and MST. Three SUC transcripts belonging to three distinct clades were present (Figure S3A). Among sugar transporters in fig fruits, SWEET was the most abundant family with 11 transcripts identified (Table S2). Phylogenetic analysis showed that the SWEET genes in fig belong to four different subfamilies based on the classification in *Arabidopsis* (Feng & Frommer, 2015): clades I (*FcSWEET7–9*, *11*), II (*FcSWEET4*, *10*), III (*FcSWEET1–3*), and IV (*FcSWEET5*, *6*) (Figure S3B). The remaining sugar transporters were assigned to seven subfamilies of the MST family and the alignment with corresponding proteins from Arabidopsis, tomato and grape revealed fig sugar transporters in each subfamily (Figure S3C–I).

3.4 | Expression patterns of sugar metabolism genes in female fig during ripening

Tissue-specific relative expression patterns of the sugar metabolism genes were determined for "Brown Turkey" and "Figaro15" and are presented as a heat map in Figure S4; genes showing patterns of interest are presented graphically in Figure 4. In general, the two varieties showed similar expression patterns and trends, leading to some general conclusions. The invertase inhibitor genes *FcC/VIF4* and *5* seemed to be the exception, with strong upregulation during ripening in "Brown Turkey" and a less pronounced developmental pattern in "Figaro15."

First, for most of the genes, the developmental patterns of expression for the pulp and flower tissues were similar and, in most cases, the genes were more highly expressed in the former. *FcCWIN1* and *FcC/VIF4* may be considered more pulp-enriched genes. *FcSUS4* was the exception to this pattern, with higher expression in the inflorescence tissue.

Second, with respect to the various enzyme families that may play a role in the breakdown of imported Suc, all of them (*VIN*, *CWIN*, *CIN* and *SUS*) had representatives with significant and developmentally increasing expression in the sugar-accumulating tissue. Accordingly, we cannot discern from the expression patterns what the Suc-unloading pathway and metabolism might be. The two invertase genes, *FcCIN2* and *FcVIN1*, were the only sugar metabolism genes for which differential expression in the inflorescence of the two varieties could possibly be related to the different sugar-accumulation patterns shown in Figure 2, in which "Figaro15" accumulated more Suc than "Brown Turkey" in the inflorescence during the R3–R4 stage.

Finally, some genes were more strongly related to ripening than others, and some were particularly strongly upregulated during the sugar-accumulation stage; these are clearly presented in Figure 4. In contrast to the general pattern of increasing expression during ripening, there were a few sugar metabolism genes that displayed down-regulation of transcription, and this was most clearly seen in the expression pattern of *FcC/VIF1* in both tissues of the two varieties, which might also contribute to an increase in invertase activity.

3.5 | Expression patterns of sugar transporter genes in female fig during ripening

To further describe the rapid sugar-accumulation process in fig fruit, 39 candidate sugar transporters belonging to nine subfamilies were

