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Cupuassu (*Theobroma grandiflorum* [Willd. ex Sprengel] Schumann) Fruit Development: Key Genes Involved in Primary Metabolism and Stress Response

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Abstract: Cupuassu (*Theobroma grandiflorum* [Willd. ex Sprengel] Schumann) seeds constitute the raw material for oil extraction and fabrication of *cupulate* (product similar to chocolate). However, fungal diseases such as witches' broom caused by *Moniliophthora perniciosa* have interfered with the large-scale development of cupuassu plantations. Cupuassu genetic breeding programmes focus on a variety of biotechnological tools or approaches to select genes related to quality or resistance mechanisms. In this study, we used expression and interactomics analyses of preselected genes involved in fruit quality and/or resistance to better understand the molecular and physiological mechanisms associated with these plant processes. It was found that (i) resistant and susceptible cupuassu genotypes showed different pulp characteristics as well as gene expression patterns; (ii) monosaccharide and carbohydrate transport pathways were enhanced during fruit maturation; (iii) sugar accumulation participated in signal transduction associated with fruit development and stress response in maturing fruits; and (iv) maturing pulp and seeds showed increased phospholipid metabolism and translocation, as well as immune system activation. The *TgSTP1*, *TgWRKY33*, *TgCZF1*, and *TgUBA1* genes in cupuassu and the orthologues of DIN10, CNI1, and TET8 identified by the interactomics approach may be good candidates for marker-assisted selection in breeding programmes focusing on both fruit quality and resistance/tolerance to biotic/abiotic stress.

Keywords: *Theobroma grandiflorum*; fruit quality; *Moniliophthora perniciosa*; interactomics; signal transduction

Citation: Ferraz dos Santos, L.; Santana Silva, R.J.; Falcão, L.L.; Alves, R.M.; Marcellino, L.H.; Micheli, F. Cupuassu (*Theobroma grandiflorum* [Willd. ex Sprengel] Schumann) Fruit Development: Key Genes Involved in Primary Metabolism and Stress Response. *Agronomy* **2022**, *12*, 763. https://doi.org/10.3390/agronomy12040763

Received: 23 February 2022
Accepted: 21 March 2022
Published: 22 March 2022

1. Introduction

Cupuassu (*Theobroma grandiflorum* [Willd. ex Spreng.] Schum) is one of the most important fruit species of the Amazon region, and the expansion of cupuassu plantations could play an important social, economic, and environmental role in the considered areas [1]. Cupuassu fruit has drupe and berry characteristics, an elongated shape with rounded ends, a length of 12 to 25 cm, and a diameter of 10 to 12 cm. It contains seeds organized on five vertical rows and embedded in a yellowish-white fibrous pulp with acidulated taste and pleasant smell [2]. Cupuassu seeds constitute the raw material for oil extraction used in the cosmetics industry and for the production of "cupulate", a product similar to chocolate [3]. Cupuassu pulp presents high levels of vitamin C and pectin, among other healthy and valuable compounds [4], and is used in the fabrication of sweets, juices, ice cream, etc. [5].

Even though good results have been obtained during approximately 30 years of cupuassu breeding in the Brazilian Amazon, some difficulties, such as fungal diseases, have interfered with the large-scale development of cupuassu plantations [6,7]. In this context, the fungus *Moniliophthora perniciosa* (Stahel) Singer, responsible for witches' broom disease, is considered the main problem encountered by cupuassu producers. This disease has generated important productivity losses in commercial, consortium, or individual cupuassu plantations [8]. *Moniliophthora perniciosa* mainly affects the meristematic regions of the leaves, stems, flowers, and fruits, causing hypertrophy of the organs of interest. The infection of the flower cushions results in an increase in their size during the early drying phase. In young fruits, the infection promotes abnormal formation and stops the growth and mummification of the fruits, which do not reach maturity and remain attached to the plant [9]. When infection occurs later during fruit development, maturation may be complete; however, the pulp and seeds may be compromised [9]. The genetic breeding programme of cupuassu focuses on aggregate biotechnological tools or approaches, such as molecular markers or functional studies [10–12], to select genes related to quality or resistance mechanisms. Such approaches will provide information about the functions and regulation of the genes involved in fruit developmental processes and/or biotic stress resistance; they can be used for marker-assisted selection in breeding programmes to create genotypes with superior characteristics that accumulate in a shorter period of time.

Here, we focused on the analysis of candidate gene expression in the pulp and seeds of young, maturing, and mature fruits from cupuassu genotypes presenting resistance vs. susceptibility to witches' broom disease, as well as differences in quality. The candidate genes were selected from previous data [10,11] based on their relation to the fruit quality determination process, as well as to pathogen resistance, at different levels of the involved signalling pathways. Elements related to transcription (transcription factor SAC51, zinc finger CCCH domain-containing protein, WRKY transcription factor), RNA processing and translation (polyadenylate-binding proteins), signalling (ubiquitin-activating enzyme), sugar transport and accumulation (sugar carrier protein C), lipid production and metabolism (e.g., phospholipid-transporting ATPase 1), biotic and abiotic stress responses (disease resistance protein RPS2, ABC transporter), and development (profilin) were examined. In a previous study, we showed that these genes contained polymorphic simple sequence repeat (SSR) markers useful for cupuassu selection [10]. The molecular mechanisms involved in the processes of biotic and abiotic stress, as well as metabolic pathways, are complex and demand more research.

In this context, the hypothesis of the work was that these previously identified genes participated in an integrated way to the cupuassu fruit quality and/or to plant stress responses. To better understand the relationship between the molecules involved in cupuassu fruit development, some computational methods may be used, such as interactomics (i.e., protein–protein interaction [PPI] network building), which provides a framework for assembling models of biological systems from molecular data [13]. The use of interactomics and homology between genes and proteins provides a basis for the functional association of genes [14]. Such an approach using PPI networks based on model organisms has already been successfully used to understand *T. cacao*-pathogen interactions or *M. perniciosa* development, among others [15,16]. This approach allows for a better understanding of the molecular and physiological mechanisms and processes in the plant. We sought to contribute functional information about the genes and mechanisms involved in fruit quality and stress responses in *T. grandiflorum*. The present report provides functional information (expression, interactomics) about these genes and their involvement in the formation of physiological characteristics that is important for cupuassu producers.

