Plant Mol Biol (2016) 91:513–531
DOI 10.1007/s11103-016-0485-4

TOMATO AGAMOUS1 and *ARLEQUIN/TOMATO AGAMOUS-LIKE1* MADS-box genes have redundant and divergent functions required for tomato reproductive development

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Received: 5 August 2015 / Accepted: 20 April 2016 / Published online: 28 April 2016
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Abstract Within the tomato MADS-box gene family, *TOMATO AGAMOUS1* (*TAG1*) and *ARLEQUIN/TOMATO AGAMOUS LIKE1* (hereafter referred to as *TAGL1*) are, respectively, members of the euAG and PLE lineages of the AGAMOUS clade. They perform crucial functions specifying stamen and carpel development in the flower and controlling late fruit development. To gain insight into the roles of *TAG1* and *TAGL1* genes and to better understand their functional redundancy and diversification, we characterized single and double RNAi silencing lines of these genes and analyzed expression profiles of regulatory genes involved in reproductive development. Double RNAi lines did show cell abnormalities in stamens and carpels and produced extremely small fruit-like organs displaying some sepaloid features. Expression analyses indicated that *TAG1* and *TAGL1* act together to repress fourth whorl sepal development, most likely through the *MACROCALYX* gene. Results also proved that *TAG1* and *TAGL1* have diversified their functions in fruit development: while *TAG1* controls placenta and seed formation, *TAGL1* participates in cuticle development and lignin biosynthesis inhibition. It is noteworthy that both *TAG1* and double RNAi plants lacked seed development due to abnormalities in pollen formation. This seedless phenotype was not associated with changes in the expression of B-class stamen identity genes *Tomato MADS-box 6* and *Tomato PISTILLATA* observed in silencing lines, suggesting that other regulatory factors should participate in pollen formation. Taken together, results here reported support the idea that both redundant and divergent functions of *TAG1* and *TAGL1* genes are needed to control tomato reproductive development.

Keywords Functional diversification · Redundancy · Reproductive development · *Solanum lycopersicum* · *TAG1* · *TAGL1*

Electronic supplementary material The online version of this article (doi:10.1007/s11103-016-0485-4) contains supplementary material, which is available to authorized users.

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Introduction

The reproductive development program of higher plants comprises several processes from floral meristem determination and floral bud generation to fruit development and ripening, all of them leading to seed formation and dispersal to ensure progeny survival. Transcriptional control of reproductive development involves several gene families with the MADS-box family being one of the most important as its members are widely conserved across angiosperm species and play key roles in most reproductive developmental processes (Ng and Yanofsky 2000; Gramzow and Theissen 2010). Several duplication and diversification events have affected MADS-box family during its evolutionary history. Some of these changes took place before the divergence of plants, animals, and fungi resulting in two main functional types (Alvarez-Buylla et al. 2000b). Type II MADS-box genes from land plants

subsequently diverged into two other groups, MIKC* and MIKC[c] (Henschel et al. 2002). Extensive studies in *Arabidopsis* demonstrated that the MIKC* MADS-box genes have conserved partially redundant roles in the development of the male gametophyte, where they are mainly expressed (Verelst et al. 2007a, b; Adamczyk and Fernandez 2009). However, the MIKC[c] MADS-box genes regulate sporophytic development and particularly floral organ identity (Becker and Theissen 2003).

Floral development is regulated by gene functions acting alone or in combination so as to specify organ identity in the four floral whorls, as defined by the widely known ABC model (Bowman et al. 1991; Coen and Meyerowitz 1991). Thus, A-function genes determine sepal identity in whorl 1, combined functions A/B and B/C determine petal and stamen identities in the second and third whorl, respectively, and C-function genes regulate carpel development in the fourth whorl. The ABC model also establishes mutually antagonistic functions of A and C genes, for the proper development of the four floral organs (Bowman et al. 1991; Coen and Meyerowitz 1991). In fact, *Arabidopsis* mutant plants affected in the A-function gene *APETALA1* lack sepal organs in the first floral whorl (Mandel et al. 1992), and mutants of the C-function gene *AGAMOUS* (*AG*) promoted homeotic conversion of carpels into sepals which in turn initiated the development of a new flower in the fourth floral whorl (Bowman et al. 1989). In addition, expression studies performed in *Arabidopsis* and petunia have shown that appropriate transcriptional levels of A and C class genes are required for the maintenance of B-function genes (Gómez-Mena et al. 2005; Heijmans et al. 2012).

Ancestral functions of *AG* genes have been suggested as regulators of male and female reproductive organs (Theissen et al. 2000; Kramer et al. 2004). However, several duplication and diversification events in the AG lineage have favoured the acquisition of new roles across angiosperm evolutionary development (Ng and Yanofsky 2000; Becker and Theissen 2003; Gramzow and Theissen 2010). For example, a recent duplication in the AG clade resulted in the euAG and PLENA (PLE) sub-lineages within the core eudicots (Kramer et al. 2004), an event that has been studied in diverse species. In *Antirrhinum majus*, *PLE* is necessary for stamen and carpel development, whereas *FARINELLI* (euAG lineage) seems to be involved only in pollen development (Carpenter and Coen 1990; Bradley et al. 1993; Davies et al. 1999). In *Arabidopsis*, *AG* (euAG lineage) establishes stamen and carpel identities and also controls floral meristem determinacy (Yanofsky et al. 1990; Favaro et al. 2003). Two paralagous genes in *Arabidopsis*, *SHATTERPROOF1* and *SHATTERPROOF2* (*SHP1* and *SHP2*), resulting from a duplication in the PLE lineage, are required for dehiscence zone formation during late stage fruit development, indicating that novel functions have been acquired by these PLE derived genes to regulate fruit development (Liljegren et al. 2000; Pinyopich et al. 2003). In further support of an ancestral role for *PLE*-like genes in late fruit development, a conserved role of *PLE*-like genes in regulating fruit dehiscence has recently been proposed in another dry-fruited species *Nicotiana benthamiana* (Fourquin and Ferrándiz 2012).