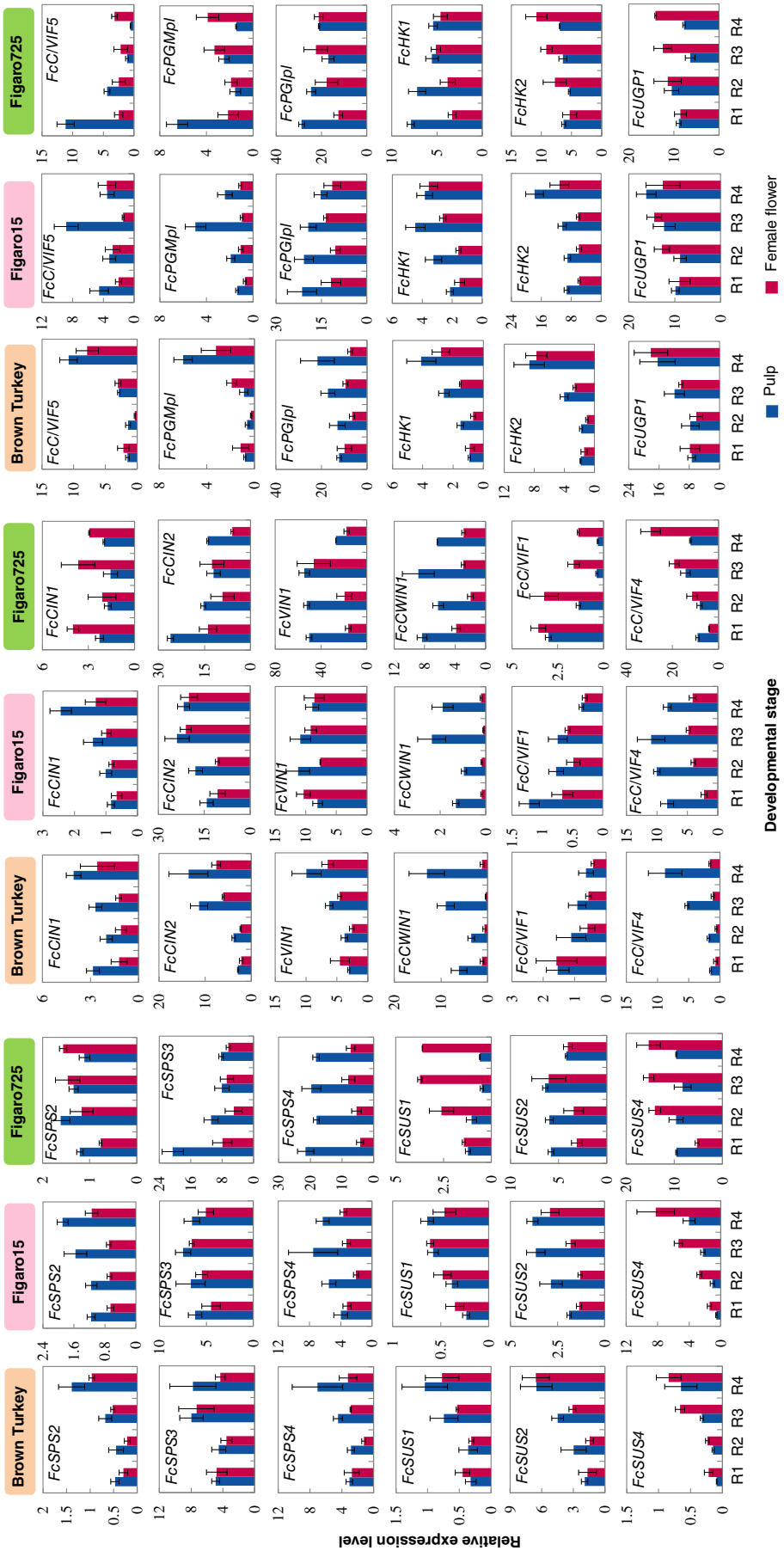

FIGURE 4 Expression patterns of predicted sugar metabolism genes in pulp and female flowers of female figs "Brown Turkey" and "Figaro15", and male fig "Figaro725." For developmental stages, see legend of Figure 1. Each value is the mean ± SE of three biological replicates and one biological replicate consists of three fruits

examined in the pulp and female flowers of "Brown Turkey" and "Figaro15", and their relative expression patterns are shown in Figure S5; genes showing patterns of interest are presented graphically in Figure 5. As with the sugar metabolism genes, there were general similarities in expression patterns between the two varieties. In the SUT/SUC family, both *FcSUC1* and *FcSUC3* showed upregulation during ripening. With regard to *SUC1*, we saw an interesting differential pattern of temporally earlier upregulation in the pulp of "Figaro15" compared with that of "Brown Turkey." This differential pattern was also observed for a number of additional transporter genes, such as *FcSWEET9*, *SFP2* and *5*, and *TMT2*.