2. Materials and Methods

2.1. Plant Materials

Samples were collected from the cupuassu genotypes 174 (resistant to witches' broom disease) and 1074 (susceptible), which originate from Coari and Paratins (Amazonas

state), respectively, that were maintained in the Active Germplasm Bank of the EMBRAPA Amazônia Oriental (Pará, Brazil) (Figure 1a–c; Table 1) and selected based on the breeding programme of this institution. For the pulp quality analyses, five mature cupuassu fruits were harvested from three different plants (n = 15) of both cupuassu genotypes. For the expression analysis, seeds and pulp were collected from young, maturing, and mature fruits (Figure 1b–e), taking the following precautions to avoid contamination between the seed and pulp tissues: (i) the pulp was carefully separated from the seeds; (ii) the seed coat was removed to avoid any contamination with the pulp material; and (iii) the collected samples were immediately frozen in liquid nitrogen and then kept at −80 °C until utilisation (Figure 1f). For each developmental stage and for each tissue, three biological samples were collected (one biological unit = one fruit); a total of nine seed samples (three units × three developmental stages) and nine pulp samples (three units × three developmental stages) were obtained.

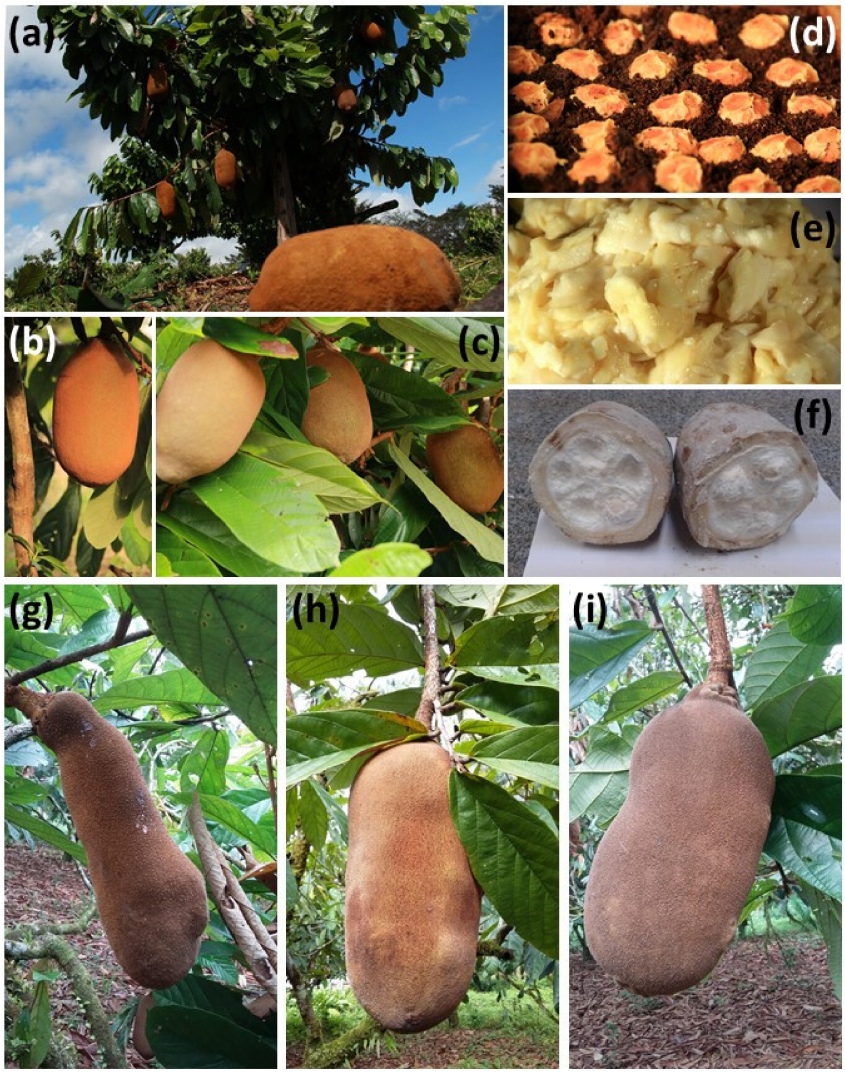

Figure 1. Cupuassu plant material used in this study. (**a**) General view of the cupuassu orchard from EMBRAPA Amazônia Oriental (PA, Brazil); (**b**) mature fruit from the 1074 genotype (susceptible to witches' broom disease); (**c**) mature fruit from the 174 genotype (resistant to witches' broom disease); (**d**) cupuassu seeds (in the sowing); (**e**) fresh cupuassu pulp (after separation from the seeds); (**f**) frozen cupuassu fruit with pulp and seeds; (**g**) young fruit from the 1074 genotype; (**h**) maturing fruit from the 1074 genotype; and (**i**) mature fruit from the 1074 genotype. Photos: Ronaldo Rosas (EMBRAPA).

Table 1. Cupuassu plant material characteristics. AM: Amazonas; ATT: titratable acidity; R: resistant; S: susceptible; SST: total soluble solids; and ST: total solids.

Genotype	Origin	Physiochemical Characteristics of the Pulp						Response to Witches' Broom Disease
		pH	SST	ATT	Humidity	ST	SST/ATT	
174	Coari-AM	3.5	13.1	1.5	83.9	16.1	8.7	R
1074	Parintins-AM	3.5	10.7	1.8	86.0	14.0	5.9	S

2.2. Cupuassu Pulp Quality Analyses

For the evaluation of the pulp characteristics (Brix, acidity, humidity, and pH), 20 g of pulp from each fruit was collected (Figure 1e) and analyzed as previously described [17]. The Brix was determined using a refractometer PR-101 (ATAGO, Tokyo, Japan). The total acidity, expressed in citric acid percentage, was determined by titration using 0.1 N NaOH. The pH was determined using a Horiba F-21 pH meter (Horiba, São Paulo, Brazil). For the determination of humidity, the samples were oven dried at 105 °C to a constant weight.

