MuMADS1 and MaOFP1 regulate fruit quality in a tomato ovate mutant

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Introduction

MADS-box genes play important roles in nearly every aspect of plant growth and development (Benlloch et al., 2009; Ferrario et al., 2006; Kaufmann et al., 2009; Liu et al., 2013). MADS-box transcription factors (TFs) are considered to be the most powerful TFs identified for regulating fruit development and ripening (Karlova et al., 2014). Most investigations regarding the function of MADS-box TFs in fruit development and ripening have focused on the model plant tomato (Solanum lycopersicum), while little information is available about other plants, particularly the important tropical fruit banana.

MADS-box transcription factors act via a complex network of protein–DNA and homo- or heterodimeric protein–protein interactions (Tonaco et al., 2006). MADS-box TFs bind other proteins through the K-domain (Kaufmann et al., 2005; Liu et al., 2015a,b; Smaczniak et al., 2012a). These TFs often bind as multimers. Subsequently, the two dimers interact to form a tetramer and loop the DNA that lies between the two CArG boxes (Melzer et al., 2009; Smaczniak et al., 2012b). These complexes contain not only MADS domain proteins, but also other non-MADS-box proteins such as SEUSS, histone fold protein NF-YB and ubiquitin-activating (UBA) enzyme E1 protein MuUBA (Liu et al., 2013; Masiero et al., 2002; Sridhar et al., 2006).

The OvATE gene was first cloned by positional cloning in tomato and was demonstrated to encode a hydrophilic protein with a putative bipartite nuclear localization signal and a C-terminal domain of approximately 70 amino acids, which was designated as the OVATE domain, indicating that the gene belongs to the ovate family of proteins (OPF) (Liu et al., 2002; Wang et al., 2011; Yu et al., 2015). As a member of a plant-specific transcription factor family, the OvATE protein was first identified in tomato as the prime controller of fruit appearance (Ku et al., 1999; Liu et al., 2002; Monforte et al., 2014; Tsaballa et al., 2011; Wang et al., 2015; Wu et al., 2015). The impacts of the ovate mutation on fruit appearance differ from elongation to creating pear- or globe-shaped fruits according to the genetic context with the ovate modification (Gonzalo and van der Knaap, 2008). This indicates that OVATE cannot be held accountable for the noted phenotype and likely is in contact with various genes in an epistatic way (Azzi et al., 2015). This ovate pear-formed phenotype was enhanced by a genomic DNA fragment containing the OvATE gene as well as its ectopic overexpression, causing its reversion to the production of globe-shaped fruits (Liu et al., 2002). Thus, the ovate modification is probably a loss-of-function mutation from a negative controller of plant development whose purpose needs to be determined (Azzi et al., 2015). A transcriptional activity examination of Arabidopsis OPFs (AtOPFs) in protoplasts indicates that they function as transcription repressors (Wang et al., 2007, 2011). Functional categorization of OPFs from various plant species, such as Arabidopsis, rice, tomato, melon and pepper, implies that OPFs control numerous portions of plant initiation and maturation, including ovule progression (Pagnussat et al., 2007), vascular progression (Schmitz et al., 2015) and secondary cell wall development (Li et al., 2011), which is probably made via the connection with various kinds of TFs, such as KNOX and BELL classes, and/or via immediate control...
of the expression of target genes, namely gibberellin-20-oxidase (GA20ox) and GSK3-like kinase (Hackbusch et al., 2005; Wang et al., 2016; Yang et al., 2016).

We previously confirmed a novel role for an OFP in regulating banana fruit ripening. The banana OFP1 (MaOFP1) interacted with a banana MADS-box protein MuMADS1 to take on antagonistic parts in ethylene-prompted postharvest maturation in banana (Liu et al., 2015b). However, the banana fruit ripening process also constitutes a process for banana fruit quality formation. Whether MuMADS1 and MaOFP1 possess novel roles in fruit quality formation remains unclear. Herein, MuMADS1 and MaOFP1 were transformed into a tomato ovate mutant, and the phenotype and physiology of the transformants were analysed. The function of MuMADS1 and MaOFP1 in regulating fruit quality was further investigated by transcriptional analysis.