In tomato, the floral phenotype of antisense transgenic lines suggested that the euAG gene *TOMATO AGAMOUS 1* (*TAG1*) could be involved in specifying stamen and carpel identities, as well as in floral meristem determinacy (Pnueli et al. 1994). Recently, characterization of *TAG1* RNAi silencing lines showed that alterations in carpel development only included the loss of floral meristem determinacy; neither homeotic transformations nor other cell identity changes occurred in the lines during carpel development (Pan et al. 2010). This suggests that *AG* genes from *Arabidopsis* and tomato have diverged in their carpel identity related functions. The fact that a complete loss of carpel identity has not been observed in *TAG1* knock-down lines also suggested that other factors are necessary for C-function in tomato, with *PLE*-like genes being the first option to consider. Indeed, euAG and PLE lineage genes were found to share C-function specification in two other Solanaceae species, *N. benthamiana* and petunia (Kapoor et al. 2002; Fourquin and Ferrándiz 2012; Heijmans et al. 2012), and a similar role for these genes has been suggested in tomato (Vrebalov et al. 2009; Gimenez et al. 2010).

The representative gene of PLE lineage in tomato, *TOMATO AGAMOUS LIKE1* (*TAGL1*), has acquired novel functions with respect to its *Arabidopsis SHP* homologues (Vrebalov et al. 2009). Pericarp of fruit from *TAGL1* silencing plants showed altered cellular and structural properties associated with the expression of genes regulating the cell cycle and lignin biosynthesis (Gimenez et al. 2010), which confirmed the important role played by *TAGL1* in fleshy fruit expansion (Itkin et al. 2009; Vrebalov et al. 2009). Moreover, several ripening characteristics such as ethylene production and fruit stiffness, as well as carotenoid metabolism, also depend on the transcriptional activity of *TAGL1* (Itkin et al. 2009; Vrebalov et al. 2009; Gimenez et al. 2010).

The crucial role of *TAGL1* in fruit ripening has been demonstrated not only in tomato but also in other fleshy fruited species such as grapevine and peach (Boss et al. 2001; Tani et al. 2007; Tadiello et al. 2009; Mellway and Lund 2013). These studies have also shown a key role for the *PLE* orthologs in fruit ripening, suggesting that *PLE* genes could have more relevant roles in this process than in reproductive organ specification or in early stages of fruit development (Tadiello et al. 2009; Mellway and Lund 2013). Likewise, recent studies in *N. benthamiana*, whose fruits are dry capsules, have shown that PLE lineage genes have conserved their contribution to the fruit dehiscence in addition to their

roles in carpel identity. These results suggest that late processes occurring during fruit development of dry and fleshy species, i.e. dehiscence and ripening may have a common evolutionary origin (Fourquin and Ferrándiz 2012).

In tomato, the lack of stable null mutants has prevented a detailed analysis of the functional interactions of *TAG1* and *TAGL1* genes. In this work, we performed a detailed functional analysis of the *TAG1* and *TAGL1* tomato genes through the phenotypic and molecular characterization of single and double RNAi silencing lines. This allowed us to demonstrate cooperative functions of both genes not only in the specification of carpel identity but also in pollen maturation, thus unravelling their respective contributions to C-function in addition to their specific role in fruit development and ripening.

Results

TAG1 and *TAGL1* MADS-box genes are, respectively, members of the euAG and PLE lineages, which resulted from the duplication of tomato AG clade (Kramer et al. 2004). Both perform important functions during flower and fruit development, as in fruit ripening of this model species (Pnueli et al. 1994; Itkin et al. 2009; Vrebalov et al. 2009; Gimenez et al. 2010; Pan et al. 2010). However, the lack of stable null mutants of *TAG1* and *TAGL1* genes and the infertility of *TAG1* knock-down lines reported so far (Pnueli et al. 1994; Pan et al. 2010) have hindered the generation of double mutants, which in turn has prevented further studies of their roles during tomato reproductive development. To gain insight into the functional overlapping and divergence between both MADS-box transcriptional factors, we have generated tomato lines which silenced *TAG1* and *TAGL1* genes simultaneously. With this aim, we selected and crossed two RNAi parent lines showing the lowest level of gene expression, i.e. *TAG1* RNAi 46a and *TAGL1* RNAi 12b lines (Fig. 2j, k). Double *TAG1–TAGL1* RNAi lines were further characterized together with single RNAi lines, and comparative developmental and gene expression analyses were performed.

TAGL1 and *TAG1* are differentially expressed during fruit development and ripening

Expression studies (Fig. 1) supported the expression patterns of *TAG1* and *TAGL1* genes previously described during fruit development and ripening stages of wild-type (WT) plants (Gimenez et al. 2010; Pan et al. 2010). In situ hybridization assays showed overlapping expression of *TAG1* and *TAGL1* genes in floral buds, being both early expressed in carpel and stamen primordia (Fig. 1a–d). However, time-course experiments showed differences in the expression levels of both genes throughout fruit development and ripening. While transcript levels of *TAGL1* were maintained throughout fruit development, from flower anthesis day (AD) to fruit red ripe (10 days after breaker stage, BR+10) stages (Fig. 1h), *TAG1* expression was detected in AD but decreased at early stage of fruit development before increasing at ripening (Fig. 1g). In the transgenic lines generated in this work, expression analyses showed that relative to WT, *TAG1* was up-regulated at breaker (BR) and BR+10 stages in *TAGL1* RNAi plants (Fig. 1g), whereas *TAGL1* expression was not affected in *TAG1* RNAi plants (Fig. 1h). This suggests that *TAG1* may be up-regulated to compensate for the lack of *TAGL1* expression. As expected, the double silencing lines showed no expression of the targeted *TAG1* and *TAGL1* genes (Fig. 1g, h).

