Open Archive TOULOUSE Archive Ouverte (OATAO)

OATAO is an open access repository that collects the work of Toulouse researchers and makes it freely available over the web where possible.

This is an author-deposited version published in: http://oatao.univ-toulouse.fr/
Eprints ID: 18643

To link to this article: DOI: 10.1093/jxb/erx303
URL: http://dx.doi.org/10.1093/jxb/erx303

To cite this version: Huang, Baowen and Routaboul, Jean-Pierre-Amans and Liu, Mingchun and Deng, Wei and Maza, Elie and Mila, Isabelle and Hu, Guojian and Zouine, Mohamed and Frasse, Pierre and Vrebalov, Julia T and Giovannoni, James J and Li, Zhengguo and van der Rest, Benoît and Bouzayen, Mondher. Overexpression of the class D MADS-box gene Sl-AGL11 impacts fleshy tissue differentiation and structure in tomato fruits. (2017) Journal of Experimental Botany, vol. 68 (n° 17). pp. 4869-4884. ISSN 0022-0957

Any correspondence concerning this service should be sent to the repository administrator: staff-oatao@listes-diff.inp-toulouse.fr
Abstract

MADS-box transcription factors are key elements of the genetic networks controlling flower and fruit development. Among these, the class D clade gathers AGAMOUS-like genes which are involved in seed, ovule, and funiculus development. The tomato genome comprises two class D genes, Sl-AGL11 and Sl-MBP3, both displaying high expression levels in seeds and in central tissues of young fruits. The potential effects of Sl-AGL11 on fruit development were addressed through RNAi silencing and ectopic expression strategies. Sl-AGL11-down-regulated tomato lines failed to show obvious phenotypes except a slight reduction in seed size. In contrast, Sl-AGL11 overexpression triggered dramatic modifications of flower and fruit structure that include: the conversion of sepals into fleshy organs undergoing ethylene-dependent ripening, a placenta hypertrophy to the detriment of locular space, starch and sugar accumulation, and an extreme softening that occurs well before the onset of ripening. RNA-Seq transcriptomic profiling highlighted substantial metabolic reprogramming occurring in sepals and fruits, with major impacts on cell wall-related genes. While several Sl-AGL11-related phenotypes are reminiscent of class C MADS-box genes (TAG1 and TAGL1), the modifications observed on the placenta and cell wall and the Sl-AGL11 expression pattern suggest an action of this class D MADS-box factor on early fleshy fruit development.

Key words: Cell wall, fleshy tissue, fruit development, MADS, tomato.

Introduction

MADS-box genes belong to a large family of transcription factors present in all plant species and are reported to control development of organs such as flowers, ovules, seeds, leaves, and roots (Riechmann and Meyerowitz, 1997; Ng and Yanofsky, 2001; De Folter et al., 2006; Deng et al., 2012; Xu et al., 2016). In flower development, they have
been subdivided into five different classes (A, B, C, D, and E genes) that are important for specifying sepalas (A, E), petals (A, B, E), stamens (B, C, E), carpels (C, E), and ovules (D, E). Several MADS-box genes have been reported to affect tomato fruit development and ripening: class A FUL1 and FUL2 (Bemer et al., 2012; Shima et al., 2014), class C TAG1 (Prueli et al., 1994) and TAGLI (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010), class E RIN, TM29, and MADS1 (Vrebalov et al., 2002; Ampomah-dwamena et al., 2002; Dong et al., 2013), and non-classified FYFL (Xie et al., 2014). These transcription factors may directly or indirectly interact with target DNA as complexes of varying composition to regulate fruit development and ripening (Karlova et al., 2014). Recently, ChIP approaches uncovered target genes for some of the MADS-box proteins; that is, RIN binds to at least 241 direct targets, resulting in both their positive and negative regulation. Consistent with its role in climacteric fruit ripening, RIN binds to genes involved in ethylene biosynthesis (ACS2 and ACS4) and perception (NICR), as well as cell wall-remodeling genes (Martel et al., 2011; Qin et al., 2012; Fujisawa et al., 2013; Zhong et al., 2013). In addition, the FUL1/FUL2/RIN complex can bind to different target genes such as those involved in the flavonoid and carotenoid biosynthesis pathways (Fujisawa et al., 2013; Zhong et al., 2013).

TAGI and TAGLI, the two tomato members of the class C MADS-box gene family, are orthologous to Arabidopsis AGAMOUS (AG) and SHATTERPROOF1/2 (SHP1/SHP2), respectively. TAGI RNAi-mediated down-regulation led to stamen defects and loss of floral organ determinacy, as evidenced by the nested flowers-in-flower (Prueli et al., 1994) or fruit-in-fruits phenotypes (Pan et al., 2010). TAGLI down-regulation resulted in ripening inhibition and reduced pericarp thickness (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010). Double RNAi silencing of tomato TAGI and TAGLI indicated that these two genes have both redundant and divergent functions in regulating carpel identity and pollen development (Pan et al., 2010; Giménez et al., 2016). This tomato subfunctionalization of class C MADS-box genes is reminiscent of Arabidopsis and other Angiosperms where AG, SHP1, and SHP2 exert overlapping functions such as floral meristem determinacy and the ability to promote reproductive organ development (Dreni and Kater, 2014), while SHP1 and SHP2 specifically control valve margin identity and development of the dehiscence zones (Liljegren et al., 2000).

SI-AGLI1 and SI-MBP3 are highly related to the AGAMOUS family and belong to the class D MADS-box genes. They are putative orthologs of the Arabidopsis SEEDSTICK (STK) gene (Pinyopich et al., 2003; Mizzotti et al., 2012) and of the petunia Floral Binding Proteins 11 and 7 (FBP11 and FBP7) genes (Angenent et al., 1995; Colombo et al., 1995). Simultaneous down-regulation of FBP7/FBP11 by co-suppression (Angenent et al., 1995) or by transposon insertion (Heijmans et al., 2012) leads to carpel-like structures instead of ovules and to aberrant seed development, indicating that the FBP7/FBP11 gene pair has a unique function in seed development. Redundantly with other AG clade members, they also specify ovule identity (Pinyopich et al., 2003; Heijmans et al., 2012). In Arabidopsis, STK mutant shows defects in ovule development including reduced fruit and seed size, and an abnormal funiculus that disturbs seed spacing and dispersal at fruit maturation (Pinyopich et al., 2003). In the seed coat, STK protein also regulates cell wall strengthening and flavonoid accumulation. For instance, it may repress BAN/NR, the main biosynthetic gene leading to proanthocyanidin (PA) accumulation and control endothelium development and differentiation (Mizzotti et al., 2014). STK has also been reported to repress some genes involved in pecin maturation and glucomannan or cellulose deposition in seed coat or columella (Ezquer et al., 2016).

Recent work on fruit crop species provided evidence for the potential impact of class D MADS-box genes on fruit quality traits. It was shown that the palm tree STK ortholog, SHELL, controls the development of the thick coconut-like shell surrounding the kernel with consequences on oil yield and composition (Singh et al., 2013). In grapevine, an STK ortholog, ViAGLI1, has been shown to be a key gene to control fruit seedlessness in addition to its previously described roles in ovule patterning (Mejia et al., 2011; Ocarez and Mejia, 2016; Malabarba et al., 2017). In addition, SI-AGLI1, the putative ViAGLI1 ortholog, was reported potentially to control seed formation (Ocarez and Mejia, 2016). However, these observations were based on the phenotype displayed by tomato T0 RNAi lines, and the global impact on fruit development has not been documented.