Results

Identification of transgenic plants

To additionally examine the parts played by MuMADS1 and MaOFP1, 35S: MuMADS1 (pCAMBIA1302–MuMADS1), 3SS: MaOFP1 (pCAMBIA1302–MaOFP1) and 35S: MuMADS1+35S: MaOFP1 were transformed into tomato ovate mutants. More than three independent transgenic lines confirmed for transgene integration were recovered, and two independent lines for each transformant were chosen for their transgene homozygosity in the T1 generation from the DNA gel blot evaluation and were expressed at higher levels in MO transgenic lines than in the WT mutant. The number of DEGs was 1395 in WT vs. ovate, while 842 (502 up and 340 down) and 904 (58 up and 404 down), respectively (Figure 2b and c). The number of DEGs was only 286 in WT vs. MuMADS1- or MaOFP1-transformed fruits and 212 (135 up and 77 down) in WT vs. MuMADS1+MaOFP1-transformed fruits, with 209 up-regulated and 77 down-regulated genes (Figure 2d). Conversely, the DEGs in the ovate vs. MuMADS1-transformed fruits and MaOFP1-transformed fruits were 84 (58 up and 26 down) and 342 (135 up and 207 down), respectively (Figure 2e and f), and 530 (287 up and 243 down) in the ovate vs. MuMADS1 + MaOFP1-transformed fruits (Figure 2g). These results suggest that the largest number of DEGs existed in WT vs. ovate. With the transformation of MuMADS1 or MaOFP1 into ovate, the DEGs in the WT vs. transformed fruits decreased. When MuMADS1 and MaOFP1 of the expression of target genes, namely gibberellin-20-oxidase (GA20ox) and GSK3-like kinase (Hackbusch et al., 2005; Wang et al., 2016; Yang et al., 2016).

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were co-expressed in ovate, the DEGs were reduced to their lowest number. Alternatively, with the transformation of MuMADS1 or MaOFP1 into ovate, the DEGs in the ovate vs. transformed fruits increased. When MuMADS1 and MaOFP1 were co-expressed in ovate, the DEGs in the ovate vs. transformed fruits increased the most. These results suggest that the expression of MuMADS1 and MaOFP1 in ovate reduces the differences in expressed genes in WT vs. transformants and increases the differences in expressed genes in ovate vs. transformants. Briefly, MuMADS1 and MaOFP1 can regulate and recover the DEGs in ovate.

**Gene annotation and function classification**

A total of 4253 DEGs were obtained from seven assemblies, and 437 of the genes were novel. The nonredundant (Nr), Swiss-Prot, Gene Ontology (GO), Clusters of Orthologous Groups (COG), Eukaryotic Orthologous Groups (KOG), evolutionary genealogy of genes: Nonsupervised Orthologous Groups (eggNOG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were introduced to annotate the DEGs. Of these, 4252 (99.98%) were matched to the Nr database, while the smallest number of DEGs was found in the KEGG database (Table S3). To further appraise the completeness of the RNA-Seq data, COG classifications were performed with the DEGs. In the assembly of the WT vs. ovate mutant, a total of 557 DEGs were annotated in 25 COG categories (Figure 3a). The category of ‘general function prediction only’ (150, 18.43%) ranked highest, followed by ‘replication, recombination and repair’ (79, 9.71%) and ‘transcription’ (73, 8.97%). The ‘signal transduction mechanism’ (72, 8.85%) and ‘carbohydrate transport and metabolism’ (64, 7.86%) categories ranked fourth and fifth, respectively. With the transformation of MuMADS1 or MaOFP1 into the ovate mutant, the DEGs in WT vs.
transformed fruits decreased. For example, in the assemblies of WT vs. MuMADS1 transformants and WT vs. MaOFP1 transformants, the category of ‘carbohydrate transport and metabolism’ decreased to 44 (8.73%) and 45 (8.46%), respectively (Figure 3b and c), while in the assembly of WT vs. MuMADS1 + MaOFP1 transformants, it decreased to 10 (4.74%); Figure 3d). Conversely, and c), while in the assembly of WT vs. MuMADS1 + MaOFP1 transformants, the number of DEGs in ovate vs. the transformed fruit gradually increased. For example, in the assemblies of ovate vs. MuMADS1 transformants, ovate vs. MaOFP1 transformants and ovate vs. MuMADS1 + MaOFP1 transformants, the category of ‘carbohydrate transport and metabolism’ gradually increased to five (14.29%), 16 (8.65%) and 21 (6.89%), respectively (Figure 3e, f and g).