Double *TAG1–TAGL1* silencing lines showed developmental alterations of reproductive floral organs

Compared to WT plants, morphological abnormalities or homeotic alterations were not observed in floral buds or in mature floral organs of *TAG* RNAi and *TAGL1* RNAi lines (Fig. 2a–c, e–g), even though expression levels of *TAG1* and *TAGL1* were significantly diminished in the respective lines (Fig. 2j, k). In accordance, scanning electron microscopy (SEM) analyses performed in flowers at AD stage did not show significant identity changes of epidermal cells covering floral organs of single RNAi plants (Fig. 3a–c, e–h, j–l). In double RNAi lines, floral organs were normal in appearance with the exception of whitish coloured stamens instead of the characteristic yellow colour (Fig. 2d, h, i). However, SEM analyses revealed some developmental alterations affecting the third (stamens) and fourth (carpels) floral whorls of double *TAG1–TAGL1* RNAi plants. Epidermal cells of stamens were larger and more rounded than WT ones, and they seemed to show less cell adhesion (Fig. 3a, d), likely due to changes in cell shape. In addition, epidermal cells of the style (whorl 4) lacked the surface folds specific to adult cells (Fig. 3f); instead, their morphology resembled cells at early stages of cell differentiation (Fig. 3e, i). These results indicated that simultaneous down-regulation of *TAG1* and *TAGL1* altered developmental features of reproductive floral organs.

Simultaneous repression of *TAG1* and *TAGL1* inhibited fruit development

Tomato fruit development is characterized by an active cell division phase followed by a cell growth and differentiation phase, both affecting carpel tissues. Fruits produced by tomato plants silencing *TAG1* were smaller and weighed less than WT plants not only at mature green (MG) stage (Fig. 4a, b;

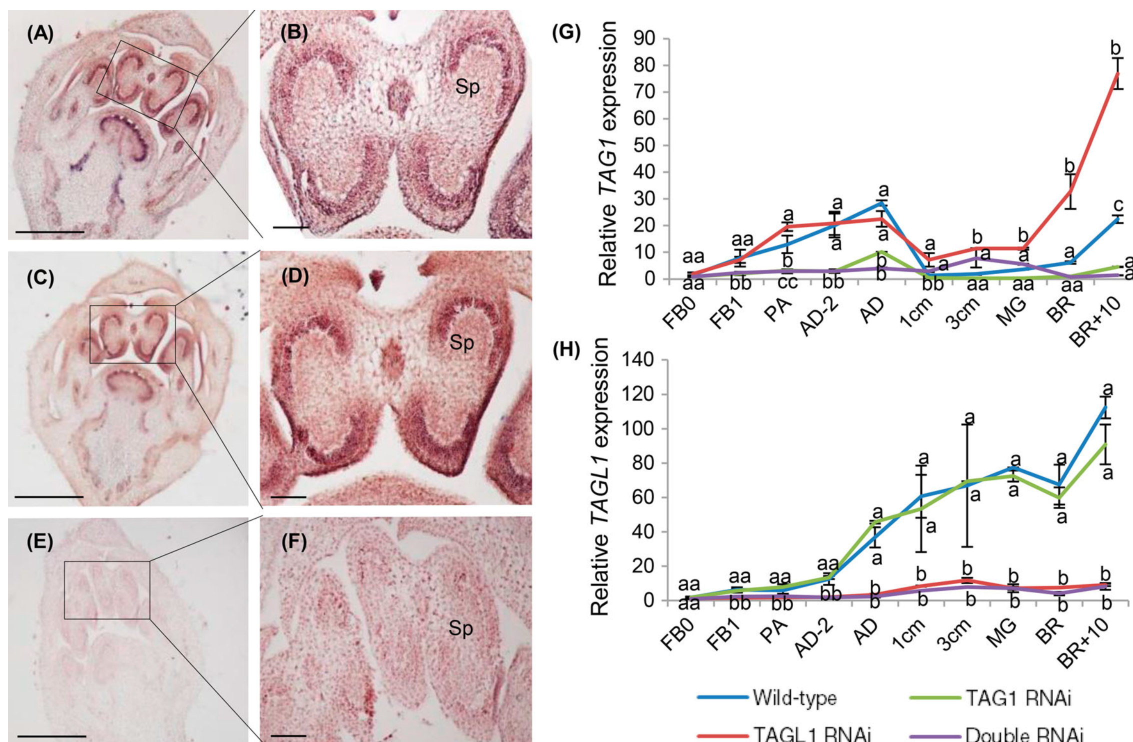


Fig. 1 Expression analyses of *TAG1* and *TAGL1* genes. **a–f** Tissue sections of WT floral buds (cv. Moneymaker, MM) were hybridized with *TAG1* antisense (**a**), *TAGL1* antisense (**c**) and *TAGL1* sense (**e**) probes. Details of stamens from **a**, **c** and **e** are shown in **b**, **d** and **f** respectively. *Scale bars* represent 500 µm in (**a**, **c**, **e**) and 100 µm in (**b**, **d**, **f**). Sp: sporogenous tissue. **g–h** Relative expression of *TAG1* (**g**) and *TAGL1* (**h**) genes in flowers and fruits from WT, *TAG1* RNAi, *TAGL1* RNAi and double RNAi plants at several stages of reproductive development: flowers at floral bud 0 (FB0), floral bud 1 (FB1), pre-anthesis (PA), 2 days before anthesis day (AD-2); anthesis day (AD), 1 cm-wide fruits (1 cm), 3 cm-wide fruits (3 cm), and fruits at mature *green* (MG), breaker (BR) and 10 days after breaker (BR+10) stages. *Data* are means of three biological replicates ± standard error of the mean. Statistical analysis was performed by comparing data from floral tissues at the same developmental stage. Values followed by the *same letter* (**a**, **b**, **c**) are not statistically significant ($P < 0.01$)