To address further the functional significance of SI-AGLI1, formerly called Le-TAGLI1 or SI-TAGL11 (Itkin et al., 2009; Vrebalov et al., 2009), both overexpressing and down-regulated tomato lines were generated and analyzed. To uncover further the physiological significance of SI-AGLI1, we report here that the ectopic expression of this gene results in dramatic modifications in flower and fruit organization. In particular, the conversion of the sepalas into a carpel-like fleshy organ, and the enhanced fruit softness and sugar content are indicative of major metabolic reorientations as validated by genome-wide gene expression profiling.

Materials and methods

Plant materials

Tomato (Solanum lycopersicum cv MicroTom) seeds were sown on 0.5× Murashige and Skoog (MS) medium (pH 5.9) with 0.8% (w/v) agar and were transferred to soil after 2 weeks and maintained in a culture chamber (14 h day/10 h night cycle, 25/20 °C day/night temperature, 80% relative humidity). Development and ripening measurements refer to days post-anthesis (DPA) or breaker (BR) stage. Flowers at the ‘anthesis stage’ were determined according to the change of petal color (deep yellow) and to a slight elongation observed either in wild-type (WT), SI-AGLI1 RNAi, or SI-AGLI1OE plants, which coincided with the acquisition of the pollination capacity. Fruits at the BR stage were determined according to the yellow color change observed on mature green fruits.

Pollination assay

Flower buds were emasculated before dehiscence of anthers according to Wang et al. (2005). Hand cross-pollination was performed on emasculated flowers 1 d prior to anthesis.
Transgenic plants with altered *SI-AGL11* expression were obtained via *Agrobacterium*-mediated transformation as described in Hao et al. (2015), and transformed lines were selected on kanamycin (70 mg l⁻¹).

For *SI-AGL11* down-regulation, a construct enabling RNAi silencing was designed using the pHHELLSGATE-12 system (Invitrogen). The *SI-AGL11* 3′ end was introduced in sense and antisense orientation after amplification with the forward 5′-ACATGTTAAGACTGCACTACC-3′ and reverse 5′-GGCCCAAAAAATTTTGAAAGATGAGC-3′ primers and intermediated cloning into the pDONOR207.

For *SI-AGL11* overexpression, the full length was amplified by PCR with the forward 5′-ATGGGTCCAGGAAGAGATGAGC-3′ and reverse 5′-TTACCTTTTGTGATCAGGAGACAA-3′ primers and inserted into the Small site of a modified 35S-PLP100 vector containing the Cauliflower mosaic virus (CaMV) 35S promoter and the Nos terminator (Hu et al., 2014). Clone orientation and sequence were confirmed by sequencing before introduction into the CS8 Agrobacterium strain.

**RNA extraction and quantitative real-time PCR (qPCR)**

Five individual fruits at each developmental stage were harvested and frozen in liquid nitrogen. Total RNA samples were isolated using Trizol (Invitrogen) according to the manufacturer’s instructions and were treated with DNase I (Invitrogen). The first-strand cDNA synthesis was performed using 1 μg of total RNA with an Omniscript Reverse Transcription kit (Qiagen). qPCR was performed in a 10 μl reaction volume using the SYBR Green PCR Master Mix on an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Primers used for PCR amplification are listed in Supplementary Table S2 at JXB online. Three independent RNA isolations were used for cDNA synthesis and each cDNA sample was subjected to RT-PCR analysis in triplicate. Actin was used as the internal reference (Lavdal and Lillo, 2009).

**Subcellular localization of *SI-AGL11* proteins**

A *SI-AGL11*-green fluorescent protein (GFP) C-terminal fusion was generated and introduced into a pGreen vector backbone containing the 35S CaMV promoter. A pGreen-GFP empty vector was used as a cytoplasmic control. Alternatively, a yellow fluorescent protein (YFP) N-terminal fusion was achieved by introducing *SI-AGL11* in the pEarlyGate104 vector (Earley et al. 2006). The nucleus control 35S:RFP-N7 was constructed including the N7 nuclear targeting signal in the expression clone pH7WGR2.0. Tobacco (*Nicotiana tabacum*) BY-2 cell protoplasts were transferred according to Leclercq et al. (2005) and fluorescence was followed by confocal microscopy as described previously (Audran-Delalande et al., 2012).

**Ethylene and 1-MCP treatment**

Ethylene and 1-methylecyclopropene (1-MCP) treatments on fruits were performed in a 22 liter glass container. For ethylene treatment on fruit, breaker stage fruits were treated with ethylene gas (50 μl l⁻¹) for 24 h. 1-MCP treatments (1.0 μg l⁻¹) were applied to 3 d post-breaker (turning) fruits for 72 h. Control fruits were incubated in air instead of ethylene or 1-MCP.

**Firmness measurement**

Fifteen fruits from each *SI-AGL11OE* line and the WT were harvested at different development stages (from DPA10 to BR+10). The firmness was then assessed using Harpenden calipers (British Indicators Ltd) as described by Ecarnot et al. (2013).

**Water loss**

Ten fruits from the WT and three *SI-AGL11OE* lines were harvested at the breaker stage. Fruits were placed at room temperature for 10 d, and fresh weight was recorded every day. Water loss was calculated as a percentage of fresh weight difference between the starting weight and each individual measurement.

**Soluble sugar and starch determination**

Fruits and sepalas were harvested and frozen in liquid nitrogen. After grinding the fruits into a frozen powder, the samples were incubated with 80% ethanol (10 mM HEPES-KOH pH 7.4) at 80 °C for 15 min, as described by Sonnewald et al. (1991). After centrifugation (15 min 16 000 g), supernatants were kept for soluble sugar determination: ethanol was removed with a centrifugal vacuum concentrator and, after appropriate dilution in water, glucose, fructose, and sucrose were determined using the Sucrose/Fructose/Glucose Assay Kit (Megazyme). For starch, the pellet was rinsed with 80% ethanol (80 °C, 15 min of incubation) and centrifugation. Starch was determined using the Total Starch HK Assay Kit (Megazyme) following the manufacturer’s instructions.

**Histological observations**

To observe fruit anatomy and determine the number of cells layers, fruits were hand-cut and stained using a 30 s bath of 0.05% (w/v) aqueous toluidine blue O (Sigma-Aldrich) followed by two rinses with distilled water. After mounting in water with a cover slip, observations were performed in an Axio Zoom V16 microscope (Zeiss). To assay defects in synthesis/release of mucilage, dry seeds were hydrated in water for 2 h, stained in 0.03% solution of ruthenium red (Sigma) for 30 min at room temperature, and then rinsed with water. To visualize the seed coat, immature and dry seeds were incubated for 30 min to 1 h in 1% vanillin (Sigma) in 6 M HCl. For starch visualization, fruit sections were stained in a 0.5× Lugol solution (1% I₂ and 2% KI in water) with a 10 s bath. The excess stain was removed by gently tipping on a paper tissue, and rinsing with distilled water.