2.3. RNA Extraction and cDNA Synthesis

Total RNA was extracted from the samples described in 2.1 using the cethyltrimethylammonium bromide (CTAB) method [18,19] with modifications [20]. The cupuassu tissues were macerated in liquid nitrogen, and the extraction was carried out with a buffer containing 2% CTAB (*w*/*v*), 2 M NaCl, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, pH 8.0, 2% PVP 10,000 (*w*/*v*), and 2% β-mercaptoethanol (*v*/*v*). The samples were then treated with chloroform, and the total RNA was precipitated with 2 M LiCl overnight at 4 °C. RNA was resuspended in SSTE buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0), extracted with 1 volume of phenol, extracted twice with chloroform/isoamyl alcohol (24:1), and then precipitated with 2.5 volumes of 100% ethanol. After resuspension in DEPC water, the RNA was quantified by a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Afterwards, 1 µg of RNA was treated with DNase I according to the manufacturer's recommendations (Fermentas Life Sciences, São Paulo, Brazil). The integrity of the RNA was checked on a 1% agarose electrophoresis gel. cDNA synthesis was performed in a final volume of 20 µl using the cDNA RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's recommendations (Thermo Scientific, Waltham, MA, USA). The cDNAs were quantified by a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and 100 ng/µL of each cDNA sample was used for qPCR analysis.

2.4. Sequence Selection, Functional Annotation and Interactomics Analysis

The selection of *T. grandiflorum* target sequences was based on previous studies aiming to identify polymorphic markers in cupuassu expressed sequence tags (ESTs) [10]; those genes are related to pulp and seed quality, as well as to the resistance to witches' broom (locus name; Table 2). The *T. grandiflorum* nucleotide sequences (available in Table S1) were submitted to the ORFfinder (NCBI; https://www.ncbi.nlm.nih.gov/orffinder/, accessed on 12 September 2021) program to identify the corresponding proteins. Because some sequences have a truncated open reading frame (ORF), they were compared to the genome of *Theobroma cacao* [21], a species that is closest to *T. grandiflorum*. To confirm the homology between the sequences and perform subsequent Gene Ontology analysis, the sequences from *T. grandiflorum* and their corresponding counterparts from *T. cacao* were compared with the *Arabidopsis thaliana* genome (TAIR10; [22]) using the BLASTp tool [23]. To avoid annotation errors, three reciprocal BLAST comparisons were made: (i) *T. grandiflorum* vs. *T. cacao*/*T. cacao* vs. *T. grandiflorum*; (ii) *T. grandiflorum* vs. *A. thaliana*/*A. thaliana* vs. *T. grandiflorum*; and (iii) *T. cacao* vs. *A. thaliana*/*A. thaliana* vs. *T. cacao*. These comparisons confirmed the homology between the genes, even for incomplete proteins. Finally, the genes in *A. thaliana* that were homologous to those in *T. grandiflorum* were used for

Gene Ontology analysis (GO; [24]). GO identification was performed by a direct search of the *A. thaliana* genome (TAIR10) using the gene ID as input. For the identification of the gene names and families, as well as the functional annotation, the UniProt database (accessed on 20 October 2021) [25] was used; the proteins were named according to the gene family of their respective homologues. For the construction of interaction networks, the IDs of the homologous sequences in *A. thaliana* were used as the input into the Cytoscape 3.8.2 tool; these data were imported directly from the String database [26] with the StringAPP plug-in [27] using the String default parameters (protein query, medium confidence score 0.40). Then, a functional enrichment of the network was made through the String Enrichment option. The definition of clusters was made using the AutoAnotate [28] and ClusterMaker [29] plugin and the MCL cluster and WordCold algorithm, with max words = 3 and an adjacent word bonus = 8.

Table 2. *Theobroma grandiflorum* genes used in this study and their orthologues in *T. cacao* and *A. thaliana*.

Locus Name [1]	*T. grandiflorum* Gene Name	Putative Function	*T. cacao* Orthologue [2]	*A. thaliana* Orthologue
C692	*TgPAB4*	Polyadenylate-binding protein 4	Tc01v2_p005300.1	PAB2
C339	*TgSCA51*	Transcription factor SAC51-like	Tc08v2_p005820.1	AT5G50010
C70	*TgPRF1*	Profilin-1	Tc09v2_p008530.1	PRF1
C203	*TgRBP45B*	Polyadenylate-binding protein RBP45B	Tc06v2_p017980.1	RBP45B
C366	*TgALA1*	Phospholipid-transporting ATPase 1	Tc10v2_p010150.1	ALA1
C28	*TgSTP1*	Sugar carrier protein C	Tc06v2_p016570.1	STP1
C345	*TgCZF1*	Zinc finger CCCH domain-containing protein 29	Tc10v2_p001340.1	CZF1
C5718	*TgNL*	Disease resistance protein RPS2	Tc07v2_p005320.2	At4g26090
C733	*TgUBA1*	Ubiquitin-activating enzyme E1 1	Tc09v2_p029770.1	UBA1
C193	*TgWRKY33*	WRKY transcription factor	Tc09v2_p030160.1	WRKY33
C4546	*TgABC1*	ABC transporter C family	Tc02v2_p000950.1	ABCC1

[1] As described in [10]. [2] from CocoaGenDB (https://cocoa-genome-hub.southgreen.fr/ accessed on 22 October 2021) and [21].

2.5. Primer Design and Quantitative PCR

Primers were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi, accessed on 15 January 2020) and the following parameters: amplicon size, 80–150 bp; GC content, 30–60%; primer size, 20–23 bp; and annealing temperature, 57–62 °C (Table S2). To avoid cross-reaction between genes, the amplified regions were checked for the following characteristics: size difference, melting temperature, GC content, and GC/AT ratio [30] (Table S3). Expression analysis by qPCR was performed as previously described [31] using the standard settings of the Stratagene MX3005P system (Agilent Technologies, Santa Clara, CA, USA). The qPCR consisted of 100 ng/µL cDNA, 0.3 µM each primer (Table S2) and 1X Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) in a total volume of 12.5 µL. The cycling conditions were as follows: 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 35 s, and 72 °C for 30 s, with detection of the fluorescent signal at the end of each extension cycle. To verify that each primer pair produced only a single PCR product, a dissociation analysis was carried out under the following cycling conditions: 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. The PCR product was analysed with MxPro QPCR software (Agilent Technologies, Santa Clara, CA, USA). The gene expression level was analysed in three biological samples, with three technical repetitions for each biological sample (cDNA) from both the 174 and 1074 genotypes. The expression level was calculated

by the comparative ΔCt method ($2^{-\Delta Ct}$) using GAPDH and MDH as reference genes (average of expression values from both genes) as previously described [31]. Experiments included a negative control (no cDNA template). Real-time data acquisition was performed by the Stratagene MX3005P system with MxPro QPCR software (Agilent Technologies, Santa Clara, CA, USA), which provided the values for cycle threshold (Ct) and fluorescence. Amplification efficiency (E) was evaluated using Miner 2.2 software [32]. Statistical analysis was made using the SASM-Agri software [33] which tested the experiments as a completely randomized design. *t* test and F test (ANOVA) were applied with a critical value of 0.01. The Scott–Knott ($p \leq 0.01$) test was employed for mean separation when *F* values were significant.