Table 1) but at all other developmental stages. Although the expression of cell cycle genes were not altered in *TAG1* RNAi pericarps (Fig. 4m), *TAG1* RNAi fruits displayed a small reduction in pericarp thickness (Table 1), which may be related to their decreased size. Transverse sections of these fruits at MG stage showed a complete inhibition of seed development (Fig. 4e, f), although ovules were observed at early stages of carpel development (Fig. 7b). In addition, *TAG1* repressed fruits lacked placenta development and developed thick septa separating fruit locules (Fig. 4e, f). In contrast, fruit weight and size were not altered in *TAGL1* RNAi plants, placenta tissue was fully developed and fruits were completely fertile (Fig. 4a, c, e, g; Table 1). However, a decreased thickness of fruit pericarp was observed in *TAGL1* silenced plants (Fig. 4e, g; Table 1), which agreed with decreased expression of cell cycle genes *Cyclin-dependent Kinase A* (*CDKA1*) and *Cyclin A* (*CycA1*) (Fig. 4m), both involved in early development of tomato fruit (Joubes et al. 1999; Joubes et al. 2000). Furthermore, phloroglucinol staining showed that repression of *TAGL1* promoted a greater deposition of lignin in fruit pericarp (Fig. 4i, k), as previously reported by Gimenez et al. (2010). It is interesting to note that none of these characteristics, i.e. pericarp lignification and expression of cell cycle genes, were altered in *TAG1* RNAi fruits (Fig. 4i, j, m), suggesting that *TAG1* and *TAGL1* regulate different aspects of fruit development in tomato.

To gain insight into the functional divergence of *TAG1* and *TAGL1* genes during fruit development, we further characterized the fruit of the double RNAi lines silencing both genes. Dual repression of both MADS box genes led to a complete lack of fruit setting, although fruit-like organs did develop. The development of these pseudo-fruits was initiated independently of pollination occurrence and 60 days later than either WT or single RNAi fruits. In addition, fruit development in the double RNAi lines was blocked at early stages, with repression of *TAG1* and *TAGL1* having additive and synergistic effects. In fact, the final fruit size of double RNAi lines was significantly smaller by nearly three times than WT fruits (Table 1). They also weighed less by 22-fold than WT fruits and fivefold less than *TAG1* RNAi fruits (Fig. 4a, d; Table 1). In addition, thickness of fruit pericarp was severely reduced and lignin deposition was highly