Among the SWEET, six genes representing clades I (*FcSWEET8*, *9*, *11*), III (*FcSWEET2*, *3*) and IV (*FcSWEET5*) were significantly expressed in the ripening fruit. In particular, the clade III members *SWEET2* and *3*, as well as clade I members *SWEET9* and *11* were developmentally upregulated. In contrast, the clade IV *SWEET5* showed a sharp downregulation during ripening, and its expression was also pulp-specific.

Among the large MST family, patterns of upregulation could be observed for *FcSTP1*, *5* and *6*, *TMT1* and *2*, and *VGT1*. An earlier upregulation in R3 in the pulp of "Figaro15" compared with R4 in 'Brown Turkey' was also observed for these genes.

With respect to the relative expression values among the upregulated transporter genes, the following represented the most highly expressed genes in the R3–R4 stages: *FcSUC1* and *SUC3*, *SWEET3* and *SWEET11*, *STP1* and *STP5*, and *TMT2*. Of interest, the strongly expressed and upregulated *FcSTP1* and *5* and *SWEET11* were relatively pulp-specific.

3.6 | Expression patterns of sugar metabolism and transporter genes in male fig during ripening

A comparison of gene expression between the sugar-accumulating female fruit and the nonsugar-accumulating male fruit was expected to be very informative in discerning which genes are indeed specifically associated with the ripening-related sugar-accumulation pattern (Figure S6). Interestingly, most of the sugar metabolism genes were differentially regulated between the female and male fruit (Figure 4). With the exception of *FcC/VIF1* and *4*, all the sugar metabolism genes that showed a developmental increase in expression during the R3 and R4 stages of the female fruit showed either a decrease or no change in expression in the maturing male fruit. This was especially evident when contrasting the expression levels in the respective pulp tissues, which is the predominant sugar-accumulating tissue in the female fruit.

A somewhat similar picture emerged from the comparison of sugar transporter gene expression in the female versus male figs, although the developmental differences were less striking. Approximately half the transporter genes that showed developmental upregulation in the sweet female fruit showed similar developmental patterns in the male fruit (Figure 5). These included, for example, *SWEET2* and *3*, and *STP1*, which were markedly upregulated in the sweet fruit but, interestingly, were similarly upregulated in the male fruit. Nevertheless, many of the transporter genes did show upregulation patterns only in the female fruit, that is, *SUC1*, *SWEET9* and *11*, the SFP family, *STP5* and *6*, and *VGT1* and *INT2*.

4 | DISCUSSION

4.1 | Sugar accumulation in female figs compared with other fruit

Fig fruits undergo a rapid ripening process (phase III), generally taking less than a week for the summer crop, and approximately 90% of the total sugar content is accumulated during the last few days of ripening. Our results are consistent with earlier reports showing that the hexose sugars Glc and Fru are the major sugars accumulated during this short period (Trad et al., 2012; Vemmos et al., 2013). Thus, the fig pulp represents an extreme case of sugar accumulation by plant sink tissue. According to the results presented here, the pulp accumulated ~50 mg sugar per gram fresh pulp in a single day, leading to a net increase of ~3 g sugar in the 25 g of pulp tissue within only 2 days. A comparison with sugar-accumulation rates of other fruit and nonfruit tissues indicates the uniqueness of the sugar-accumulation phase in the individual fig fruit.

Sugar concentration in the figs in this study reached ~15% of the pulp fresh weight or ~ 50% of the dry weight. While other high-sugar fruit can also reach these and even higher levels, as in grapes, their period of sugar accumulation is much longer, and thus, the sugar-accumulation rate is much lower than that for figs. Sugar accumulation in 'Shiraz' grapes, for example, was reported to reach 150 mg g fw^{-1}, but this accumulation took place over a period of 8 weeks (Davies & Robinson, 1996), translating to ~3 mg g fw^{-1} day^{-1}, compared with ~50 mg g fw^{-1} day^{-1} for fig. Similarly, sugar accumulation occurs over a period of weeks in other fruit, for example, apple (Janssen et al., 2008; Li et al., 2012), cherry (Gao et al., 2003) and pineapple (Singleton & Gortner, 1965), in which ripe fruit sugar levels are in the range of those of fig (Coombe, 1976). This appears to hold true in general for high-sugar perennial tree fruits, and even more so for high-sugar annual fruits. For example, sweet melon sugar levels can reach 80 mg g fw^{-1} but the sharp accumulation of sugar during the ripening period contributes about half of that, ~40 mg g fw^{-1}, which accumulates over abour 10 days, for a daily accumulation rate of ~4 mg g fw^{-1} (Burger & Schaffer, 2007; Dai et al., 2011).