3. Results

3.1. Gene Expression during Fruit Maturation

Generally, the gene expression in young fruits in both the pulp and seeds (Figures 2 and 3) did not present significant differences between genotypes, except for TgUBA1 and TgABC1, which showed a higher expression in the 1074 genotype pulp (Figure 2), and TgUBA1, which showed a higher expression in the 174 genotype seeds (Figure 3). During the maturing fruit stage, most of the genes presented a higher expression in the 174 genotype (in both pulp and seeds; Figures 2 and 3, respectively); the few exceptions were: (i) TgCZF1, which was not differentially expressed in maturing fruit regardless of the genotype or tissue (Figures 2 and 3), and (ii) TgPRF1 and TgSCA51, which were not differentially expressed in maturing seed samples (Figure 3). In mature fruits, differential expression between genotypes was also observed, but this was not the case for all genes and was mainly found in the pulp (six genes in the pulp, two genes in seeds; Figures 2 and 3, respectively). Except for TgPRF1 (all maturation stages in both pulp and seeds; Figures 2 and 3) and TgWRK33 (pulp of mature fruits; Figure 2), all of the genes were upregulated (Figures S1 and S2). TgPRF1 was downregulated in both genotypes, but at a higher level in the 1074 pulp (Figure S1).

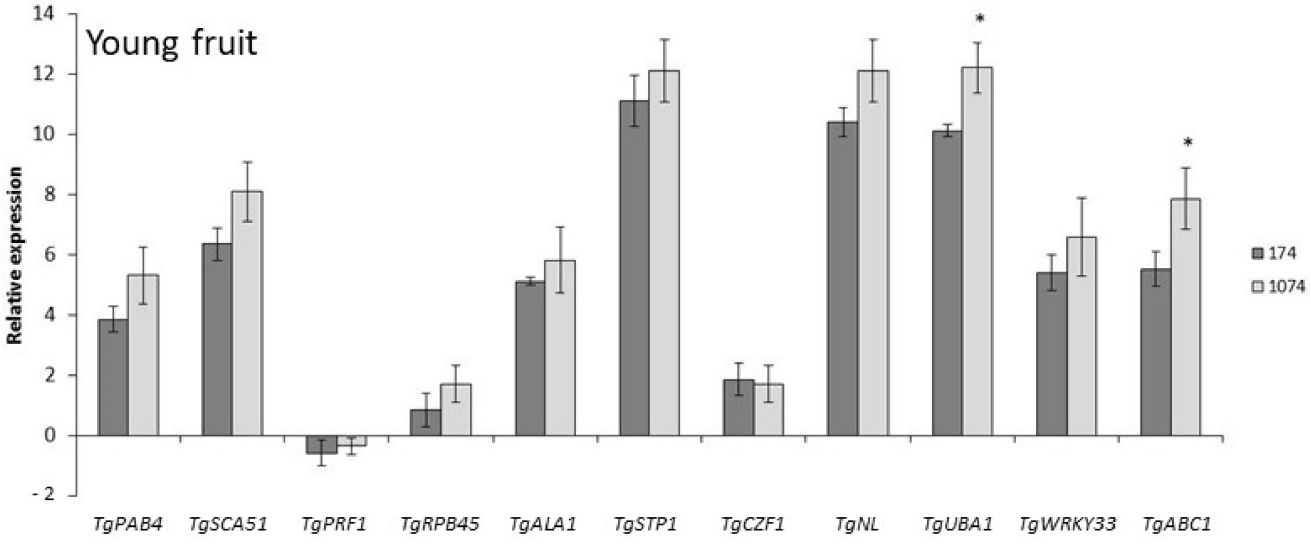

Figure 2. *Cont.*

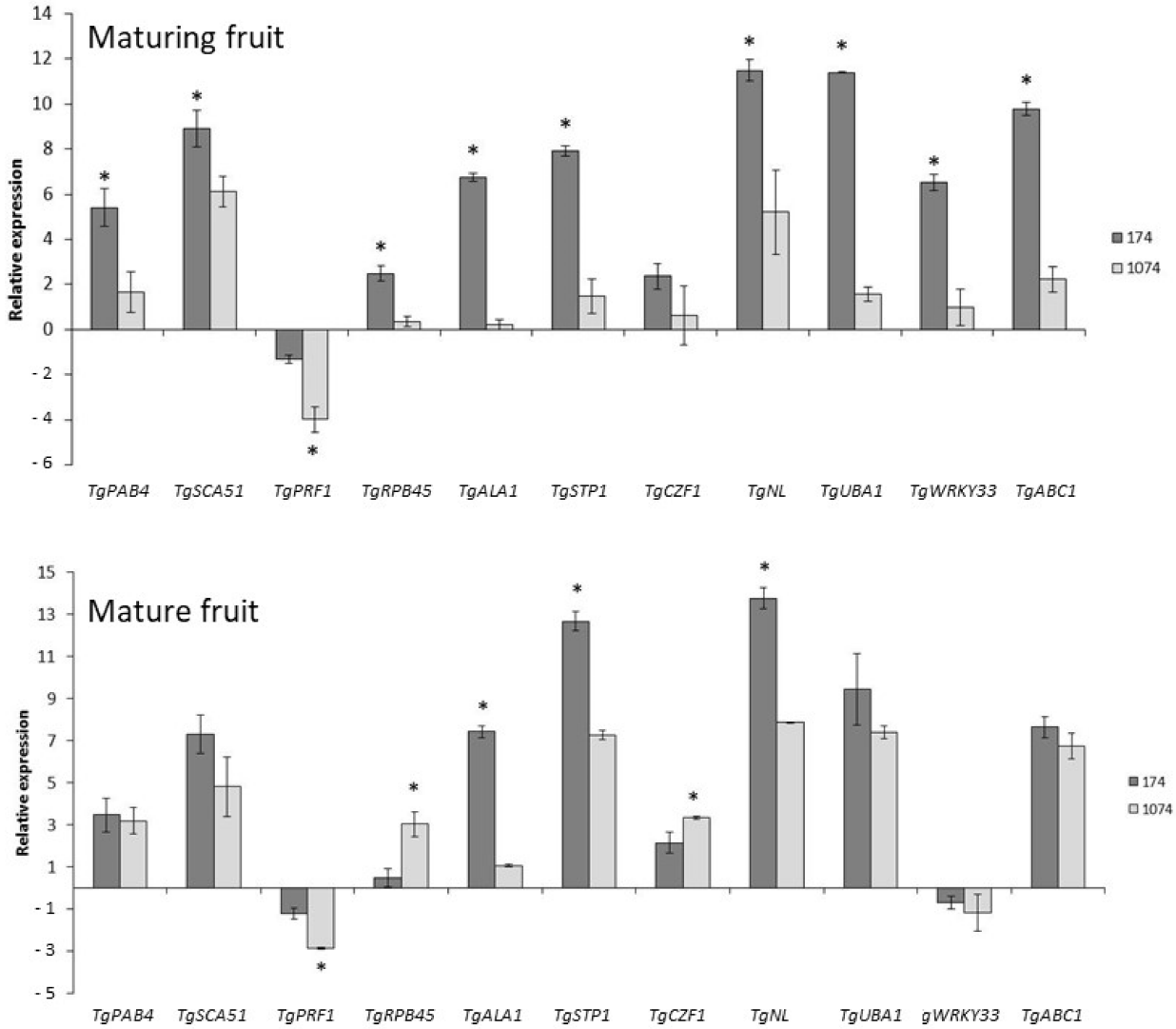

Figure 2. Relative expression of selected genes in pulp samples from young, maturing, and mature fruits of contrasting cupuassu genotypes. (*) indicates significant differences between 174 and 1074 genotype samples by *t* test ($p \leq 0.01$).