Unigenes that were successfully annotated to the GO database were classified into three principal categories for the seven assemblies, including ‘cellular component’, ‘molecular function’ and ‘biological process’, which were further subdivided into 53 categories (Table S4; Figure S2). Of these, the categories that were most represented in the ‘biological process’ principal category included ‘immune system process’ [WT vs. ovate: 10 (0.40%); WT vs. MuMADS1 transformants: eight (0.32%); WT vs. MaOFP1 transformants: eight (0.32%); WT vs. MuMADS1 + MaOFP1 transformants: six (0.24%); ovate vs. MuMADS1 transformants: 0; ovate vs. MaOFP1 transformants: 0; ovate vs. MuMADS1 + MaOFP1 transformants: six (0.24%)], followed by ‘signalling’ [WT vs. ovate: 13 (0.30%); WT vs. MuMADS1 transformants: 10 (0.23%); WT vs. MaOFP1 transformants: 11 (0.26%); WT vs. MuMADS1 + MaOFP1 transformants: 10 (0.23%); ovate vs. MuMADS1 transformants: 0; ovate vs. MaOFP1 transformants: one (0.02%); ovate vs. MuMADS1 + MaOFP1 transformants: six (0.14%)]. Within the ‘cellular component’ principal category, unigenes [WT vs. ovate: one (2.13%); WT vs. MuMADS1 transformants: 0; WT vs. MaOFP1 transformants: one (2.13%); WT vs. MuMADS1 + MaOFP1 transformants: 0; ovate vs. MuMADS1 transformants: 0; ovate vs. MaOFP1 transformants: one (2.13%); ovate vs. MuMADS1 + MaOFP1 transformants: one (2.13%); ovate vs. MuMADS1 + MaOFP1 transformants: one (2.13%); ovate vs. MaOFP1 transformants: one (2.13%)].

Pathway enrichment analysis of DEGs

To identify the active pathways in the transformants, the obtained unigenes were mapped to the canonical reference pathways in the KEGG database. KEGG pathway enrichment examination was performed to classify the biological tasks of the DEGs. Specific enrichment of genes was obtained for 98, 85, 88, 47, 22, 61 and 74 pathways for WT vs. ovate, WT vs. MuMADS1 transformants, WT vs. MaOFP1 transformants, WT vs. MuMADS1 + MaOFP1 transformants, ovate vs. MuMADS1 transformants, ovate vs. MaOFP1 transformants and ovate vs. MuMADS1 + MaOFP1 transformants, respectively (Table S5). The top 50 enriched pathways are displayed in Figure 4. ‘Starch and sucrose metabolism’ was the most frequently noted and had 28 (11.07%) DEGs in the WT vs. ovate, 23 (9.09%) DEGs in WT vs. MuMADS1 transformants, 16 (6.32%) DEGs in WT vs. MaOFP1 transformants, five (1.98%) DEGs in WT vs. MuMADS1 + MaOFP1 transformants, one (0.40%) DEG in ovate vs. MuMADS1 transformants, three (1.19%) DEGs in ovate vs. MaOFP1 transformants and four (1.58%) DEGs in ovate vs. MuMADS1 + MaOFP1 transformants, respectively. In the assembly of WT vs. ovate, the second largest term was ‘carbon metabolism’, which included 25 (8.68%) DEGs. In the assemblies of WT vs. MuMADS1 transformants and WT vs. MuMADS1 + MaOFP1 transformants.
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MuMADS1 + MaOFP1 transformants, the ‘carbon metabolism’ term changed from 14 (4.86) and three (1.04%) DEGs, respectively. This result indicated that ‘starch and sucrose metabolism’ and ‘carbon metabolism’ constituted the primary metabolic pathways that they changed with the integration of exogenous genes and that they might be more active during tomato fruit quality formation.

**Gene expression**

Setting a $P < 0.01$ and $\log_2$ (fold change) $\geq 1$, the DEGs of the assemblies involved in sugar and cell wall metabolism were identified as being up-regulated or down-regulated (Table S6). In the WT vs. ovate assembly, 73 genes were up-regulated and 13 genes were down-regulated. When the ovate mutant was transformed, the DEGs gradually decreased. There were 28, 39 and 15 genes that were up-regulated, and 26, 6, and 2 genes were down-regulated in the assemblies of WT vs. MuMADS1 transformants, WT vs. MaOFP1 transformants and WT vs. MuMADS1 + MaOFP1 transformants, respectively. Furthermore, the DEGs gradually increased in the assemblies of ovate vs. MuMADS1-transformants, ovate vs. MaOFP1 transformants and ovate vs. MuMADS1 + MaOFP1 transformants with one, five and eight up-regulated, and two, 12 and 15 down-regulated genes, respectively (Table S6, Figure S).