Vegetative tissues selected for unusually high sugar accumulation, such as sugar beetroots (Bell et al., 2017) and sugarcane stems (Moore & Maretzki, 2017), have similarly high levels of sugar, reaching more than 15 mg Suc g fw^{-1} in both tissues. However, the high storage levels in these tissues are as well a consequence of an extended period of sugar accumulation, lasting weeks.

Our results also showed that the increase in soluble sugar levels of the ripening fig fruit was primarily due to a net import of photosynthate during this short period, rather than to the synthesis of soluble sugars from previously stored reservoirs in the fruit. A rough estimation based on sugar levels in fruit ripened on the tree compared with those in fruit ripened off the tree suggested that about

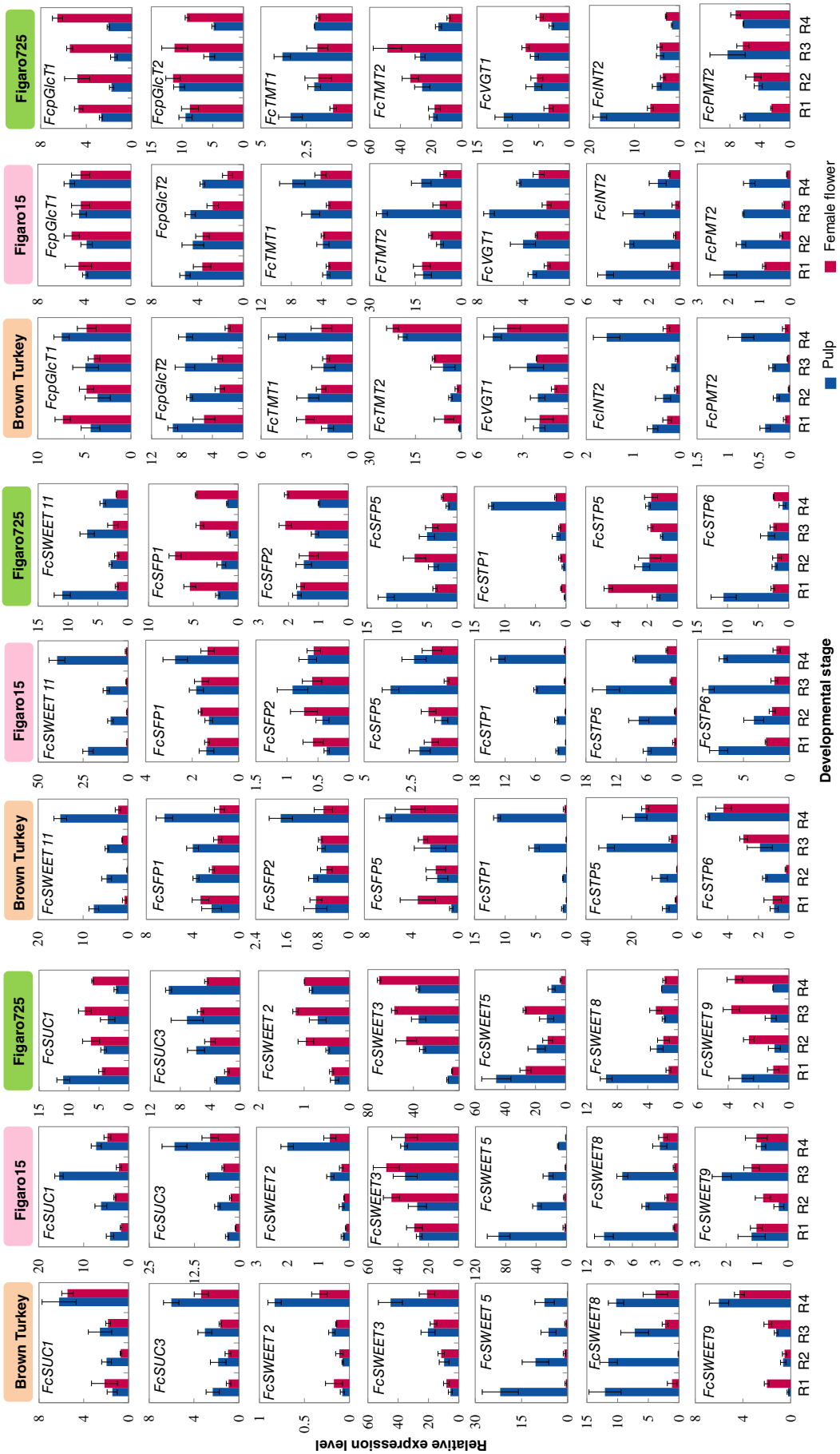

FIGURE 5 Expression patterns of predicted sugar transporter genes in pulp and female flowers of female figs "Brown Turkey" and "Figaro15" and male fig "Figaro725." For developmental stages, see legend of Figure 1. Each value is the mean ± SE of three biological replicates and one biological replicate consists of three fruits

85% of the net increase was due to net import. Among sugar-accumulating plant tissues, the range of soluble sugar levels due to a net increase in dry matter derived from import versus the conversion of sugars from previously deposited storage material is wide. For example, in strawberry fruit (Hubbard et al., 1991; Souleyre et al., 2004), sugar accumulation is predominantly due to import during the accumulation period, with negligible synthesis of sugars from transiently stored starch. On the other hand, the sharp increase in soluble sugar levels of ripening fruit such as banana (Hubbard et al., 1990) is largely due to the conversion of stored starch into sugars. The metabolic pathways associated with these different sugar-accumulation strategies will differ accordingly.