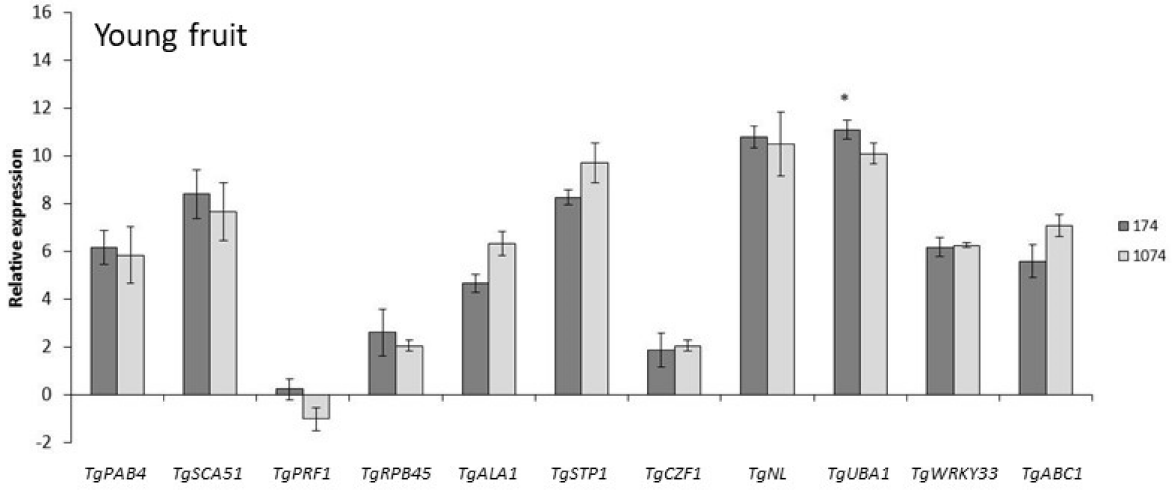

Figure 3. *Cont.*

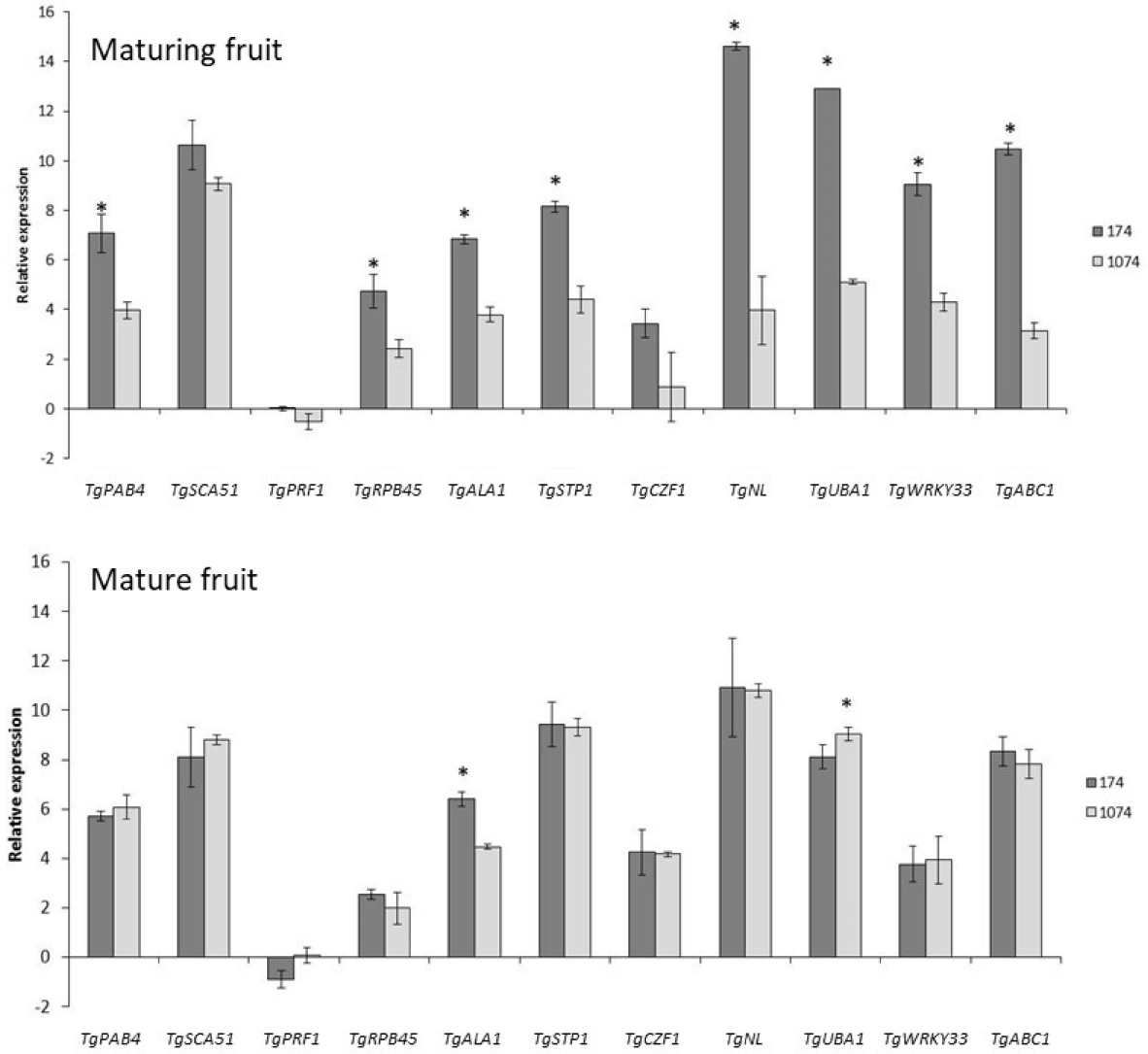

Figure 3. Relative expression of selected genes in seed samples from young, maturing, and mature fruits of contrasting cupuassu genotypes. (*) indicates significant differences between 174 and 1074 genotype samples by *t* test ($p \leq 0.01$).

3.2. Interactomics and Ontology

The ontologies of the TgWRKY33 and TgCZF1 homologues showed that these proteins may be involved in several biological processes, such as biotic and abiotic stresses, as well as in the regulation of transcription (Table S4). Based on their correlated functions and coexpression connections, these proteins were grouped into Cluster A, which contained seven other proteins (TET8, SZF1, CNI1, STZ, RHL41, RDUF1, and RDUF2; Figure 4). In this cluster, the edges between the proteins mainly corresponded to coexpression and experimental relationships (Figure 4). The ontology found for the TgRP45B homologue was related to mRNA metabolism, such as mRNA binding, poly A tail binding, and ozone response (Table S4); this gene was found in Cluster B and had a direct connection with AT4G36010, which was linked to Cluster A (Figure 4). Cluster B contained eight other proteins that were linked mainly by coexpression edges: AT4G36010, AT1G77700, AT5G02140, AT1G73620, AT5G40020, AT4G36000, AT2G26810, and TLP-3 (Figure 4). The TgRP45B homologue is also directly connected to AT1G19320 from Cluster C. This cluster contained 12 proteins, in which the TgSTP1 homologue had a global ontology related to the transmembrane transport of monosaccharides and carbohydrates (Table S4). The Cluster C proteins were connected with each other mainly by coexpression or text-mining