Specifically, of the 86 DEGs, 45 involved in sugar metabolism, including **cellulose synthase A catalytic subunit 5 (Soly9g005560.1)** and **galacturonosyltransferase 8-like (Soly9g013560.1)**, were differentially expressed in the WT vs. ovate assembly, which suggested that these genes might be the putative targets of OVATE in the regulation of fruit firmness (Tables S6, S7).

Considering the compensation of MuMADS1, MaOFP1 and MuMADS1 + MaOFP1 in the ovate mutant, the putative target genes regulated by MuMADS1, MaOFP1 and MuMADS1 + MaOFP1 should constitute DEGs that do not overlap between the WT vs. ovate and the WT vs. MuMADS1 transformants, WT vs. ovate and the WT vs. MaOFP1 transformants, and the WT vs. ovate and the WT vs. MuMADS1 + MaOFP1 transformants. Therefore, the DEGs regulated by MuMADS1, MaOFP1 and MuMADS1 + MaOFP1 included 44 DEGs (25 DEGs involved in carbohydrate metabolism and 19 involved in cell wall metabolism), 60 DEGs (29 DEGs involved in carbohydrate metabolism and 31 involved in cell wall metabolism) and 83 DEGs (44 DEGs involved in carbohydrate metabolism and 39 involved in cell wall metabolism), respectively. As shown in Figure 6 and Table S7, the coregulated DEGs regulated by OVATE, MuMADS1, MaOFP1 and MuMADS1 + MaOFP1 included 30 DEGs, with 14 DEGs involved in sugar metabolism and 16 involved in cell wall metabolism. The specific DEGs regulated by OVATE, MuMADS1, MaOFP1 and MuMADS1 + MaOFP1 were 56, 14, 30 and 53, respectively. Moreover, the numbers of coregulated DEGs between the OVATE and MuMADS1 + MaOFP1, MuMADS1 and OVATE, MaOFP1 and OVATE, MuMADS1 and MuMADS1 + MaOFP1, MaOFP1 and MuMADS1 + MaOFP1, MuMADS1 and MaOFP1 were 72, 44, 51, 42, 47 and 35, respectively (Figure 6; Table S7).
Verification of gene expression by qRT-PCR

To confirm the RNA-Seq results, five DEGs involved in sucrose and cell wall metabolism were selected for qRT-PCR analysis, including plastidic hexokinase (Solyc04g081400.2), putative beta-glucosidase 41 (Solyc07g063880.2), UDP-glucose 6-dehydrogenase 1 (Solyc02g067080.2), UDP-glucuronate 4-epimerase 1-like (Solyc12g010540.1) and galacturonosyltransferase (Solyc06g083310.2). The expression levels of the selected DEGs revealed by qRT-PCR were generally consistent with those from the RNA-Seq analysis at the three developmental stages, which indicated that the results of the RNA-Seq analysis showed a high degree of correlation with those of the qRT-PCR (Figure 7).

Discussion

Fruit shape and quality are common botanical phenomena in nature. Banana and tomato are typical climacteric fruits. To some extent, they enjoy a common quality formation mechanism whereby the peculiar fruit quality properties can be strongly modified by TFs. The quality of tomato fruit is defined by a set of properties, including fruit appearance, flavour and texture. Flavour is defined as the combination of taste and odour. An intense taste is the result of an increase in gluconeogenesis, hydrolysis of polysaccharides, a decrease in acidity and accumulation of sugars (Prasanna et al., 2007), while textural characteristics are primarily controlled by the cell wall structure in addition to cuticle properties, cellular turgor and fruit morphology (Vicente et al., 2007). In recent years, the quality of tomato fruit has been investigated at both the genetic and biochemical levels to obtain new varieties with improved taste (Carli et al., 2011; Causse et al., 2003; D’Esposito et al., 2017).

OVATE was the first fruit shape gene identified by positional cloning (Ku et al., 1999; Liu et al., 2002). Changes in the fruit shape of the ovate tomato mutant have been frequently studied (Monforte et al., 2014; Tsaballa et al., 2011; Wang et al., 2015; Wu et al., 2015), but little to no research has focused on fruit quality. To date, this constitutes the first report on the role of
OVATE in regulating fruit firmness and sugar accumulation. We transformed MuMADS1 and MaOFP1 into tomato ovate plants, and the formation of transgenic lines was confirmed by Southern blotting and mRNA transcription-level measurements (Figure S1). The fruit phenotype and quality detection results indicated that the co-expression of MuMADS1 and MaOFP1 could regulate fruit shape and quality, which was consistent with the reports that the OVATE protein and MADS-box protein regulate fruit shape and quality (Ireland et al., 2013; Karlova et al., 2014; Wang et al., 2015). However, the DEGs involved in regulating fruit shape were not enriched in the present study. This may be attributed to the fact that fruit shape patterning by MuMADS1 or MaOFP1 is established well before anthesis, and the DEGs regulated by MuMADS1 or MaOFP1 could not be detected at the RM stage. This is consistent with the reports of van der Knaap and Tanksley (2001), which showed that the ovary is very elongated at the time of anthesis and gradually becomes less elongated during fruit development. Additionally, Liu et al. (2002) also discovered that the OVATE transcript can be detected in tomato flowers 10 days before anthesis and begins to decrease in developing fruit 8 days after anthesis.