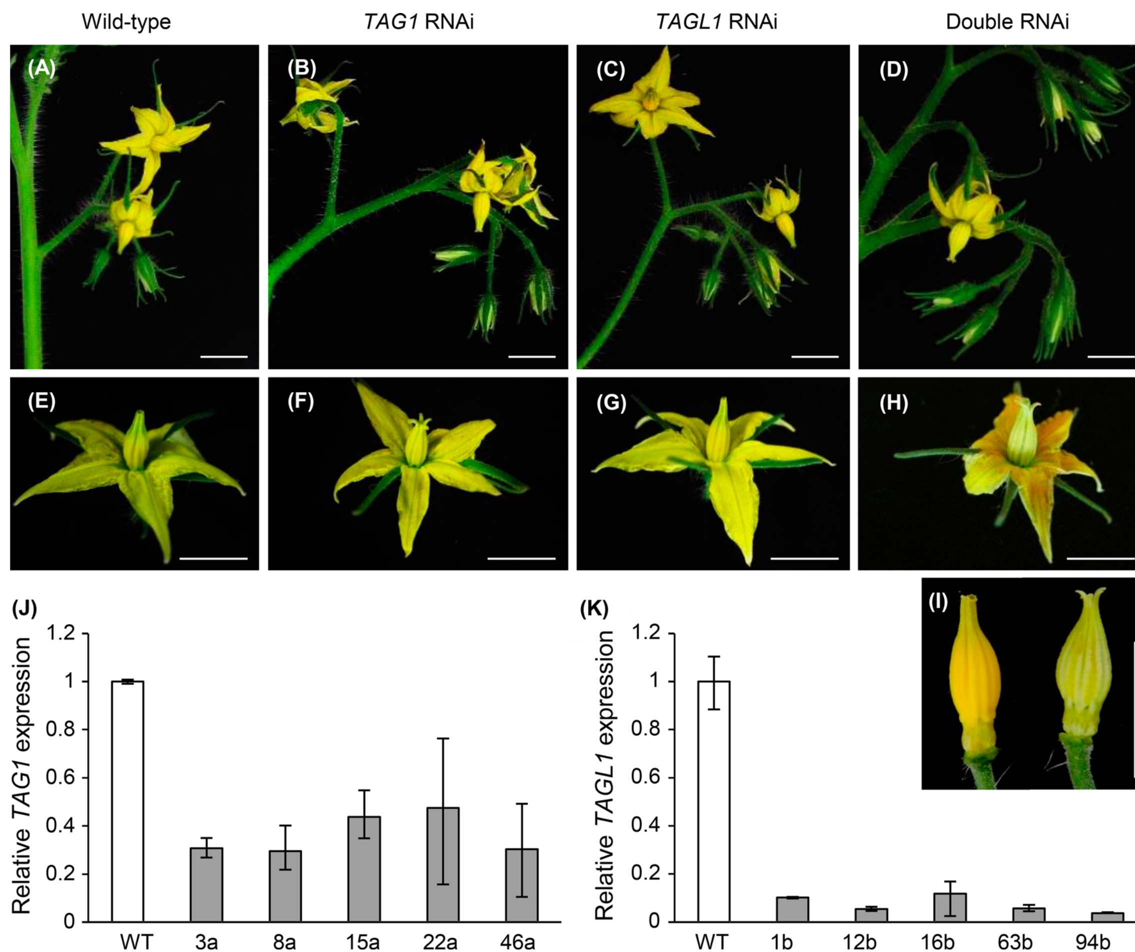


Fig. 2 Flower development and gene expression analyses in *TAG1*, *TAGL1* and double *TAG1–TAGL1* silencing lines. **a–h** Inflorescence architecture (**a–d**) and flower morphology at anthesis day stage (**e–h**) of WT (**a**, **e**), *TAG1* RNAi (**b**, **f**), *TAGL1* RNAi (**c**, **g**) and double silencing (**d**, **h**) plants. **i** Isolated staminal cone from WT and double RNAi flowers in order to observe *colour* changes of double RNAi stamens. *Scale bars* in (**a–i**) = 1 cm. **j** Relative expression of *TAG1* in flowers at anthesis day stage from WT (*white bar*) and several *TAG1* silencing lines (*grey bars*). **k** Relative expression of *TAGL1* in flowers at anthesis day stage from WT (*white bar*) and several *TAGL1* RNAi lines (*grey bars*). *Data* are means of three biological replicates ± standard error of the mean

increased as compared to WT fruits (Fig. 4h, l; Table 1). Both of these characteristics showed even stronger differences relative to WT than those observed in *TAGL1* RNAi plants (Fig. 4). However, placenta tissue seemed to develop normally in double RNAi fruits as observed in *TAGL1* RNAi fruits (Fig. 4g, h), although seed formation was completely avoided, as occurred in *TAG1* RNAi fruits (Fig. 4f, h). Reduced pericarp thickness is consistent with the down-regulation of *CDKA1* and *CycA1*, which is also observed in *TAGL1* RNAi fruits (Fig. 4m) but not in TAG1 RNAi, indicating that additional fruit-growth factors regulated by *TAG1* repression should participate in fruit development.

At the tissue level, dual repression of *TAG1* and *TAGL1* altered the cell division and growth pattern of carpel tissues, which agreed with the inhibition of fruit growth described above (Fig. 5a, b). Thus, at the floral bud stage, cells and tissues forming carpel organs showed similar morphology and layer distribution to WT ones (Fig. 5d, e). However, significant differences were observed later at AD stage: while WT carpel cells initiated growth by active cell division, double RNAi carpels were arrested in division and no evidence of growth was observed (Fig. 5g, h). This growth arrest affected mainly exocarp and endocarp tissues and was even more evident 10 days after anthesis (AD+10) (Fig. 5i, j); however, vascular tissues developed normally. At MG stage, WT fruits showed clear differentiation of cell layers that form fruit pericarp (i.e. epidermis, collenchyma and parenchyma) and suppression of development of vascular bundles (Fig. 5k). It is noteworthy that none of these developmental features were observed in double RNAi fruits (Fig. 5l), confirming their inability to grow and properly develop. Indeed, double silencing of *TAG1* and *TAGL1* prevented differentiation of collenchyma cell layers below the external epidermis and hence, exocarp