edges (Figure 4). Proteins from Cluster A in particular were connected to proteins from Cluster D: RDUF1 and RDUF2 were directly linked to UBA1, AT1G53930, and AT2G20050 by experimental edges (Figure 4). Cluster D also contained the TgALA1 and TgPAB2 homologues involved in the metabolism and translocation of phospholipids and mRNA regulation and translation initiation, respectively (Table S4). The TgUBA1 homologue was also directly linked (experimental edge) to AT3G24530 from Cluster H, which contained the TgPRF1 homologue and showed ontologies related to the development of leaves, lateral roots, and inflorescences (Table S4). Clusters E, F, and G are independent of each other and of the previously described clusters; the edges in these three clusters corresponded mainly to coexpression or text-mining relationships. Cluster E contained the TgCZF1 homologue (AT5G500010) as well as 10 other proteins and was related to responses to biotic (response to chitin and fungi) and abiotic (cold and hypoxia) stresses (Figure 4 and Table S4). In Cluster F (Figure 4), the TgABCC1 homologue was grouped with 10 other proteins with ontologies related to the transmembrane transport of abscisic acid (Table S4). The TgNL homologue (AT4G26093) was grouped with 10 other proteins in Cluster G, which was related to the defence response to pathogens and responses to biotic and abiotic stresses (Figure 4, Table S4).