OVATE protein and MADS-box protein regulate fruit shape and quality, which was consistent with the reports that the OVATE protein and MADS-box protein regulate fruit shape and quality (Ireland et al., 2013; Karlova et al., 2014; Wang et al., 2015). However, the DEGs involved in regulating fruit shape were not enriched in the present study. This may be attributed to the fact that fruit shape patterning by MuMADS1 or MaOFP1 is established well before anthesis, and the DEGs regulated by MuMADS1 or MaOFP1 could not be detected at the RM stage. This is consistent with the reports of van der Knaap and Tanksley (2001), which showed that the ovary is very elongated at the time of anthesis and gradually becomes less elongated during fruit development. Additionally, Liu et al. (2002) also discovered that the OVATE transcript can be detected in tomato flowers 10 days before anthesis and begins to decrease in developing fruit 8 days after anthesis.

Figure 5 Heatmap of DEGs. (a) WT vs. ovate; (b) WT vs. MuMADS1 transformants; (c) WT vs. MaOFP1 transformants; (d) WT vs. MuMADS1 + MaOFP1 transformants; (e) ovate vs. MuMADS1 transformants; (f) ovate vs. MaOFP1 transformants; (g) ovate vs. MuMADS1 + MaOFP1 transformants. T04, T05, T06, three lines of transformants; T07, T08, T09, three lines of MuMADS1 + MaOFP1 transformants.

The fruit quality of the ovate mutant was poor, with the fruit firmness and soluble solids decreased (Figure 1), which was compensated for by the transformation of MuMADS1 and MaOFP1 into the ovate mutant. This result was further demonstrated by the KEGG enrichment analysis, whereby ‘starch and sucrose metabolism’ and ‘carbon metabolism’ were the primary metabolic pathways. The changed number of DEGs in the ‘starch and sucrose metabolism’ and ‘carbon metabolism’ pathways may have resulted in the inferior quality of the ovate tomato fruit.

The results of the de novo transcriptome analysis demonstrated that MuMADS1 and MaOFP1 could compensate for the phenotype of ovate in two ways. Firstly, the total number of DEGs in the WT vs. ovate mutation ranked highest (Figure 1), which suggests that the OVATE gene regulates the expression of many genes. In the transformation of MuMADS1 and MaOFP1 into the ovate mutant, the number of DEGs gradually decreased in the assemblies of the WT vs. MuMADS1 transformants or WT vs. MaOFP1 transformants. When MuMADS1 and MaOFP1 were cotransformed into the ovate mutant, the number of DEGs dropped to their lowest value. Secondly, the gene annotation and function classification (Figure 2), ‘carbohydrate transport and
metabolism’ (64, 7.86%) in the WT vs. ovate mutant ranked highest. In the transformation of MuMADS1 and MaOFP1 to ovate mutant, ‘carbohydrate transport and metabolism’ was decreased to 44 (8.73%) and 10 (4.74%) in the assembly of WT vs. MuMADS1 transformants and WT vs. MuMADS1 + MaOFP1 transformants, respectively. We have summarized the distribution of DEGs regulated by MuMADS1, MaOFP1, MuMADS1 + MaOFP1 and OVATE based on the RNA-Seq data as shown in Figure 6 and Table S7. OVATE regulated the largest number of target genes in the control of fruit quality, followed by the co-expression of MuMADS1 and MaOFP1, which could nearly compensate for the ovate mutation. Either MuMADS1 or MaOFP1 could partially regulate the expression of genes involved in carbohydrate and cell wall metabolism to control fruit sugar accumulation and softening. MuMADS1 is not a homolog of tomato OVATE. However, MuMADS1 shared almost all target genes such as lysosomal beta-glucosidase-like and pectate lyase 8 with OVATE, MaOFP1 and MuMADS1 + MaOFP1. Similarly, MaOFP1 shared most target genes with OVATE and MuMADS1 + MaOFP1. MADS-box genes have been shown to play a role in the formation of fruits (Tadiello et al., 2009). The suppression of the homologous SEPALLATA1-like genes MADS8 and MADS9 in the fleshy fruit apple (Malus x domestica) leads to a change in ovary locule shape from a “tear drop” to a more open triangular shape by strongly reducing the cortex layer (Ireland et al., 2013). Banana MuMADS1 is an AGAMOUS MADS-box gene. Our previous study indicated that MuMADS1 is closely related to fruit ripening (Liu et al., 2009, 2015b), which suggests that MuMADS1 plays an important role in controlling fruit quality. Therefore, an explanation for the partial compensation of the ovate mutation in tomato by MuMADS1 is that MuMADS1 itself plays a role in regulating fruit shape and quality in a similar manner as OVATE, by regulating sugar and cell wall metabolism, or by partially independent mechanisms. Our previous results demonstrated that MuMADS1 could interact with MaOFP1 to regulate banana fruit ripening (Liu et al., 2015b). The DEGs regulated by MuMADS1 + MaOFP1 were far greater in type and number than those of either MuMADS1 or MaOFP1, as shown in Figure 6 and Table S7, which might be the reason that overexpression of both genes in the ovate mutant results in an additive effect.