**RNA-Seq analyses and data processing**

Global expression of tomato genes was determined by replicated strand-specific Illumina RNA-Seq. Paired-end RNA sequencing (2 × 150 nucleotides) was carried out using the Truseq Illumina SBS Kit V4 and the Genotoul Hiseq 2500 platform (http://get.genotoul.fr/). For each line (WT and *SI-AGL11OE-L2*), RNA was extracted from DPA10 fruits and sepalas of three biological replicates. Prior to sequencing, purified RNA quality was checked with the Agilent 2100 Bioanalyzer (rin >8.5). Raw paired-end RNA-Seq sequences in FASTQ format were analyzed as follows. Low-quality reads were removed with the FASTQ quality filter using the FASTX toolkit version 0.0.13 (http://hannon-lab.cshl.edu/fastx_toolkit/). Trimmed reads were then mapped to the *S. lycopersicum* reference genome and gene annotation (ITAG2.4: Tomato Genome Consortium, 2012) using TopHat-2.0.14 (Trapnell et al., 2009) calling bowtie 2.1.0 (Langmead and Salzberg, 2012).

The differential expression analysis has been carried out with the DESeq2 R package with default settings (Love et al., 2014). The normalization method used by default (LRE) agrees with the assumption that <50% of genes are regulated and <50% of genes are down-regulated between two given conditions (Mazé et al., 2013;
Maza, 2016). The false discovery rate (FDR) is controlled by the Benjamini–Hochberg method; genes were declared as differentially expressed genes (DEGs) if the adjusted P-value was <0.05.

All statistical analyses have been performed with the R software (https://www.r-project.org). The multidimensional scaling analysis (MDS) has been performed with the cmdscale function of the stats R package. This analysis coincides with the principal component analysis (PCA) in the present case where we calculate the Euclidean distance between samples. Expression data were visualized using the MAPMAN 3.5.1 software (Thimm et al., 2004).

Accession numbers
All RNA-Seq data were placed in the European Nucleotide Archive (ERR1904925–ERR1904937).

Results
SI-AGL11, a class D MADS-box gene mainly expressed during early fruit development

Four AGAMOUS-like genes— TAG1 (Solyc02g071730), TAGLI (Solyc07g055920), SI-AGL11 (Solyc11g028020), and SI-MBP3 (Solyc06g064840)—were found in the tomato genome. Based on a phylogenetic analysis (Supplementary Fig. S1) and consistent with previous functional characterization, TAG1 and TAGLI belong to the class C SHP/Plena lineage that comprises both the AGAMOUS and SHATTERPROOF members (Pnueli et al., 1994; Vrebalo et al., 2009). The two other genes, SI-AGL11 and SI-MBP3, are highly similar to the petunia FBP11 and FBP7 genes and belong to class D function. This D lineage also contains the Arabidopsis SEEDSTICK (STK, At4g09960) gene, which is involved in seed development and seed abscission (Pinyopich et al., 2003). Amino acid sequence comparison indicates that SI-AGL11 and SI-MBP3 share 91% identity, whereas the sequence conservation falls to 56–59% identity when comparing SI-AGL11 with TAG1 and TAG1.

The expression of SI-AGL11 determined by qPCR revealed a clear preferential expression in flower and fruit, especially at early stages of fruit development, and a weak expression in vegetative organs (Fig. 1A). Within the fruit organ, SI-AGL11 expression is high in the central part of the fruit, with steady expression levels in the seed and in the ‘inner tissues’ that comprise the septum, the locular tissue, the placenta, and the columella (Fig. 1C). Moreover, the expression pattern of SI-MBP3 seems similar to that of SI-AGL11, as it is also expressed in young fruits with high levels in the seed and the ‘inner tissues’ (Fig. 1B). These data are in agreement with the expression patterns established in silico using the TomExpress database (Zouine et al. 2017; http://gfb.toulouse.inra.fr/tomexpress/) that combines a large number of RNA-Seq expression studies (Supplementary Fig. S2). Interestingly, the four AGAMOUS-like genes exhibit their maximum expression level at different developmental stages: TAG1 reaches its maximum in bud and opened flower, SI-AGL11 and SI-MBP3 in young fruits, and TAGLI at the beginning of ripening (breaker stage), thus suggesting that despite their ancestral origin, the different AGAMOUS-like genes have evolved by acquiring temporalspecific expression patterns.

To gain insight into the subcellular localization of SI-AGL11, two different fusion constructs, the C-terminal 35S-SI-AGL11-GFP and the N-terminal 35S-YFP-SI-AGL11, were transiently expressed in tobacco BY-2 protoplasts. For both constructs, the fluorescence signals were found mainly in the nucleus but also extended to the cytoplasmic compartment (Supplementary Fig. S3). Considering the putative function of SI-AGL11 as a transcription factor, these data suggest that it might undergo important regulation at the post-translational level.

SI-AGL11 down-regulation results in a limited effect on seeds

To investigate the functional significance of SI-AGL11, we generated 12 independent tomato transgenic lines exhibiting down-regulation of the SI-AGL11 gene through an RNAi approach designed to target specifically this class D member. Transcript level analysis performed by qPCR on young fruits (DPA10 stage) confirmed that SI-AGL11 was substantially down-regulated whereas SI-MBP3, its closest class D homolog, remained unaffected (Fig. 2B). Out of the 12 RNAi lines generated, none exhibited visually detectable phenotypes, either in the vegetative organs or in the fruits, where SI-AGL11 is normally expressed (Fig. 2A, C). Despite a 60–77% decrease of SI-AGL11 expression, which was confirmed by qPCR for nine RNAi lines (Supplementary Fig. S4), all transgenic lines produced seeded fruits, in contrast to previous reports also using an RNAi strategy (Ocarez and Mejia, 2016). Nonetheless, a slight decrease in seed size and an average 20% reduction of seed weight were observed (Fig. 2C). Also, ruthenium red or vanillin staining of seeds did not reveal any change in mucilage and flavonoid accumulation in the seed coat (Supplementary Fig. S5).

SI-AGL11 overexpression induces dramatic modifications in flower and fruit organization

Since the phenotypes due to SI-AGL11 down-regulation were visually subtle and apparently restricted to seed, we generated tomato plants overexpressing the SI-AGL11 coding sequence under the control of the 35S promoter in order to gain further insight into the putative function of this tomato class D member. Fifteen independent transgenic lines, named SI-AGL11OE, were generated, and all displayed dramatic phenotypes associated with flower and fruit development (Fig. 3A).