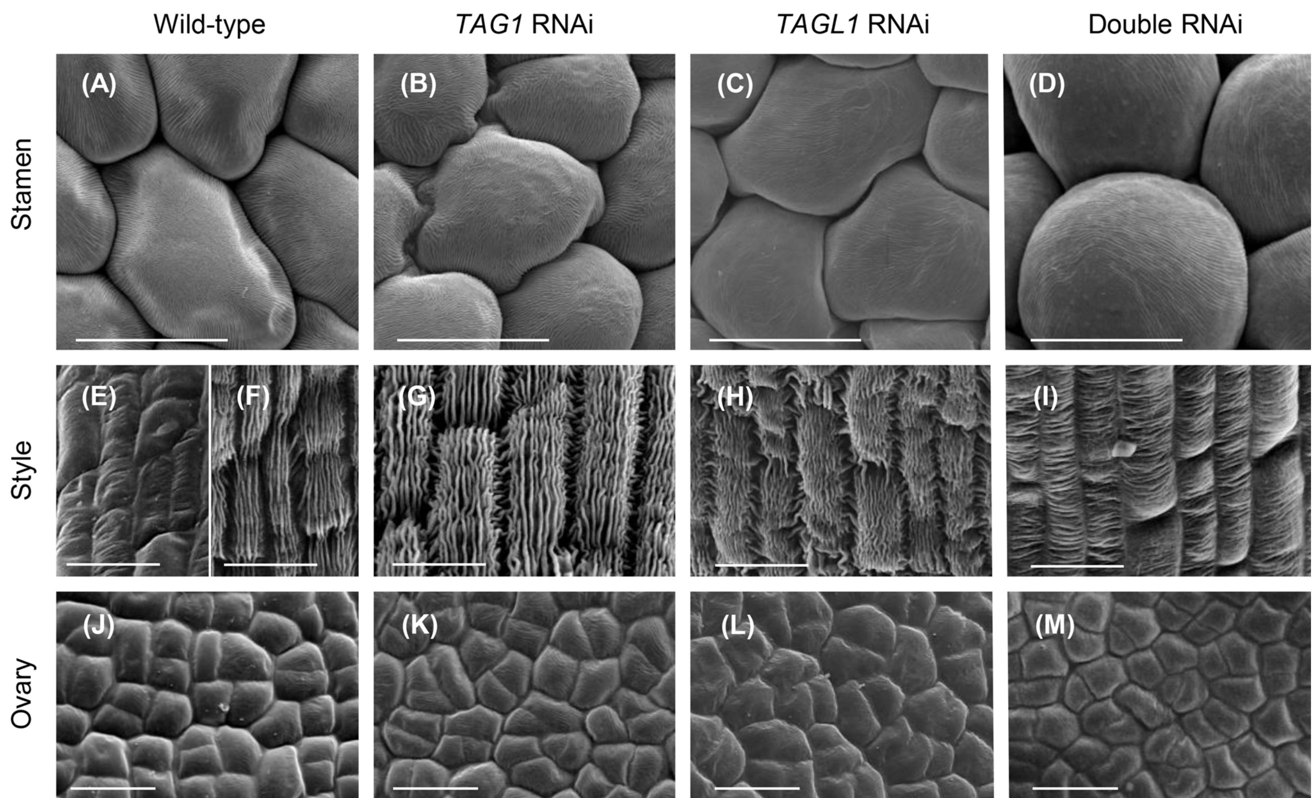


Fig. 3 Epidermal cell morphology of floral organs from tomato flowers. **a–m** Epidermal cells of stamens (**a–d**), styles (**e–i**) and carpels (**j–m**) from WT (**a**, **e**, **f**, **j**), *TAG1* RNAi (**b**, **g**, **k**), *TAGL1* RNAi (**c**, **h**, **l**) and double RNAi (**d**, **i**, **m**) tomato flowers at anthesis day (AD) stage. Epidermal cells of WT style from young flowers (5 days before AD) are shown in panel E. *Scale bars* represent 50 μm in (**a–d**) and 20 μm in (**e–m**)

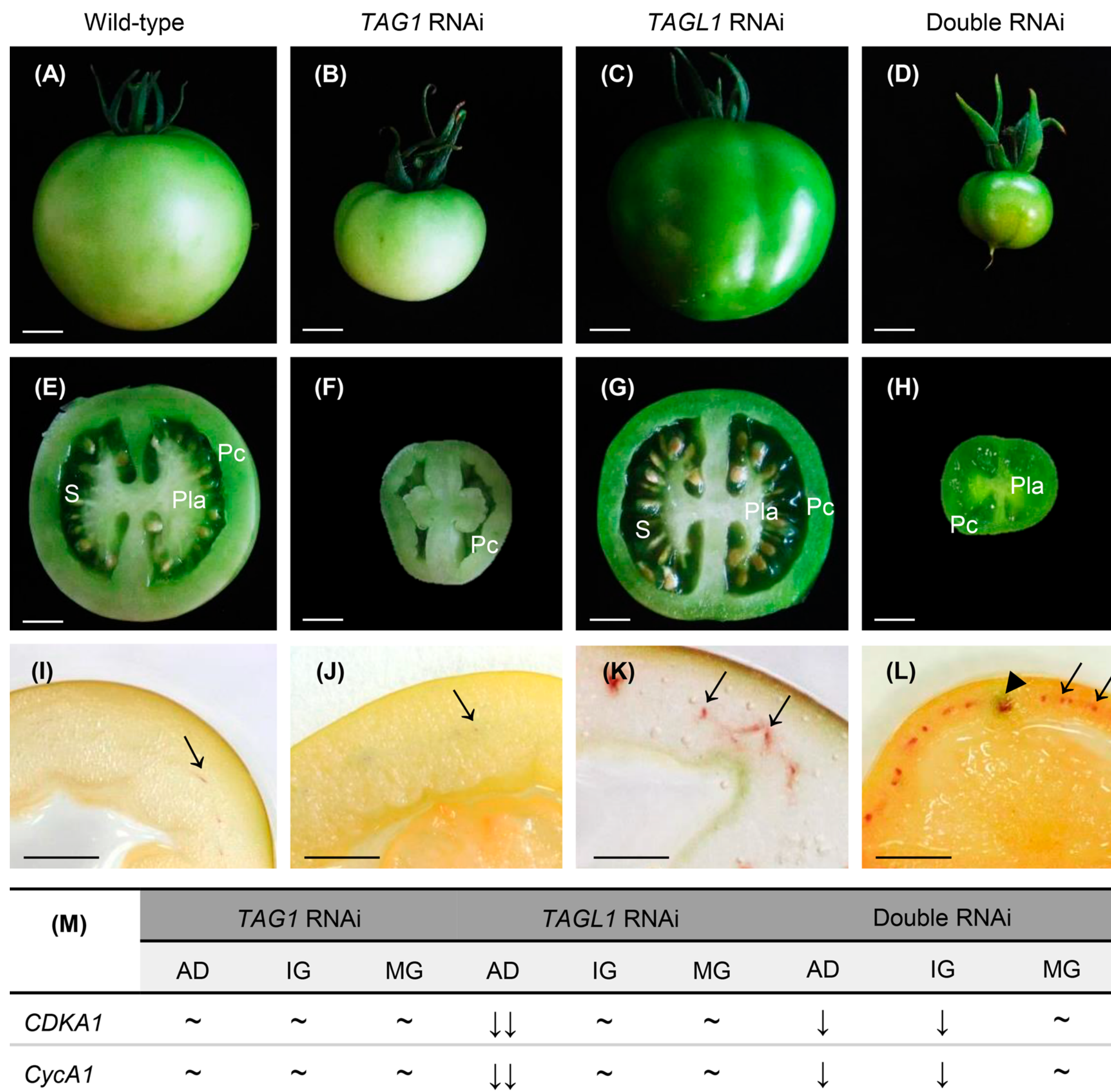
was not developed in double RNAi fruits (Fig. 5l). Moreover, the number of parenchyma cell layers was reduced by almost half (10.16 ± 0.84) respect to the wild-type (19.44 ± 0.88), which together with the smaller size of parenchyma cells resulted in a drastic decrease of pericarp thickness of double RNAi fruits (Fig. 5l).