Fruit softening is a key trait for tomato fruit, and cell wall remodelling plays a major role in the textural changes and involves the coordinated expression of a large number of genes. In tomato, >50 cell wall structure-related genes are expressed during fruit development (Tomato Genome Consortium, 2012; Minoia et al., 2016). Sucrose, a disaccharide, is an important end product of photosynthesis and is the primary carbon source for

Figure 6 Distribution of DEGs regulated by MuMADS1, MaOFP1, MuMADS1 + MaOFP1 and OVATE. (a–f), Venn diagrams showing the overlap between the OVATE and MuMADS1 + MaOFP1, MuMADS1 and OVATE, MaOFP1 and OVATE, MuMADS1 and OVATE, MuMADS1 + MaOFP1, MaOFP1 and MuMADS1 + MaOFP1, MuMADS1 and MaOFP1, respectively. The triangle indicates the 30 genes coregulated by MuMADS1, MaOFP1, MuMADS1 + MaOFP1 and OVATE.

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metabolism in the sink tissues of many plants. Sucrose must be cleaved either into UDP-glucose and fructose by sucrose synthase (SUS) or into glucose and fructose by invertase before it can be further metabolized (Dennis and Blakeley, 2000). The free hexoses, fructose and glucose, must then be phosphorylated by fructokinase (FRK) or hexokinase (HXK) before they can enter metabolic pathways (Stein et al., 2017). The tomato SlFRK2 is essential for proper xylem development, and the xylem vessels in the stems of SlFRK2 antisense plants have thinner xylem secondary cell walls with cells that are narrower and deformed (Damari-Weissler et al., 2009). In this study, plastidic hexokinase (Solyc04g081400.2) was up-regulated in the WT vs. ovate mutant, indicating that it contributes greatly to fruit cell wall biosynthesis in the ovate mutant, which was highly consistent with the firmer fruit observed in ovate than in WT. This up-regulation could be suppressed by the co-expression of MuMADS1 and MaOFP1 in ovate, which suggests that this gene may be regulated by MuMADS1 and MaOFP1. The increased firmness associated with the ovate mutation might be due to its increased cell wall polysaccharide content, which may result in the reduction of soluble carbohydrates (composed of mainly sucrose, glucose and fructose; Figure 7).

β-Glucosidase (BGL; EC 3.2.1.21) is a typical cellulase that acts synergistically to hydrolyse cell wall cellulose to glucose (Ma et al., 2016). The down-regulation of β-glucosidase (Solyc07g063880.2) might be the main factor accounting for the increase in fruit firmness and decreased glucose in the ovate mutant. However, this down-regulation could be compensated for by the co-expression of MuMADS1 and MaOFP1 in the ovate mutant, suggesting that MuMADS1 and MaOFP1 act synergistically to regulate the expression of β-glucosidase (Solyc07g063880.2). In this study, another β-glucosidase named lysosomal beta-glucosidase-like (Solyc04g015560.2) displayed a different expression pattern (Figure 5), suggesting that different members of the β-glucosidase family with different properties exist in tomato fruit.