In all these lines, the expression of TAG1, TAGLI, and SI-MBP3 assessed at the transcript level showed no significant alteration, with the exception of TAGLI which displayed a slight decrease in transcript accumulation in one of the SI-AGL11OE lines (Supplementary Fig. S6). No major alteration of vegetative development was observed except a minor reduction in plant size in some lines only visible in adult plants since young plants were unaffected (Fig. 3A; Supplementary Fig. S7). In contrast, SI-AGL11OE plants demonstrated severe phenotypes visible at early flower bud stages with defects in sepal development. The sepals were light green, swollen, and
failed to open at anthesis (Fig. 3A). In the most severe lines, the sepals virtually enclosed the ovary, thus preventing pollen dispersion and leading to the development of seedless fruit (Supplementary Fig. S8). Three lines exhibiting a 20- to 30-fold increase in *Sl-AGL11* transcript level (Fig. 3B) were selected for further characterization. In the *Sl-AGL11OE* lines, as the fruit entered the ripening process, the sepals evolved like a fleshy fruit, turning orange and then red, suggesting that they differentiated into a succulent organ that shared most fruit attributes. Besides sepals, the flower peduncles in *Sl-AGL11OE* plants were nearly glabrous with few trichomes, and underwent swelling and ripening (Supplementary Fig. S9). In contrast to the sepal, no major difference was observed in petal structure (Fig. 3A). Another remarkable feature of the *Sl-AGL11OE* plants is the lack of an activated abscission zone at the middle of the pedicel which prevents the fruit from dropping from the plant at the end of the ripening process (Supplementary Fig. S9). Fruit development was also dramatically affected in *Sl-AGL11OE* plants, with reduced fruit size and weight (Supplementary Fig. S10A). Histological observations on fruit sections stained with toluidine blue revealed dramatic modifications in both pericarp and inner tissues, including gel, placenta, and columella. The fruit size was reduced and the pericarp was typically thinner in *Sl-AGL11OE* fruits, with smaller cells even though the number of cell layers was slightly higher (Supplementary Fig. S10B). In the inner part of the *Sl-AGL11OE* fruits, we observed a marked reduction of the locular space that was restricted to a thin ‘jelly’ surrounding the seeds (Fig. 3C), while the relative area corresponding to the placenta was increased (Fig. 3D). Moreover, *Sl-AGL11OE* lines did not exhibit any delay in flowering initiation (Supplementary Fig. S11). The fruits of *Sl-AGL11OE* lines produced few or no

---

**Fig. 1.** Expression pattern of *Sl-AGL11*. (A) *Sl-AGL11* expression in different tissues determined by qPCR. Ro, root; St, stem; Le, leaf; Bud1, Bud8, 1 mm and 8 mm long flower buds; Fl, opened flower at anthesis; DPA4, DPA10, DPA15, DPA20, fruit at 4, 10, 15 and 20 d, respectively, after anthesis; MG, mature green fruit; BR, fruit at breaker stage; BR+3, BR+7: fruits 3 d and 7 d after the BR stage. Values are means ± SD of three biological replicates. (B) *Sl-AGL11* expression in different DPA15 fruit tissues. ‘Inner tissues’ comprise columella, placenta, septum, and locular tissue. Values are means ± SD of three biological replicates. (C) ‘Inner tissues’ in young tomato section including columella, septum, placenta, and locular tissue.
seeds. However, manual flower cross-pollination with WT pollen restored almost normal seed development (Supplementary Fig. S12). Cross-fertilization of emasculated WT flowers could not restore normal seed development, revealing pollen deficiency in Sl-AGL11OE plants (Supplementary Fig. S12). Therefore, the selected transgenic lines were maintained and multiplied as hemizygous lines by cross-pollination with WT pollen and subsequent antibiotic selection of seedlings.

Sl-AGL11OE fruits and sepals undergo a ripening-like process that is ethylene dependent

Since Sl-AGL11OE fruit development was altered and plant sepals differentiated into fleshy tissues, we examined the ripening dynamics of Sl-AGL11OE fruit and fleshy sepals. Compared with WT fruit, color change in Sl-AGL11OE occurs more slowly, suggesting a delay in the onset of ripening (Fig. 4A). Accordingly, the peak of climacteric ethylene was delayed by 5–6 d in Sl-AGL11OE fruits while the amount of ethylene produced was enhanced 3-fold (Fig. 4B). In addition, exogenous ethylene treatment proved to be efficient in inducing ripening and, conversely, treatment with 1-MCP, an inhibitor of ethylene perception, prevented ripening, thus confirming that fruit and succulent sepals in Sl-AGL11OE plants behave as climacteric organs (Fig. 4C). We then examined the expression of a set of key ripening genes including the ethylene synthesis genes ACC oxidase1 (ACO1) and ACC synthase2 (ACS2), as well as two major regulators of climacteric ripening Ripening Inhibitor (RIN) and Non-Ripening (NOR) genes. For all four ripening-associated genes, the expression level increased during ripening until the BR+7 stage, where it was significantly higher than in WT fruits (Fig. 4D), fully consistent with the pattern of ethylene production.

Sl-AGL11 overexpression has dramatic effect on fruit firmness

In addition to the acquisition of fleshy sepals, another remarkable feature displayed by the Sl-AGL11OE fruit consists of a dramatic decrease in firmness starting at an early stage of fruit development well before ripening (Fig. 5A). When they reach the ripening stage, the fruits become difficult to handle.

**Fig. 2.** Phenotype of tomato lines with RNAi-mediated down-regulation of Sl-AGL11. (A) Observation of plants, fruits, and seeds in three representative independent lines. (B) Relative down-regulation level of Sl-AGL11 monitored by qPCR on young fruits (DPA10 stage). Values are means ± SD of three biological replicates. (C) Quantification of mean weight and size of fruits (n=15) and seeds (n=120) in three representative independent lines compared with the wild type (WT). Values are means ± SD. Statistical significance determined by Student’s t-test: *0.01<P<0.05; **0.001<P<0.01; ***P<0.001. L1–L3 are three independent Sl-AGL11-RNAi lines. (This figure is available in colour at JXB online.)
and often burst upon manipulation. Measurement of firmness by Harpenden calipers (Fig. 5B) confirmed that this loss of firmness occurred very early during fruit development. In addition to the enhanced softness, other modifications may affect the cuticle as upon harvest the Sl-AGL11OE fruits display accelerated water loss compared with control WT fruits (see Supplementary Fig. S13). We therefore monitored the expression of four cell wall-related genes known to be involved in ripening-associated cell wall modifications: polygalacturonase PG2A (Grierson et al., 1986), β-galactosidase β-GAL4 (Smith et al., 2002), expansin EXP1 (Brummell et al., 1999), and pectate lyase PL2 (Uluisik et al., 2016). We also monitored three additional genes whose expression was highly affected in the ‘Sl-AGL11OE-fruit’ versus ‘WT-fruit’ RNA-Seq experiment described below: xyloglucan-endosyltransferase XTH1 (Solyc01g099630), pectin acetyl transferase PAE-like (Solyc08g005800), and cellulose synthase CS-like (Solyc07g051820). In agreement with the RNA-Seq data, the expression of the four cell wall-related genes more commonly associated with ripening (EXP1, PG2A, β-GAL4, and PL2) showed no major difference between WT and Sl-AGL11OE fruits at DPA10 and their transcript levels showed an increase only at the onset of fruit ripening (Fig. 5C). In contrast, XTH1, PAE-like, and CS-like expression was dramatically reduced in Sl-AGL11OE fruit samples (Fig. 5C), suggesting that the enhanced softness exhibited by Sl-AGL11OE fruits may originate from early modifications in fruit cell wall differentiation.