Thus, in comparison to other sugar-accumulating plant organs, the fig fruit is distinguished by a period of extreme sugar accumulation, in terms of both the short length and the high rate of import and accumulation. This phenomenon can be understood in the context of the unusual ripening pattern of fig fruit on the tree. In contrast to other fruit, where multiple fruits ripen simultaneously and therefore compete with each other for source carbohydrates, in fig, fruit ripening occurs individually. A single fruit per shoot ripens and only after it is fully ripe does the next fruit on the shoot start to ripen. Thus, there is little inter-sink competition on the individual shoot during sugar accumulation, allowing for short but focused sugar import into each individual fruit. Unfortunately, this strategy of fruit production does not allow for the mechanical or once-over harvesting that is used for most other fruit trees.

4.2 | Significance of sugar metabolism and transporter expression

To shed light on the different sugar-accumulation patterns in the pulp compared with the inflorescence tissues, tissue-specific expression patterns of putative sugar metabolism and transporter genes were examined in the two female fig varieties. To focus on genes specifically related to the novel sugar-accumulation patterns, we compared the expression patterns of the same genes in the nonaccumulating male figure.

4.2.1 | Sugar metabolism genes

The changing patterns of carbohydrate-metabolism gene expression point to a global transition of the sugar-accumulation metabolism, rather than to a particularly striking change in the regulation of a single gene. This seems to be an appropriate generalization for developmental patterns of sugar accumulation in sink tissue. Global transitions in sugar-metabolism gene expression characterize other sugar-accumulating sinks whose transcriptomes have been studied, including, for example, grapes (Degu et al., 2014), apple (Janssen et al., 2008; Li et al., 2012) and melon fruit (Dai et al., 2011).