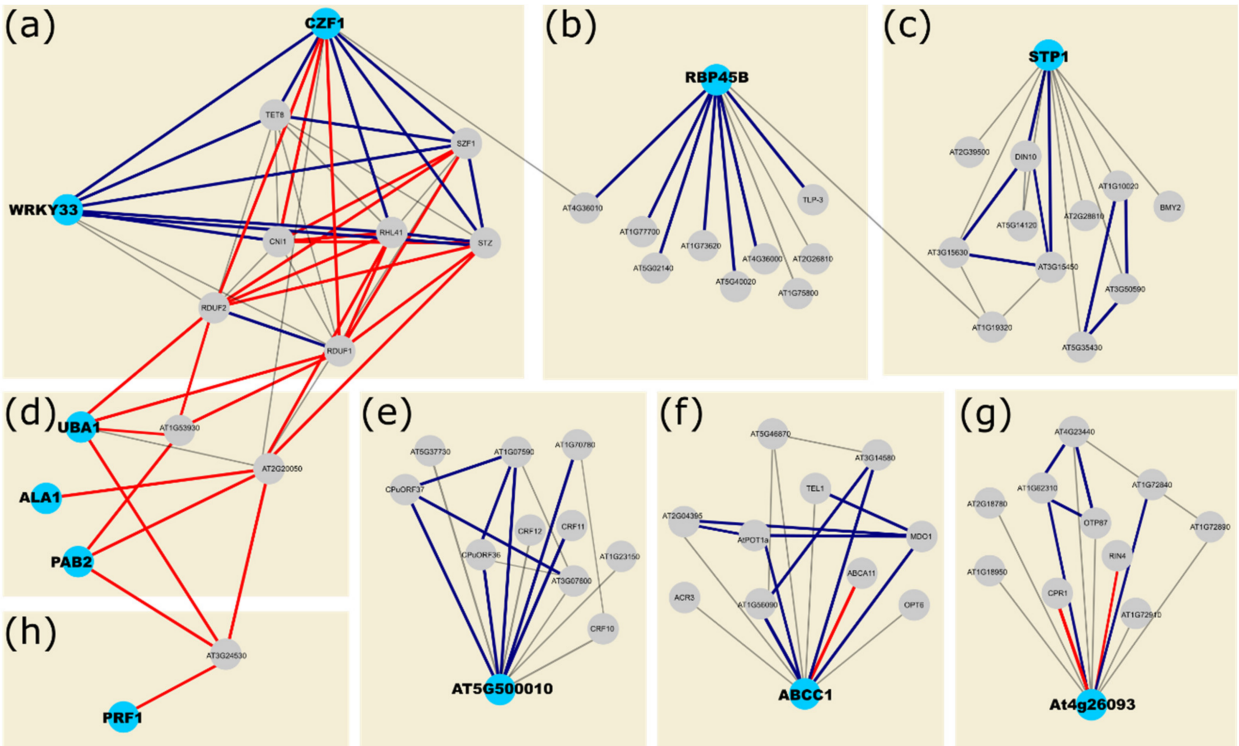

Figure 4. Protein-protein interaction (PPI) network of *Arabidopsis thaliana* protein orthologues with sequences expressed in cupuassu pulp and seeds (Table 2). (**a**) Functional cluster corresponding to biotic and abiotic stresses and transcriptional regulation. (**b**) Functional cluster corresponding to mRNA metabolism. (**c**) Functional cluster corresponding to the transmembrane transport of monosaccharides and carbohydrates. (**d**) Functional cluster corresponding to metabolism and translocation of phospholipids. (**e**) Functional cluster corresponding to responses to biotic (response to chitin and fungi) and abiotic (cold and hypoxia) stresses. (**f**) Functional cluster corresponding to the transmembrane transport of abscisic acid. (**g**) Functional cluster corresponding to defence response to pathogens and responses to biotic and abiotic stresses. (**h**) Development of leaves, inflorescences, and lateral roots. Blue nodes correspond to initial sequences (used in expression analysis), and grey nodes were aggregated by the String program to build the network. Thick red, thick dark blue, and thin grey edges correspond to experimental, coexpression, and text-mining relationships between nodes, respectively.

4. Discussion

4.1. Cupuassu Genotypes Showed Different Pulp Characteristics and Gene Expression Patterns

Even though the 174 and 1074 cupuassu genotypes showed similar external fruit appearance through fruit maturation (Figure 1), the physicochemical characteristics analysis showed that genotype 174 presented a sweeter pulp (SST/ATT of 8.7) than genotype 1074 (SST/ATT of 5.9) (Table 1). Sweet or less acidic pulp can prevent the addition of too much sugar during the fabrication of bonbons or jams, and consequently generates products considered healthy by consumers. Interestingly, the expression of the studied genes (Table 2), which were previously selected by the molecular and genetic analysis of several cupuassu genotypes [10], also strongly differed between the two genotypes: almost all of the genes from the 174 genotype showed a higher expression than those in the 1074 genotype, mainly in the maturing fruit (Figure 2). This pattern was also observed in the seeds (Figure 3). Specifically, the expression of *TgSTP1*, a gene related to sugar transport, was higher in the 174 maturing and mature pulp and in maturing seeds than in the tissues from the 1074 genotype (Figures 2 and 3). Six of the analysed genes (*TgPAB4*, *TgPRF1*, *TgRBP45B*, *TgSTP1*, *TgCZF1*, and *TgUBA1*) had orthologues in *T. cacao* that were expressed in seed, testa, and/or pulp EST libraries [21] (Table S5), indicating that these sequences may have a similar function and action in both related species. The TgPAB4, TgPRF, and TgCZF1 *T. cacao* orthologues were expressed in both seed and pulp EST libraries (Table S5), corroborating our results of similar expression patterns in cupuassu tissues (Figures 2 and 3). The similar expression patterns in the pulp and seeds associated with the previous identification of polymorphic markers for these genes [10] may direct selection for both fruit parts, especially since they are both commercially and economically valuable [34].

4.2. Monosaccharide and Carbohydrate Transport Pathways Were Enhanced in Cupuassu Fruit during Maturation

Cluster C corresponded to proteins related to monosaccharides and carbohydrate transport and contained the TgSTP1 homologue and other closely linked genes, such as DIN10, BMY2, and AT3G50590 (Figure 4). In Arabidopsis, STP1 belongs to a highly conserved gene family that mediates hexose transport and is expressed in specific tissues or developmental stages [35]. The transcriptional expression of STP1, as well as DIN10, is known to be regulated by sugar; particularly, *AtSTP1* was strongly downregulated within minutes after the level of sugar increased [30]. In *T. grandiflorum* pulp, STP1 showed high expression in the maturing and mature stages, particularly in the 174 genotype, in which the mature stage was the sweetest [30] (Figure 2, Table 1). In Arabidopsis suspension cells, dark-inducible (DIN) gene expression was modulated under sugar starvation [36]. Sugar starvation involves phosphorylation and dephosphorylation events, and the inhibition of type 1 and 2A protein phosphatases decreased DIN10 expression in sugar-depleted cells [36]. AT3G50590 belongs to the WD40 repeat-like superfamily protein; the WD motif was found in rice and Arabidopsis glucose-inducible genes [37]. In another study, the WD40 protein AtGHS40 from Arabidopsis played a role in ABA-mediated glucose signalling during early seedling development [38]. The WD40 protein is known to be part of the MBW (R2R3-MYB, bHLH, and WD40) ternary protein complex, whose gene expression is stimulated by sucrose and methyl jasmonate in *Brassica juncea* [39]. BMY2 (beta-amylase), which is involved in the hydrolysis of (1,4)-alpha-D-glucosidic linkages in polysaccharides, was also found in Cluster C. It is important to highlight that in plants, sugars not only serve as energy and metabolic resources, but also modulate several fundamental processes during the plant life cycle. Sugars may have hormone-like activity [37,40] and may participate in the plant defence response against pathogens [41–43]. Thus, *TgSTP1*, which was more highly expressed in the cupuassu-resistant genotype (Figures 2 and 3), appeared to be a good candidate due to its possible role in both fruit maturation and defence mechanisms against microorganisms.