Tomato fruits lacking *TAG1* and *TAGL1* expression displayed some sepal characteristics

Phenotype and microscopy analyses of double RNAi fruit described above revealed a low degree of tissue differentiation as well as developmental abnormalities in cell size and tissue composition, which mainly affected collenchyma and parenchyma layers (Fig. 5k, l). Additionally, vascular tissues, which are usually scarcely developed at late stages of WT fruit development (Fig. 5k), showed a high degree of development in double RNAi fruit (Fig. 5l), suggesting that dual silencing of *TAG1* and *TAGL1* modifies vascular development in tomato fruit. It is noteworthy that the cellular and tissue characteristics of double RNAi fruit were quite similar to those of WT sepals, where there are a discrete number of parenchyma cell layers (10.83 ± 1.25), two epidermal layers, and vascular tissues (Fig. 5f, l).

In addition to cell and tissue similarities between double RNAi fruits and WT sepals, the former also displayed an external dark green line along the middle of the carpel surface (Fig. 5b), which was not observed in single RNAi or in WT fruits (Fig. 5a). Phloroglucinol staining of pericarp tissue sections showed that this external feature corresponds to a set of vascular bundles similar to those forming the central primary vein characteristic of WT leaves and sepals (Fig. 6a–c). Indeed, lignin accumulated in the vascular bundles of the primary and secondary veins ectopically developed in the pericarp of double RNAi fruits (Fig. 6c, f). These features of vascular tissue development and lignin accumulation were never observed in WT fruit (Fig. 6b, e), but they are common during WT leaf and sepal development (Fig. 6a, d). As happened in normal sepals, this primary vein was maintained during fruit development of double RNAi fruits, and it was even visible as a greenish thickening at ripening stages (Fig. 9d).

Taken together, results showed that the cellular features, tissue layer distribution, and vascular pattern found during double RNAi fruit development were similar to those of WT sepals, indicating that dual repression of *TAG1* and *TAGL1* prevented an appropriated fruit development and conferred sepal characteristics to developing fruits. Such



(M)	TAG1 RNAi			TAGL1 RNAi			Double RNAi		
	AD	IG	MG	AD	IG	MG	AD	IG	MG
CDKA1	~	~	~	↓↓	~	~	↓	↓	~
CycA1	~	~	~	↓↓	~	~	↓	↓	~

Fig. 4 Fruit development and gene expression analyses in *TAG1*, *TAGL1* and double *TAG1–TAGL1* silencing lines. **a–l** External morphology (**a–d**) and equatorial sections (**e–h**) of tomato fruits at mature *green* (MG) stage from WT (**a**, **e**), *TAG1* RNAi (**b**, **f**), *TAGL1* RNAi (**c**, **g**) and double RNAi lines (**d**, **h**). Phloroglucinol staining of lignin in transversal sections of tomato fruit pericarps from WT (**i**), *TAG1* RNAi (**j**), *TAGL1* RNAi (**k**) and double RNAi (**l**) plants. *Arrows* point to vascular bundles and arrowhead to ectopic primary vein. *Scale bars* represent 1 cm in (**a–h**) and 5 mm in (**i–l**). *Pc* pericarp; *Pla* placenta; *S* seeds. **m** Schematic representation of expression analyses of *CDKA1* and *CycA* genes in fruits from single and double RNAi lines. *Downward arrows* indicate down-regulation. Changes of gene expression were indicated by one (twofold to tenfold) or two (tenfold to 50-fold) *arrows*. Similar expression levels were indicated by ∼ *symbol*. *AD* anthesis day; *IG* immature *green*; *MG* mature *green*

features of double RNAi tomato fruits could be due to changes in the expression of *MACROCALYX* (*MC*), an A-class MADS-box gene involved in sepal development (Vrebalov et al. 2002). Results showed that *MC* transcriptional level in single *TAG1* RNAi and *TAGL1* RNAi plants did not differ from the WT; in all cases, *MC* expression remained constant during fruit development (Fig. 6g). However, a greater increase of *MC* expression was detected in double RNAi plants at early stages of fruit development, reaching the maximum level at MG stage, as also happened in wild type sepals (Fig. 6g).