Sl-AGL11-overexpressing fruit accumulate more sugar

Preliminary observations based on the staining of Sl-AGL11OE fruits with iodine suggested important
modifications in starch and sugar accumulation (Fig. 6A). Monitoring starch evolution in fruits confirmed a 2-fold increase in \textit{Sl-AGL11OE} green tomatoes and revealed that starch breakdown was delayed (Fig. 6A, B). Soluble sugars were then quantified in ripening fruits. In ripe fruit (BR+12 stage), glucose and fructose were higher in \textit{Sl-AGL11OE} fruits, with a 1.5- and 2-fold increase, respectively (Fig. 6C). Notably, while sucrose was found at trace levels in WT fruits, its concentration reached up to 28 g kg\(^{-1}\) at the late ripening stages (BR+12) in \textit{Sl-AGL11OE} fruits (Fig. 6C). Sepals of \textit{Sl-AGL11OE} plants also contained high concentrations of starch and soluble sugars, further confirming that the
conversion into a fleshy organ implies similar metabolic reorientations to those occurring in genuine fruit tissues (Fig. 6C).

**Genome-wide transcriptomic profiling of Sl-AGL11OE fruit and sepals**

As the major histological and physiological changes in *Sl-AGL11OE* lines were observed during early fruit development, we performed a global gene expression profiling of young fruits and sepals harvested at the DPA10 stage. The RNA-Seq analysis produced, after removing the low quality reads, ~325 million paired-end reads, with a total number of reads for each sample ranging from 16 million to 39 million. On average, 83% of these reads were mapped to the ITAG-2.4 tomato reference genome, producing 13.5–30 million unique mapping reads depending on the sample considered. The number of predicted genes covered with a minimal average density of 20 independent counts per kilobase was ~60% (Supplementary Data S1). DEGs between various samples and conditions were identified with the following rules: mean
Performing a PCA on normalized mRNA-Seq counts confirmed that the biological replicates clustered together in both the sepal and the fruit (Fig. 7A). More interestingly, the same PCA analysis revealed that the first axis, holding 70% of the variability, could only separate WT sepals, Sl-AGL11OE sepals, and a cluster comprising both WT and Sl-AGL11OE fruits. Yet, the fruit samples were clearly discriminated through the second and following axes. Conversely, among the genes that displayed differential expression in the WT fruit versus WT sepal (12,389), more than half (6355) were also differentially expressed in the Sl-AGL11OE succulent sepals versus WT sepals experiment (Fig. 7B). Based on this preliminary analysis, the position of Sl-AGL11OE sepals along the first axis already suggests that the conversion of sepals into a succulent organ creates a kind of intermediary organ between a vegetative sepal and a fleshy fruit.

In order to identify specific functions impacted by the over-expression of Sl-AGL11, DEGs were associated with their respective MAPMAN gene annotation category (Thimm et al., 2004). The functional categories displaying the highest over-representation were determined using a Wilcoxon rank sum test on the MAPMAN bins for the Sl-AGL11OE versus WT fruit and Sl-AGL11OE versus WT sepal experiments (Fig. 7C). When comparing WT and Sl-AGL11OE sepals or fruit, ‘Photosynthesis’, ‘RNA processing’, and ‘Cell wall’ categories were over-represented for both tissues (Fig. 7C). Consistent with the transition from a green sepal to a fleshy ripening organ, the complete set of photosynthesis-related genes is repressed in Sl-AGL11OE lines (Supplementary Fig. S14).

Since Sl-AGL11OE plants exhibited a marked softness and a different pattern of toluidine blue staining (Figs 3C, 5A), a dye known for its metachromatic properties, we focused our analyses on the expression of different cell wall genes. Out of the 306 annotated cell wall genes expressed in fruit, 134 genes (44%) were differentially expressed in the Sl-AGL11OE versus WT DPA10 fruit experiment. Strikingly, 82% of these DEGs were down-regulated (Supplementary Data S2); this proportion reached 90% when considering only DEGs with high expression and a marked difference (|log_fod|>1, Table 1).
Interestingly, several cell wall genes whose homologs are targets of Arabidopsis STK, such as cellulose synthase CESA5 and CESA2, cellulose synthase-like CSLA2, and COBRA-LIKE2 COBL2 (Ezquer et al., 2016), were down-regulated in Sl-AGL11OE fruits (Supplementary Table S1).

Discussion

In tomato, the C/D lineage of AGAMOUS-related genes consists of four paralog genes: TAG1, TAGL1, Sl-AGL11, and Sl-MBP3. Various expression studies collected in the TomExpress database (Supplementary Fig. S2) have shown that the four AGAMOUS paralogs in tomato display temporal-specific expression patterns, with the class D genes Sl-AGL11 and Sl-MBP3 being preferentially expressed in early fruit development. Expression studies confirmed the high transcript levels of Sl-AGL11 and Sl-MBP3 in developing flowers and young fruits, reaching a maximum value in the inner part of young fruits that comprises placenta, seed, and columella (Fig. 1C). A laser-assisted microdissection on young Solanum pimpellifolium fruits (Pattison et al., 2015) revealed similar expression patterns for the two class D isoforms (Sp-AGL11 and Sp-MBP3) as they are both found in seed and inner fruit tissues (seed coat, endosperm, funiculus, embryo, placenta, and septum). This contrasts with petunia dry fruit, where the expression of FBP11 and FBP7 class D genes was restricted to seeds and ovule (Colombo et al., 1997). Thus, the expression pattern observed in tomato suggests an additional role for Sl-AGL11 or Sl-MBP3 that extends beyond seed development.

The initial approach consisting of Sl-AGL11 down-regulation resulted in a subtle phenotype affecting seed size, in agreement with class D function defined as regulating ovule and seed development. However, our data clearly contrast with a previous report (Ocarez and Mejía, 2016) stating that Sl-AGL11 down-regulation leads to seedless tomato fruits. Moreover the seedlessness phenotype reported by Ocarez and
Table 1. List of cell wall-related genes differentially expressed in Sl-AGL11OE fruit

DEGs were deduced from the DPA10 fruit Sl-AGL11OE versus WT RNA-Seq experiment using the following rule: $n (\text{counts kb}^{-1}) > 20; |\log_2 \text{fold}| > 1$ and $P$-value $< 0.05$.