4.3. In Maturing Cupuassu Fruits, Sugar Accumulation Participated in Signal Transduction Associated with Fruit Development and Stress Response

Cluster A corresponded to proteins related to biotic and abiotic stresses and transcriptional regulation, and was related to Cluster C through Cluster B, which was associated with mRNA metabolism (Figure 4a–c). The main proteins from Cluster A were TgCZF1, TgWRKY33, ubiquitins (RDUF1, RDUF2, CNI1/ATL3), SZF1, STZ, and TET8. Most of them are involved in the response to osmotic stress, salt stress, high light, heat, and/or metabolic sensing, including carbon/nitrogen (C/N) balance changes [44–46]. Crosstalk, modulation, and integration between signalling pathways responding to sugars, phytohormones, light, and biotic and abiotic stress-related stimuli have been shown [40,47]. Protein complexes containing glucose-modulated master regulators play integrative and complementary roles in cellular signalling and metabolism, and are critical parts of plant signalling networks [40]. For example, CNI1/ATL3 activity was required for the plant C/N response during the seedling growth transition in Arabidopsis, and CNI1/ATL3 expression was also strongly reduced by hyperosmotic stress [46,48]. The expression of such genes depended on stress-responsive transcription factors, such as TgWRKY33, TgCZF1, and SFZ1 [45,49,50]; in maturing pulp and seed, and in mature pulp, TgWRKY33 and TgCZF1 were highly expressed, respectively, in the resistant genotype 174 (Figures 2 and 3). In higher plants, fruits are considered 'sinks', i.e., organs that consume or accumulate assimilates (e.g., simple sugars) that come from 'source' organs (i.e., leaves that export those assimilates) [51]. Communication between 'sources' and 'sinks' during plant development plays a pivotal role in controlling crop yield and affecting fruit quality [51]. Moreover, it is possible that sugar accumulation in plant cells leads to local osmotic stress, thus resulting in specific target gene regulation [40].

4.4. Maturing Cupuassu Pulp and Seeds Showed Increased Phospholipid Metabolism and Translocation, as Well as Immune System Activation

The TET8 protein found in Cluster A and indirectly related to Cluster D, which corresponded to proteins related to the metabolism and translocation of phospholipids (Figure 4c,d), was involved in the resistance of Arabidopsis to the necrotrophic pathogen *Botrytis cinerea* [52,53]. The TET8 protein participated in the plant immune response through plant extracellular vehicle (EV) formation. These EVs contain small RNAs that contribute to plant resistance [52]. Cluster D contained other proteins related to lipid transport and metabolism (Figure 4), such as ALA1 (lipid flippase), which mediates antiviral defence in Arabidopsis by enhancing the amplification of viral siRNAs [54,55]. *TgALA1* showed increased expression in the mature and maturing pulp and seeds of the resistant cupuassu genotype 174 (Figures 2 and 3). Cluster D also contained the TgUBA1 and TgPAB4 orthologous proteins (Figure 4d). UBA1 participates in the ubiquitination pathway, which is essential for the activation of some R-protein-mediated resistance responses and for basal defence in Arabidopsis [56], while PAB2 from Arabidopsis is involved in the regulation of mRNA translation and stability, as well as in imprinting during flowering [57]. Both *TgUBA1* and *TgPAB4* were highly expressed in the maturing fruit from the resistant genotype 174 (Figures 2 and 3). Not only Clusters A, B, and D (see above), but also Clusters E, F, and G contained proteins related to biotic and abiotic stress responses, even if they were not all interconnected (Figure 4). RBP45B (Cluster B, Figure 4b) was involved in mRNA processing, and in *Nicotiana tabacum*, it was related to the response to *tobacco mosaic virus* infection [58,59]. At4g26093/TgNL belonged to the NL (NBS + LRR) class of recognition pattern proteins (PRRs) that are directly involved in plant resistance mechanisms (Cluster G) [60,61], and the ontology indicated that it played a role in the response to bacteria in Arabidopsis [62]. ABBC1/TgABC1 was induced by ABA and related to the plant response to drought; its function involved flavonoid glucoside transport [63]. Most of these genes showed higher expression in maturing fruit from the resistant genotype (Figures 2 and 3).

5. Conclusions

The expression analysis and associated systems biology approach allowed us to identify some interesting genes from cupuassu fruit that were involved in signalling pathways at the intersection of carbohydrate accumulation, plant development, and the response to biotic and abiotic stress, which are all important areas for cupuassu breeding programmes. The *TgSTP1*, *TgWRKY33*, *TgCZF1*, and *TgUBA1* genes in cupuassu and the orthologues of DIN10, CNI1, and TET8 may be good candidates for marker-assisted selection in breeding programmes focusing on both fruit quality and resistance/tolerance to biotic/abiotic stress.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12040763/s1: Table S1. Sequences used in this study with putative functions. Table S2. Primers used in this study. F: forward. R: reverse. Table S3. Amplicon characteristics. Figure S1. Relative expression of selected cupuassu genes in pulp samples from the 174 and 1074 genotypes through fruit maturation. Lower- and upper-case letters indicate statistical values for the 174 and 1074 genotype samples, respectively. Different letters indicate significant differences between samples by the Scott–Knott test ($p \leq 0.01$). (*) indicates samples with only two repetitions. Figure S2. Relative expression of selected cupuassu genes in seed samples from the 174 and 1074 genotypes through fruit maturation. Lower- and upper-case letters indicate statistical values for the 174 and 1074 genotype samples, respectively. Different letters indicate significant differences between samples by the Scott–Knott test ($p \leq 0.01$). (*) indicates samples with only two repetitions. Table S4. Gene Ontology (GO) of the cupuassu sequences used in this study. Table S5. ESTs expressed in the seed, testa, and/or pulp of *T. cacao* showing orthology with the *T. grandiflorum* sequences used in this study.

Author Contributions: Conceptualization, L.H.M. and F.M.; formal analysis, L.F.d.S. and R.J.S.S.; investigation, L.F.d.S., R.J.S.S. and F.M.; resources, R.M.A., L.L.F. and L.H.M.; writing—original draft preparation, L.F.d.S., R.J.S.S. and F.M.; supervision, F.M.; project administration, F.M.; and funding acquisition, F.M. All authors have read and agreed to the published version of the manuscript.

Funding: LFS was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES; Project CAPES-EMBRAPA "Theobroma"); RJSS was funded by the Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB, project number DTE0038/2015). This research was funded by CAPES-EMBRAPA "Theobroma" and FAPESB projects, both coordinated by FM.

Data Availability Statement: The data presented in this study are available in the present article and associated Supplementary Materials.

Acknowledgments: This work was developed within the framework of the International Consortium in Advanced Biology (CIBA).

Conflicts of Interest: The authors declare no conflict of interest.

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