UDP-glucose 6-dehydrogenase is an important enzyme involved in diverting UDP-Glc to cell wall synthesis (mainly hemicellulose) (Xue et al., 2008). Within the operon, a UDP-glucose 6-dehydrogenase converts UDP-glucose to UDP-glucuronic acid and NADH in the presence of NAD+UDP-glucuronate 4-epimerase 1-like belongs to the family of short-chain dehydrogenases/reductases (Broach et al., 2012). It converts UDP-glucuronic acid to UDP-galacturonic acid, the production of which is directly provided to Golgi-localized galacturonosyltransferases during cell wall synthesis (Mølhøj et al., 2004). Therefore, the up-regulation of UDP-glucose 6-dehydrogenase (Solyc02g067080.2) and UDP-glucuronate 4-epimerase 1-like (Solyc12g010540.1) in the WT vs. ovate mutant may result in increased cell wall polysaccharide contents, which was consistent with the increased firmness observed in the ovate mutant. In the MuMADS1 and MaOFP1-transformed ovate mutant, the expressions of UDP-glucose 6-dehydrogenase (Solyc02g067080.2) and UDP-glucuronate 4-epimerase 1-like (Solyc12g010540.1) decreased, which was consistent with the decreased firmness in the MuMADS1 + MaOFP1 transformants (Figure 7).

Galacturonosyltransferase is a α-1, 4-galacturonosyltransferase that synthesizes homogalacturonan, the most abundant pectic polysaccharide (Atmodjo et al., 2011). The up-regulation of three galacturonosyltransferases (Solyc06g083310.2, Solyc02g089440.2 and Solyc07g055930.2) in WT vs. ovate suggested that these...
genes contribute to the increased fruit firmness in the ovate mutant. This up-regulatory phenomenon of three galacturonosyltransferases gradually disappeared as MuMADS1 and MaOFP1 transformed into the ovate mutation, which suggests that the expression of these genes is regulated by MuMADS1 and MaOFP1 (Figure 7).

Taken together, we suggest a mechanism by which MuMADS1 and MaOFP1 regulate fruit firmness and sugar accumulation. As shown in Figure 8, the putative target genes regulated by MuMADS1 could be placed into carbohydrate metabolism, including glycolysis (3), Calvin cycle (6), sucrose metabolism (16) and cell wall metabolism, including cellulose synthesis (2), hemicellulose metabolism (14), pectin metabolism (1) and cell wall protein (2). Starch metabolism (2) was added to the putative targets regulated by MaOFP1, and a number of other classes displayed a few more genes than those of MuMADS1. When MuMADS1 and MaOFP1 were co-expressed, the number of genes involved in glycolysis, Calvin cycle, sucrose metabolism, starch metabolism and hemicellulose metabolism increased to 4, 9, 27, 4, 28, respectively, which was much higher than that of either MuMADS1 or MaOFP1. This is the first time that a mechanism for MuMADS1 and MaOFP1 in controlling fruit sugar accumulation and softening has been proposed.

Experimental procedures

Plant materials and treatments

Tomato ovate mutants (S. lycopersicum, LA3543) and wild-type (S. lycopersicum ‘Ailsa Craig’, LA2838A) were kindly provided by the Tomato Genetics Resource Center (http://tgrc.ucdavis.edu). Plants were raised in a field environment in groups at the Institute of Tropical Bioscience and Biotechnology (Haikou, Hainan Province). Transgenic plants were produced to the T3 line, and just the homozygous transgenic plants were utilized in the quantitative analyses (Liu et al., 2015a). The fruits at various maturation periods were evaluated by labelling pollinated flowers: IMG [17 days after pollination (DAP)], MG, BR and RM (Bastias et al., 2011). Typical and transgenic plants were gathered, quickly frozen in liquid nitrogen and retained at −80 °C for later use.

Constructs and genetic transformation

pCAMBIA1302 was digested by Ncol and SpeI, and then MuMADS1 and MaOFP1 were cloned into the multiple cloning sites to obtain the transformation vectors pCAMBIA1302-MuMADS1 and pCAMBIA1302-MaOFP1. The two transformation vectors were introduced into A. tumefaciens EHA105. The two strains were used to infect the tomato ovate mutant together at a ratio of 1 : 1. The callus culture and genetic transformation procedures were based on the method described by McCormick et al. (1986).

RNA extraction and cDNA synthesis

Total RNA from every one of the tissues utilized in this evaluation was removed with an altered cetyltrimethylammonium bromide (CTAB) technique (Wan and Wilkins, 1994).