<table>
<thead>
<tr>
<th>MAPMAN category</th>
<th>Solyc number</th>
<th>Log$_2$ fold</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1: Precursor synthesis (26/56/88)*</td>
<td>Solyc08g080570</td>
<td>1.1</td>
<td>UDP-glucose 4-epimerase</td>
</tr>
<tr>
<td></td>
<td>Solyc07g014640</td>
<td>-1.1</td>
<td>Galactokinase-like protein</td>
</tr>
<tr>
<td></td>
<td>Solyc08g080140</td>
<td>-1.2</td>
<td>dTDP-4-dehydrorhamnose reductase</td>
</tr>
<tr>
<td></td>
<td>Solyc07g006220</td>
<td>-1.3</td>
<td>UDP-$\alpha$-glucuronate 4-epimerase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc09g092330</td>
<td>-1.5</td>
<td>NAD epimerase/dehydratase</td>
</tr>
<tr>
<td></td>
<td>Solyc02g084210</td>
<td>-1.7</td>
<td>GDP-mannose 4 6-dehydratase</td>
</tr>
<tr>
<td></td>
<td>Solyc03g096730</td>
<td>-2.4</td>
<td>GDP-$\alpha$-mannose pyrophosphorylase 1</td>
</tr>
<tr>
<td>10.2: Cellulose synthesis (8/24/86)*</td>
<td>Solyc04g071650</td>
<td>-1.1</td>
<td>Cellulose synthase</td>
</tr>
<tr>
<td></td>
<td>Solyc11g066820</td>
<td>-1.2</td>
<td>Cellulose synthase-like C6</td>
</tr>
<tr>
<td></td>
<td>Solyc09g008990</td>
<td>-1.5</td>
<td>Cellulose synthase-like A2</td>
</tr>
<tr>
<td></td>
<td>Solyc09g009010</td>
<td>-1.6</td>
<td>Cellulose synthase-like C1</td>
</tr>
<tr>
<td></td>
<td>Solyc06g074630</td>
<td>-1.6</td>
<td>Cellulose synthase-like C6</td>
</tr>
<tr>
<td></td>
<td>Solyc10g083670</td>
<td>-2</td>
<td>Cellulose synthase-like C2</td>
</tr>
<tr>
<td></td>
<td>Solyc12g014430</td>
<td>-2</td>
<td>Cellulose synthase</td>
</tr>
<tr>
<td></td>
<td>Solyc07g051820</td>
<td>-6.2</td>
<td>Cellulose synthase</td>
</tr>
<tr>
<td>10.5: Cell wall proteins (15/33/50)*</td>
<td>Solyc03g114860</td>
<td>-1.1</td>
<td>Alpha-1 4-glucan-protein synthase</td>
</tr>
<tr>
<td></td>
<td>Solyc03g019750</td>
<td>-2.2</td>
<td>Alpha-1 4-glucan-protein synthase</td>
</tr>
<tr>
<td></td>
<td>Solyc10g054900</td>
<td>-2.4</td>
<td>Proline-rich protein</td>
</tr>
<tr>
<td></td>
<td>Solyc07g053540</td>
<td>-3.3</td>
<td>Fasciclin-like arabinoxylan protein 4</td>
</tr>
<tr>
<td>10.6: Cell wall degradation (36/89/181)*</td>
<td>Solyc09g075360</td>
<td>1.6</td>
<td>Endoglucanase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc03g058910</td>
<td>1.1</td>
<td>Pectate lyase</td>
</tr>
<tr>
<td></td>
<td>Solyc06g073760</td>
<td>1.1</td>
<td>Beta-$\alpha$-glucosidase</td>
</tr>
<tr>
<td></td>
<td>Solyc07g049300</td>
<td>-1.1</td>
<td>Polygalacturonase</td>
</tr>
<tr>
<td></td>
<td>Solyc01g110340</td>
<td>-1.1</td>
<td>BURP domain-containing protein</td>
</tr>
<tr>
<td></td>
<td>Solyc03g116500</td>
<td>-1.2</td>
<td>Pectate lyase</td>
</tr>
<tr>
<td></td>
<td>Solyc08g068150</td>
<td>-1.3</td>
<td>BURP domain-containing protein</td>
</tr>
<tr>
<td></td>
<td>Solyc06g083580</td>
<td>-1.3</td>
<td>Pectate lyase</td>
</tr>
<tr>
<td></td>
<td>Solyc02g084990</td>
<td>-1.4</td>
<td>Polygalacturonase</td>
</tr>
<tr>
<td></td>
<td>Solyc05g005080</td>
<td>-1.5</td>
<td>BURP domain-containing protein</td>
</tr>
<tr>
<td></td>
<td>Solyc05g005560</td>
<td>-1.5</td>
<td>BURP domain-containing protein</td>
</tr>
<tr>
<td></td>
<td>Solyc04g081300</td>
<td>-1.5</td>
<td>BURP domain-containing protein</td>
</tr>
<tr>
<td></td>
<td>Solyc08g082250</td>
<td>-1.5</td>
<td>BURP domain-containing protein</td>
</tr>
<tr>
<td></td>
<td>Solyc05g014000</td>
<td>-1.5</td>
<td>Mannan endo-1 4-beta-mannosidase</td>
</tr>
<tr>
<td></td>
<td>Solyc07g064870</td>
<td>-1.6</td>
<td>Mannan endo-1 4-beta-mannosidase</td>
</tr>
<tr>
<td></td>
<td>Solyc04g008220</td>
<td>-1.6</td>
<td>Mannan endo-1 4-beta-mannosidase</td>
</tr>
<tr>
<td></td>
<td>Solyc05g005570</td>
<td>-1.7</td>
<td>Mannan endo-1 4-beta-mannosidase</td>
</tr>
<tr>
<td></td>
<td>Solyc10g009300</td>
<td>-1.7</td>
<td>Endo-1 4-beta-xylanase</td>
</tr>
<tr>
<td></td>
<td>Solyc06g064520</td>
<td>-1.7</td>
<td>Mannan endo-1 4-beta-mannosidase</td>
</tr>
<tr>
<td></td>
<td>Solyc04g072850</td>
<td>-2.2</td>
<td>Endoglucanase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc05g051260</td>
<td>-2.3</td>
<td>Endoglucanase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc01g109500</td>
<td>-2.4</td>
<td>Endo-1 4-beta-glucanase</td>
</tr>
<tr>
<td></td>
<td>Solyc05g005550</td>
<td>-4.5</td>
<td>Endoglucanase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc01g008720</td>
<td>-6.6</td>
<td>Endoglucanase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc05g052530</td>
<td>-7.2</td>
<td>Endoglucanase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc02g062320</td>
<td>-7.9</td>
<td>Endoglucanase 1</td>
</tr>
<tr>
<td>10.7: Cell wall modification (17/35/81)*</td>
<td>Solyc06g051180</td>
<td>1.8</td>
<td>Expansin</td>
</tr>
<tr>
<td></td>
<td>Solyc06g060970</td>
<td>1.2</td>
<td>Expansin</td>
</tr>
<tr>
<td></td>
<td>Solyc07g054170</td>
<td>-1.1</td>
<td>Expansin B1</td>
</tr>
<tr>
<td></td>
<td>Solyc09g010860</td>
<td>-1.6</td>
<td>Expansin</td>
</tr>
<tr>
<td></td>
<td>Solyc07g009380</td>
<td>-2.3</td>
<td>Xyloglucan endotransglucosylase 5</td>
</tr>
<tr>
<td></td>
<td>Solyc01g099630</td>
<td>-2.4</td>
<td>Xyloglucan endotransglucosylase 1</td>
</tr>
<tr>
<td></td>
<td>Solyc02g091920</td>
<td>-3.1</td>
<td>Xyloglucan endotransglucosylase 2</td>
</tr>
<tr>
<td></td>
<td>Solyc09g008320</td>
<td>-3.2</td>
<td>Xyloglucan endotransglucosylase 12</td>
</tr>
<tr>
<td></td>
<td>Solyc03g115310</td>
<td>-3.6</td>
<td>Expansin</td>
</tr>
<tr>
<td></td>
<td>Solyc07g048540</td>
<td>-6.8</td>
<td>Expansin B5</td>
</tr>
<tr>
<td></td>
<td>Solyc11g017450</td>
<td>-8.4</td>
<td>Xyloglucan endotransglucosylase 1</td>
</tr>
</tbody>
</table>
Mejia (2016) was based on the analysis of primary transfor-
mants (T0 lines) and these authors did not check whether
t heir RNAi strategy affected the second member of the class
D clade (Sl-MBP3) whose nucleic acid sequence shares 85%
identity with Sl-AGL11. The absence of a strong seed pheno-
type in our down-regulated lines is consistent with the data
reported in petunia, where a single knockout of class D FBP7
or FBP11 did not result in a seedless phenotype whereas ma-
jor seed defects were visible with simultaneous FBP7/FPB11
down-regulation (Angenent et al., 1995; Colombo et al., 1997;
Heijmans et al., 2012). Such redundancies were also reported
in Arabidopsis (Pinyopich et al., 2003) and rice (Dreni et al.,
2011). In Arabidopsis, redundant activities in the promotion
of ovule identity were suggested since ovule and seed de-
velopment were only abolished in the triple stk/shp1/shp2 mutant
(Pinyopich et al., 2003). Altogether, these data suggest a partial
redundancy among class D genes that varies among plant spe-
cies, which is consistent with the similar expression pattern of
Sl-AGL11 and Sl-MBP3 in young developing fruits.
In contrast to the down-regulated lines, Sl-AGL11-
overexpressing lines exhibited dramatic flower and fruit mod-
ifications, notably sepal swelling and conversion into a fleshy
organ that eventually underwent a typical ripening process.
Our transcriptome analyses highlighted the extent of sepal
reprogramming and confirmed that the ectopic expression of
Sl-AGL11 is the causal element as other AGAMOUS genes
remained almost unaffected. The sepal conversion into a suc-
culent organ is reminiscent of different phenotypes obtained
with ectopic expression of different class C AGAMOUS
genes in tomato such as TAG1 (Pnueli et al., 1994), TAGL1
(Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al.,
2010), peach Plena (Tadiello et al., 2009), grape VviAGL11
(Mellway and Lund, 2013), and the Ginkgo biloba GBM5
gene (Lovisetto et al., 2015). In all these studies, the sepal
identity modification is often interpreted as a partial conser-
vation of the class C function, similarly to the conversion of
sepal to carpelloid structure in Arabidopsis (Mizukami and
Ma, 1992), tobacco (Kempin et al., 1993), or petunia (Van
Der Krol and Chua, 1993; Kater et al., 1998). Yet, our data
support the idea that the C function of Sl-AGL11 is still
incomplete. This is consistent with the absence of petal modi-
fications, in contrast to tomato lines overexpressing class C
TAGI and TAGLI MADS-box genes (Pnueli et al., 1994;
Vrebalov et al., 2009). Likewise, no flowering delay occurred,
in contrast to Arabidopsis plants overexpressing AGAMOUS
or STK that exhibited mild early flowering (Mizukami and
Ma, 1997; Favaro et al., 2003). It is also important to high-
light the differences between Sl-AGL11OE tomatoes and
petunia or Arabidopsis plants overexpressing FBP11 or STK
class D genes, respectively (Colombo et al., 1995; Favaro
et al., 2003). Indeed, no ovule-like structures were found on
the Sl-AGL11OE sepal, whereas the ectopic expression of
class D genes in petunia and Arabidopsis resulted in a fail-
ure to form any carpelloid organ structure. These differences
may be interpreted as the consequence of the ‘fleshy’ back-
ground found in tomato. Moreover, the conversion of sepal
into fleshy organs suggests that Sl-AGL11 acts as a class C/D
MADS-box gene.
Since Sl-AGL11 overexpression phenotypes suggested
only a partial conservation of class C function, analyzing
the similarities and differences between Sl-AGL11OE plants
and TAGI- or TAGLI-overexpressing plants should provide
leads to uncovering a specific signature of Sl-AGL11 action.
The comparison of Sl-AGL11OE phenotypes with those
reported in TAGLI experiments (Itkin et al., 2009) reveals
similar dynamics of sepal conversion. That is, swelling starts
at the basis of the calyx in the intersepal tissue and ripening
of Sl-AGL11OE fruits and sepal matches that of TAGLI-
overexpressing fruits. Regarding sugar metabolism, the data
on Sl-AGL11OE plants converge with those reported for
TAGLI-overexpressing tomatoes which indicated an increase
in Brix (Giménez et al., 2010). The enhanced starch pheno-
type is also consistent with TAGLI RNAi experiments report-
ing a depletion of starch in the pericarp of immature fruits
(Vrebalov et al., 2009). In contrast, two features seem specific
to Sl-AGL11 overexpression and may be considered as a dis-