Some of these global changes include developmental increases in members of the four Suc-breakdown enzyme families: VIN, CWIN, CIN, SUS. Regarding the invertase inhibitors, which would presumably lead to an increase in invertase activity, *FcC/VIF1* was weakly expressed but its pattern indicated developmental downregulation. The other two putative inhibitor genes, *FcC/VIF4* and *5*, were more highly expressed but did not show striking developmental changes in expression. Moreover, we cannot predict from their protein sequences whether they interact with the VIN or CWIN genes since they could also function as pectin methylesterase inhibitors. The gene clade of *FcC/VIF4* and *5* also contains *AtC/VIF2*, which has been shown to inhibit *Arabidopsis* CWIN and VIN (Link et al., 2004). Thus, the physiological significance of these developmental changes in expression cannot be ascertained.

The genes responsible for Suc synthesis, *FcSPS*, and those of the SUS family, enzymes capable of both synthesis and cleavage of Suc, were upregulated during sugar accumulation. Although the fig fruit accumulates the breakdown products of Suc, there is likely to be a role for hydrolysis–resynthesis–hydrolysis of the translocated Suc during accumulation, and such a "futile cycle" has been reported for other sugar-accumulating fruit as well (Miron et al., 2002; Nguyen-Quoc & Foyer, 2001).

By comparing these patterns with those in the male fruit, we can conclude that there is a differential pattern of sugar-metabolism gene expression at the global level of the metabolic pathway. Practically all of the genes that showed developmental upregulation in the female fig showed no such developmental changes, or were characterized by downregulation, in the male fruit. This indicates that sugar accumulation is accompanied by a global transition in metabolic-pathway regulation and that it is unlikely that the accumulation trait is due to more limited changes in gene expression (Figure 6).

4.2.2 | Sugar transporter genes

Sugar transporters of the major facilitator superfamily in plants comprise a range of 60 distinct gene products, classified into seven subfamilies of MST and a small family of SUT/SUC (Slewinski, 2011). In 2010, the SWEET family was added to the inventory of sugar transporter families and characterized (Chen et al., 2010).

With respect to the expression patterns of the sugar transporters, there were a number of significant observations. Again, there was a global transition of transporter expression. This was indicated, for example, by the developmental increases in the two *FcSUC* genes combined with changes in various MST genes. The most striking developmental change was for the MST family members STP and TMT. *FcSTP1* expression strongly paralleled sugar accumulation specifically in the pulp, and was strongly upregulated in the final stages of sugar accumulation. *FcTMT1* and *FcTMT2* also showed significant upregulation during the final stages of development.

The STP protein family is also referred to as HT (e.g., in tomato, grape). The contribution of tomato *LeHT* genes to fruit hexose levels has been demonstrated in transgenic tomato lines silenced for *HT* genes, in which the hexose levels were significantly decreased (McCurdy et al., 2010). *STP/HT* do not appear to be striking