First-strand cDNA was combined with a SMARTTM PCR cDNA Synthesis Kit for reverse transcriptase (Clontech, Palo Alto, CA) based on the company’s directions. The primers utilized for real-time RT-PCR are revealed in Table S8.

qRT-PCR analysis

Transcriptional changes in MuMADS1 and MaOFP1, as well as key genes responding to fruit quality formation in transgenic lines and controls, were determined via qRT-PCR analysis on a Stratagene Figure 8 Model for the role of MuMADS1 and MaOFP1 in regulating fruit sugar accumulation and softening. The numbers of target genes involved in carbohydrate and cell wall metabolism regulated by MaOFP1 (the left box), MuMADS1 (the right box) and MuMADS1 + MaOFP1 (the middle box) have been summarized. Solid blue line, validated protein–protein interaction; solid blue arrows, validated regulation; dashed blue arrows, putative regulation.
Mx3000P Real-Time PCR system with SYBR® Premix Ex Taq™ (TaKaRa, Japan). The PCR amplification setup for each of the reactions was: 10 min at 95 °C, then 40 cycles of 10 s at 95 °C, 15 s at 55 °C and 30 s at 72 °C. The typical expressions of the target genes were quantified by the 2-ΔΔCT method (Livak and Schmittgen, 2001). 18S rDNA (accession number: X51576.1) was used as the internal control to normalize the expression of the target genes in tomato.

Southern blot analysis
The Southern blot analysis was conducted based on Liu et al.'s technique (2015a).

Measurements of fruit quality-related physiological indexes
Upon harvesting, three fruits per treatment were sampled for measurements among the marked fruits. After weighing, the fruit sample was measured for longitudinal and transverse diameter (cm) using a Vernier caliper (Li et al., 2016). The fruit shape index was analysed by longitudinal/transverse diameter. The taste and nutritional properties of the first ripe fruit harvested from the first truss of each tagged plant were determined. The fruit was sliced and blended after removing the skin and seeds. A hand-held ATAGO-P32 temperature compensated refractometer (ATAGO Co. Ltd, Tokyo, Japan) was used to directly read the % soluble solids (as Brix) of the blended fruit at room temperature (Pieper and Barrett, 2009).

Fruit solidity was quantified based on Li et al.’s technique (2013).

The sugar (fructose, glucose and sucrose) contents were analysed using high-performance liquid chromatography (HPLC; Waters, Milford, CT).

De novo transcriptome assembly and annotation
RM fruits of different transgenic lines including M (MuMADS1 transformants), O (MaOFP1 transformants) and MO (MuMADS1 + MaOFP1 transformants), as well as WT and ovate were collected to extract total RNA using the plant RNAeasy extraction kit (TIANGEN, Beijing, China) for transcriptome analysis. The sequencing was performed with an Illumina GAI following the manufacturer’s instructions, with three replicates for each sample. The average sequencing depth was 5.34X. Using the FASTX-toolkit, adapter sequences in the raw sequence reads were removed. Clean reads were generated after examining the sequence quality and removing low-quality sequences using FastQC. Using TopHat v. 2.0.10, clean reads were mapped to the S. lycopersicum L. (2n = 24). The transcriptome assemblies were performed by Cufflinks (Trapnell et al., 2012). Gene expression levels were calculated as Reads per Kilobase of exons model per Million mapped reads (FPKM). DESeq was used to identify DEGs (Wang et al., 2010). Significant DEGs were screened using DESeq software (Anders and Huber, 2010). The corrected P-values from this method accounting for multiple tests used the key factor, which was false discovery rate (FDR). FDR <0.01 and |log2 (fold change)| > 1 or <-1 were set as the thresholds for differential gene expression. Fold changes in the expression levels between samples were used as the criteria in the screening process. The unigen sequences were searched against the following public databases: NR database (Deng et al., 2006), Swiss-Prot (Apweiler et al., 2004) GO database (Ashburner et al., 2000), COG database (Tatusov et al., 2000) and KEGG (Kanehisa et al., 2004).

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Conflict of interest
The authors declare that they have no conflicts of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Identification of transgenic tomatoes.

Figure S2 GO classification. The DEGs corresponded to three main categories: “biological process”, “cellular component” and “molecular function”. The left-hand y-axes indicate the percentage of genes.

Table S1 RNA-Seq analysis.
Table S2 Mapped results of RNA-Seq and reference genome.
Table S3 Annotated number of DEGs.
Table S4 Analysis of GO classification.
Table S5 Analysis of KEGG classification.
Table S6 The expression data of the DEGs in different assemblies.
Table S7 The DEGs regulated by OVATE, MuMADS1, MaOFP1 and MuMADS1 + MaOFP1.
Table S8 The primer sequences used for qRT-PCR.