tinctive signature: placenta and columella hypertrophy and
the extreme softening at the early stage of fruit development.
Regarding columella and placenta hypertrophy, it is impor-
tant to mention that these tissues represent high Sl-AGL11
expression domains (Fig. 1A). While this may suggest that
class D MADS-box genes control the differentiation of
the inner tissues of tomato fruits, the biological significance
of this signature must be interpreted cautiously since ectopic
expression of MADS-box genes can act either by triggering
abnormal signaling pathways in tissues where Sl-AGL11 is
normally absent or by creating competition with endogenous
MADS-box factors within the tetramereric complex or during

### Table 1. Continued

<table>
<thead>
<tr>
<th>MAPMAN category</th>
<th>Solyc number</th>
<th>Log2 fold</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.8.1: Phosphomethylesterase (8/24/86)*</td>
<td>Solyc03g083360</td>
<td>–1.5</td>
<td>Pectinesterase</td>
</tr>
<tr>
<td></td>
<td>Solyc03g123630</td>
<td>–1.5</td>
<td>Pectinesterase</td>
</tr>
<tr>
<td></td>
<td>Solyc07g017600</td>
<td>–2.2</td>
<td>Pectinesterase</td>
</tr>
<tr>
<td></td>
<td>Solyc12g098340</td>
<td>–2.3</td>
<td>Pectinesterase</td>
</tr>
<tr>
<td></td>
<td>Solyc06g009190</td>
<td>–4.2</td>
<td>Pectinesterase</td>
</tr>
<tr>
<td></td>
<td>Solyc09g091730</td>
<td>–4.5</td>
<td>Pectinesterase</td>
</tr>
<tr>
<td></td>
<td>Solyc08g075020</td>
<td>–1.3</td>
<td>Pectinacylesterase</td>
</tr>
<tr>
<td></td>
<td>Solyc08g005800</td>
<td>–5.6</td>
<td>Pectinacylesterase-like</td>
</tr>
</tbody>
</table>

*Number of genes in each subcategory, DEGs in Sl-AGL11OE versus WT fruits/total expressed genes in fruit/total genes in tomato genome.
import in the nucleus (Smaczniak et al., 2012; Dreni and Kater, 2014).