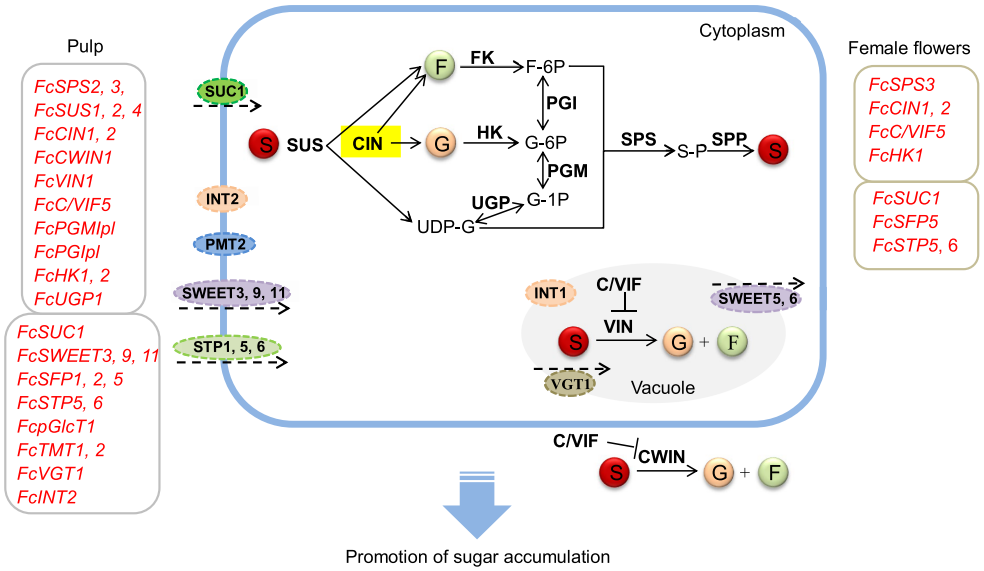

FIGURE 6 Predicted crucial genes related to rapid sugar-accumulation process in pulp and female flowers of fig fruit during ripening. Genes in red (green) color are upregulated (downregulated) in the pulp, and pink color represents upregulation in female flowers. Genes with the same change trend in both female flowers and pulp are in bold font

candidates with a major role in sugar accumulation in grape (Afoufa-Bastien et al., 2010; Lecourieux et al., 2014), with the possible exception of *VvHT8* (Lecourieux et al., 2014; Xin et al., 2013). Thus, the very strong upregulation of *FcSTP1*, particularly in the pulp tissue, is certainly worthy of further study.

The TMT are also referred to as TST. A *TST* gene was identified and implicated as responsible for the high Suc accumulation in sugar beetroots (*BvTST2*) (Jung et al., 2015), and a *TST* orthologue was associated with Suc accumulation in sweet sorghum stems (Bihmidine et al., 2016). Recently, a *TST* gene was proposed as a quantitative trait locus for sugar content in *Citrullus lanatus* fruits based on a genome-wide association study (Ren et al., 2018), and exogenous expression of a *TST* in melon fruits led to an increase in sugar levels (Cheng et al., 2018). Members of the TMT gene family show strong upregulation during pear (Li et al., 2015) and apple fruit (Li et al., 2012; Wei et al., 2014) development and ripening. Thus, there is strong support for the role of TST in sugar accumulation in high-sugar sinks, leading to its nickname of "sugar dumper" (Hedrich et al., 2015) and the role of the *FcTMT* genes are also worthy of further research attention.

Of the SWEET transporters, the clade I *FcSWEET9* and the clade III *FcSWEET3* were particularly upregulated in the ripening pulp. *FcSWEET9* is the orthologue of the Arabidopsis *AtSWEET1*, the first reported SWEET that was also characterized as a Glc efflux transporter localized to the plasma membrane (Chen et al., 2010); it is also orthologous to the tomato *Fgr* gene (Solyc04g064610) recently characterized as a Glc exporter responsible for the increased level of Fru relative to Glc in ripe tomato fruit (Shammai et al., 2018). The clade III *FcSWEET3*, homologous to clade III *AtSWEET9*, *11* and *12*, is predicted to transport Suc as do the other clade III SWEET members (Lin et al., 2014). However, although they are bidirectional transporters, SWEET are generally implicated in sugar efflux, and their role in sink sugar accumulation remains to be determined.

Interestingly, there are a number of genes whose expression was upregulated earlier in "Figaro15" than in 'Brown Turkey'. This was observed for the sugar metabolism genes *SPS4*, *CIN2*, *VIN1*, *CWIN1*, *C/VIF4*, *PGMpl* and *HK1*, as well as the sugar transporter genes *SUC1*, *SWEET9*, *SFP2*, *SFP5*, *STP6*, *TMT2* and *VGT1*. These temporal differences in expression may be related to the earlier onset of strong sugar accumulation in "Figaro15" and suggest earlier onset of the global transition to sugar accumulation.

The comparison with male fruit transporter expression provided some interesting observations. First, the potential candidates for involvement in the sharp sugar accumulation in the female fruits, particularly the clade III *SWEET2* and *3* and *STP1*, were similarly upregulated in the male fruit, thereby eliminating them as primary candidates for determining sugar accumulation. On the other hand, many of the transporter genes that were upregulated in the female fruit were not similarly upregulated in the male fruit, implying their contribution to the global transition to sugar accumulation in the female fig fruit. These include *SUC1*, the clade I *SWEET9* and *11*, and members of the SFP and STP families, as well as *VGT1* and *INT2*.

Figure 6 presents the genes involved in the global transition of sugar metabolism and transport that are unique to the developing female fruit in a comparison with the nonsweet developing male fruit.

4.3 | Complexity of fruit structure with respect to sugar accumulation

The common fig fruit bears a unique closed inflorescence structure—the syconium. This closed inflorescence produces an aggregate fruit, which is composed of small individual drupelets that develop from the ovaries enclosed in the succulent vegetative-originated receptacle (Storey, 1977). Our results showed that during the late stage of fruit ripening, striking developmental changes occur in the fig fruit: a sharp rise in sugar and water accumulation. In both varieties, the sugar accumulation occurs mainly in the pulp tissue with a lower increase in the female flower tissue (Figure 2). Recently, we showed that abscisic acid (ABA) and ethylene regulate ripening in the fig inflorescence, whereas in the receptacle, ripening is mainly directed by ABA, as in nonclimacteric fruits (Lama et al., 2019). In

strawberry, a nonclimacteric fruit, Suc functions as a ripening signal (Jia et al., 2016).

4.4 | Practical application of the research

The current market trend is characterized by an increasing demand for fresh figs. The worldwide trade of fresh figs has become possible mainly due to technological developments in postharvest fruit preservation. Nevertheless, fresh figs are highly perishable and difficult to store, even when harvested at the proper ripening stage. In fact, fig cannot be stored for more than 18 days (Flaishman et al., 2008). In this study, we found that two fig cultivars, "Figaro15" and "Brown Turkey", have different patterns of sugar accumulation. "Figaro15" showed larger and earlier Suc accumulation, which was already higher at the R3 stage (Figure 2). The different patterns of sugar accumulation could be further used in breeding programs to select early sugar-accumulating firm fruit, providing the consumer with tasty fruits that have a longer shelf life.

In summary, we analyzed the tissue-specific sugar-accumulation patterns during ripening of two female fig cultivars and one "regular" male fig (set as a control). The results showed extremely rapid and high sugar accumulation in the female fig fruit during ripening, approximately 50 mg g fw^{-1} day^{-1} during the R3–R4 stage, while sugar mainly accumulated during the R1–R2 stage in the male fig at a rate of about 20 mg g fw^{-1} day^{-1}. We also reported the putative sugar metabolism and transporter genes of fig, and potentially crucial genes involved in rapid sugar increase for female and male fig fruit during different ripening phases were predicted (Figure 6), based on a global analysis of sugar-related gene-expression patterns and comparisons between female and male figs. The alterations in the expression levels of the tested genes during ripening showed tissue-specific characteristics in both male and female figs. More significantly different gene-expression patterns during ripening were found between pulp and inflorescence in males than in females. More active genes were identified during their major sugar-accumulation stages, R1–R2 for male figs and R3–R4 for female figs. Several genes revealed pulp-specific characteristics in the female figs. We plan to use these candidate genes for genome editing to further evaluate their functions and to shift sugar accumulation to an earlier date when fruits have a certain degree of hardness for transport and storage.

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AUTHOR CONTRIBUTIONS

Moshe A. Flaishman and Arthur A. Schaffer designed the experiments. Li-Juan Chai, Kumar Lama, Reut Peer, and Yelena Yeselson conducted the experiments. Li-Juan Chai and Kumar Lama analyzed the results. Li-Juan Chai, Kumar Lama, Reut Peer, Arthur A. Schaffer, and Moshe A. Flaishman prepared the manuscript. Huiqin Ma provided help to revise the manuscript. All authors have read and approved the manuscript for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [repository name e.g "figshare"] at http://doi.org/[doi], reference number [reference number].

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