The extreme softening of Sl-AGLI11OE fruits and the different histological staining reflect major cell wall modifications. Analyzing the RNA-Seq data by the MAPMAN annotation tool identified the functional ‘Cell wall’ category as being clearly enriched in the Sl-AGLI11OE versus WT fruit experiment. Cell wall modifications have been largely studied in ripening-associated softening (Seymour et al., 2013). Among the cell wall-related genes, pectin-modifying genes, cellulose synthesis genes, and xyloglucan-modifying enzymes were particularly affected (Fig. 7C; Supplementary Fig. S14).

Strikingly, the modifications observed were not linked to the softening genes active upon fruit ripening (PG2A, β-GAL4, PL2, and EXP1) but to cell wall-related genes expressed earlier during fruit development such as XTH1, PAE-like, and CS-like. The data support the view of the acquisition of a new metabolic differentiation program leading to a different cell wall structure which induces tissue softening at early stages of fruit development. Indeed, the expression of genes known to play a major role in cell wall degradation such as polygalacturonase, β-galactosidase, expansin, and pectate lyase PL2 (Grierson et al., 1986; Brumwell et al., 1999; Smith et al., 2002; Uluisik et al., 2016) was very limited during early development of Sl-AGLI11OE fruit and therefore cannot account for the extreme softening already taking place in green fruits. In contrast, XTH, PAE, PME, and Cellulose Synthase displayed abnormal expression patterns in Sl-AGLI11OE fruits and may be considered as promising candidates for green fruit-associated cell wall modifications. Indeed, XTH has been previously reported in different fleshy fruits such as pears, litchis, kiwis, apples, and strawberries to be associated with cell wall loosening (Miedes and Lorences, 2009).

In tomato, heterologous expression of the Sl-AGLI11OE tobacco homolog reduced softening (Miedes et al., 2011). Several genes coding for pectin-modifying enzymes were also down-regulated in Sl-AGLI11OE fruits including pectin methyltransferases (Solyc06g009190, Solyc07g017600, Solyc12g009270, and Solyc03g123630) and a PAE-like gene (Solyc08g005800). The altered expression of these genes, notably those involved in pectin methylesterification, may contribute to the extreme softening of Sl-AGLI11OE fruits since pectin modification usually occurs during the expansion phase of young fruits (Terao et al., 2013). What is more, in the Arabidopsis class D MADS-box stk mutant, which displays abnormal differentiation of the cell wall matrix, the homeotic STK transcription factor directly controls a molecular network regulating cell wall properties in seed coats (Mizotto et al., 2014; Ezquer et al., 2016). This network includes AtPME16, Cellulose Synthase CESAs5 and CESAs2, Cellulose Synthase-like CSLA2, COBRA-LIKE COBL2, and MYB61. Interestingly, all the tomato closest homologs of these Arabidopsis genes (Solyc04g071650, Solyc10g083670, Solyc06g074630, Solyc11g066820, Solyc02g06577, and Solyc01g102340) were found to be differentially expressed in Sl-AGLI11OE fruits (Supplementary Table S1). Taken together, both Arabidopsis STK and tomato Sl-AGLI11 class D MADS-box genes seem to control cell wall differentiation programs.

The conversion of sepal into an intermediary organ between leaf and fleshy carpel offers a model to decipher the early mechanisms involved in the acquisition of the fleshy character. The convergences and divergences between class C and Sl-AGLI11 emphasized here might provide a useful tool to evaluate the functional evolution and action modes of the AGAMOUS family of transcription factors. Implementation of ChIP-seq strategies might allow uncovering of the conserved target genes and provide clues as to how the different isoforms have acquired their specialization during flowering plant evolution. In that perspective, the functional characterization of Sl-MBP3 becomes essential to complete the picture of the tomato class C/D MADS-box gene family. With a prospect of applications, the present study highlights the impact of Sl-AGLI11 on several fruit quality traits, notably the increase of sugar content and the modification of fruit firmness. Identifying the downstream components of Sl-AGLI11 will provide leads towards understanding the determinants of sink strength and fruit firmness, and might uncover new mechanisms controlling fruit quality and productivity that could ultimately be used in breeding programs.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Phylogenetic tree of AGAMOUS-like MADS-box genes.

Fig. S2. Expression pattern of different MADS genes (TAGL1, TAGL1, Sl-AGLI11, Sl-MBP3, RIN) deduced from the RNA-Seq data.

Fig. S3. Subcellular localization of Sl-AGLI11 protein.

Fig. S4. Morphology of nine independent tomato lines with RNAi-mediated down-regulation of Sl-AGLI11.

Fig. S5. Ruthenium red and vanillin staining of seeds in the wild type (WT) and Sl-AGLI11-RNAi line.

Fig. S6. Expression level of four Agamous genes in DPA10 fruits measured by qPCR.

Fig. S7. Plant size of Sl-AGLI11OE plants.

Fig. S8. Flower phenotypes of Sl-AGLI11OE lines.

Fig. S9. Abscission zone and pedicel swelling in an Sl-AGLI11OE plant.

Fig. S10. Fruit size, fruit weight, and number of cell layers in pericarp of Sl-AGLI11-overexpressing lines.

Fig. S11. Timing of flower initiation in WT and Sl-AGLI11OE lines.

Fig. S12. Pollination efficiency of Sl-AGLI11-overexpressing plants.

Fig. S13. Water loss and water content in tomato fruits overexpressing Sl-AGLI11.

Fig. S14. MAPMAN enrichment of different functional categories in Sl-AGLI11OE sepals and fruits.

Table S1. List of cell wall-related DEGs in Sl-AGLI11OE versus WT fruit with known homologs affected in the Arabidopsis STK mutant.

Table S2. List of primers used in this study.

Data S1. Number of counts in RNA-Seq experiments performed on fruits and sepals harvested at the DPA10 stage.
Data S2. List of differentially expressed genes in the Sl-AGLI11OE versus WT fruits harvested at the DPA10 stage.

Data S3. List of differentially expressed genes in the Sl-AGLI11OE versus WT sepals harvested at the DPA10 stage.

Acknowledgements

The authors are grateful to L. Lemampion and D. Saint-Martin for the cultivation of tomato plants, to GetPlaGe for deep sequencing, and to GenoToulBioinfo for computing facilities. This research was supported by the Labex TULIP (ANR-10-LABX-41), by the TomGEM H2020 project, by the National Key Research and Development Program (2016YFD0400101), and by the National Natural Science Foundation of China (31572175). The work benefited from the networking activities within the European COST Action FA1106. BH was the beneficiary of the ERASMUS MUNDUS program.

Author contributions

BH performed the experiments and contributed to the drafting of the article; WD and ML helped in generating the transgenic lines; IM carried out subcellular localization experiments; JV and JG contributed to the writing and the critical analysis of the results; GH, MZ, EM, and PF contributed to the RNA-Seq experiment; JMR contributed to the RNA-Seq data processing, to the critical analysis, and the drafting of the manuscript; ZL, BVDR, and MB designed the study and wrote the manuscript.

References


Maza E. 2016. In ppyro comparison of TMM (edgeR), RLE (DESeq2), and MRN normalization methods for a simple two-conditions-without-replicates RNA-seq experimental design. Frontiers in Genetics 7, 164.


Ng M, Yanofsky MF. 2001. Floral and vegetative gene expression in transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. The Plant Journal 1, 95–100.