Pollen viability is suppressed in double *TAG1–TAGL1* RNAi lines

One of the most remarkable effects of *TAG1* silencing is the development of seedless fruits from the second and following inflorescences (Figs. 4f, 9f). However, fruit fertility is not altered in *TAGL1* RNAi lines, which produced a normal or even higher number of seeds than WT plants (Figs. 4g, 9g; Table 1); contrarily, the seedless phenotype is enhanced in double RNAi plants as they completely fail to produce seeds (Figs. 4h, 9h; Table 1). SEM analyses

Table 1 Characteristics of red ripe fruits and percentage of non-viable pollen (under in vitro conditions) of wild-type and RNAi plants

	Wild-type	*TAG1* RNAi	*TAGL1* RNAi	Double RNAi
Weight (g)	85.62 ± 17.95a	19.16 ± 4.95b	80.25 ± 18.44a	3.85 ± 1.3c
Size (cm)	5.57 ± 0.46a	3.63 ± 0.55b	5.65 ± 0.53a	2.07 ± 0.24c
Pericarp thickness (cm)	0.69 ± 0.08a	0.56 ± 0.07b	0.54 ± 0.05b	0.26 ± 0.07c
Seed number	57.14 ± 47.88a	0.067 ± 0.26b	73.15 ± 27.63a	0 ± 0b
Non-viable pollen (%)	22.70 ± 11.40a	55.85 ± 13.41b	35.06 ± 10.95a	100 ± 0c
Bottom stiffness (%)	73.92 ± 6.17a	75.8 ± 6.09a	82.31 ± 9.84b	99.78 ± 0.85c
Medium stiffness (%)	73.71 ± 8.63a	77.33 ± 6.16a	97.46 ± 3.47b	99.78 ± 1.04b
Top stiffness (%)	69.14 ± 9.76a	80.73 ± 9.95b	95.85 ± 3.89c	99.22 ± 2.15c
Ethylene (nl/g.h)	8.8 ± 0.24a	27.6 ± 0.19b	1.32 ± 0.33c	0 ± 0d

Values are mean ± SE. Values followed by the same letter (a, b, c, d) are not statistically significant ($P < 0.01$)

showed that ovules of double RNAi flowers, although smaller than WT, developed normally (Fig. 7a, d), suggesting that the fruit sterility in double RNAi plants could be due to defects in pollen development. With the aim to elucidate the causes of seedless fruit formation in *TAG1* RNAi and double *TAG1–TAGL1* RNAi lines, pollen viability was analyzed in these transgenic lines through in vitro and in vivo assays. Results of in vitro analyses indicated that as expected, pollen viability was not affected in *TAGL1* RNAi lines as pollen grains displayed similar size, morphology and staining as WT pollen (Fig. 7e, g; Table 1). However, in *TAG1* RNAi flowers, the percentage of non-viable pollen significantly increased to 55.85 % (Fig. 7f; Table 1), and double RNAi flowers produced no viable pollen grains (Fig. 7h; Table 1). These results were confirmed by in vivo pollen germination assays (Fig. 7i–n), which indicated that pollen grains germinated and developed normally in self-pollinated flowers of WT and *TAGL1* RNAi flowers (Fig. 7i, j), but not in *TAG1* RNAi and double RNAi flowers, where the percentage of viable pollen was reduced and null, respectively. In addition, reciprocal crosses were performed to discriminate whether stigma reception or other ovary-dependent factors could affect pollen germination. Results showed that pollen from WT anthers germinated and developed normal pollen tubes on the stigma of *TAG1* RNAi and double RNAi flowers (Fig. 7k, m). Moreover, when *TAG1* RNAi plants were used as pollen donors, a low percentage of pollen germination was detected on the stigma of WT flowers (Fig. 7l), whereas germination of pollen grains produced by double RNAi plants was completely blocked (Fig. 7n). These results involved *TAG1* and *TAGL1* genes in pollen development and ruled out gynoecium-related factors as responsible for pollen defects found in *TAG1* RNAi and double RNAi plants.

It is known that transcriptional levels of A and C class genes are required for the maintenance of B-function genes (Gómez-Mena et al. 2005; Heijmans et al. 2012). Therefore, silencing of *TAG1* and *TAGL1* genes was checked so as to discern whether it could alter the expression of tomato B-class *Tomato APETALA3* (*TAP3*) (syn. *SlDEF*, *LeAP3*, *SL*; Kramer et al. 1998; di Martino, Pan, Emmanuel, Levy and Irish 2006; Quinet et al. 2014), *Tomato MADS box gene 6* (*TM6*) (syn. *TDR6*; Busi et al. 2003; Pnueli et al. 1991) and *Tomato PISTILLATA* (*TPI*) (syn. *SlGLO2*; Mazzucato et al. 2008) genes. No differences were found in *TAP3* transcript accumulation in both single and double RNAi flowers with respect to WT flowers (Fig. 7o). However, *TM6* expression was significantly down-regulated at all developmental stages here analyzed, while *TPI* was up-regulated in flowers of single and double RNAi lines (Fig. 7p, q), suggesting that *TM6* inhibition may be compensated by *TPI* expression levels in RNAi lines. To further analyze if expression changes of *TM6* and *TPI* are associated with stamen development and pollen viability, histological analyses of single and double RNAi flowers were performed. No alterations affecting pollinic sac development, tetrad formation and tapetum degradation were found, although some pollen grains showing a flake-like morphology and lacking the typical sculptured wall of WT pollen grains were observed at mitotic and dehiscence stages in *TAG1* RNAi and double RNAi lines (Fig. 8).

Effects of *TAG1* and *TAGL1* gene silencing on fruit ripening

Previous reports have suggested a functional role of the *TAG1* gene in fruit ripening since *TAG1* overexpression resulted in the homeotic conversion of sepals into carpel-like organs, which showed typical ripening features such as fleshy expansion, cell wall metabolism, and carotenoid accumulation (Pnueli et al. 1994; Ishida et al. 1998). In agreement with this, tomato plants expressing an antisense *TAG1* construct developed pseudocarpels, which were unable to ripen and displayed perianth organ identity (Pnueli

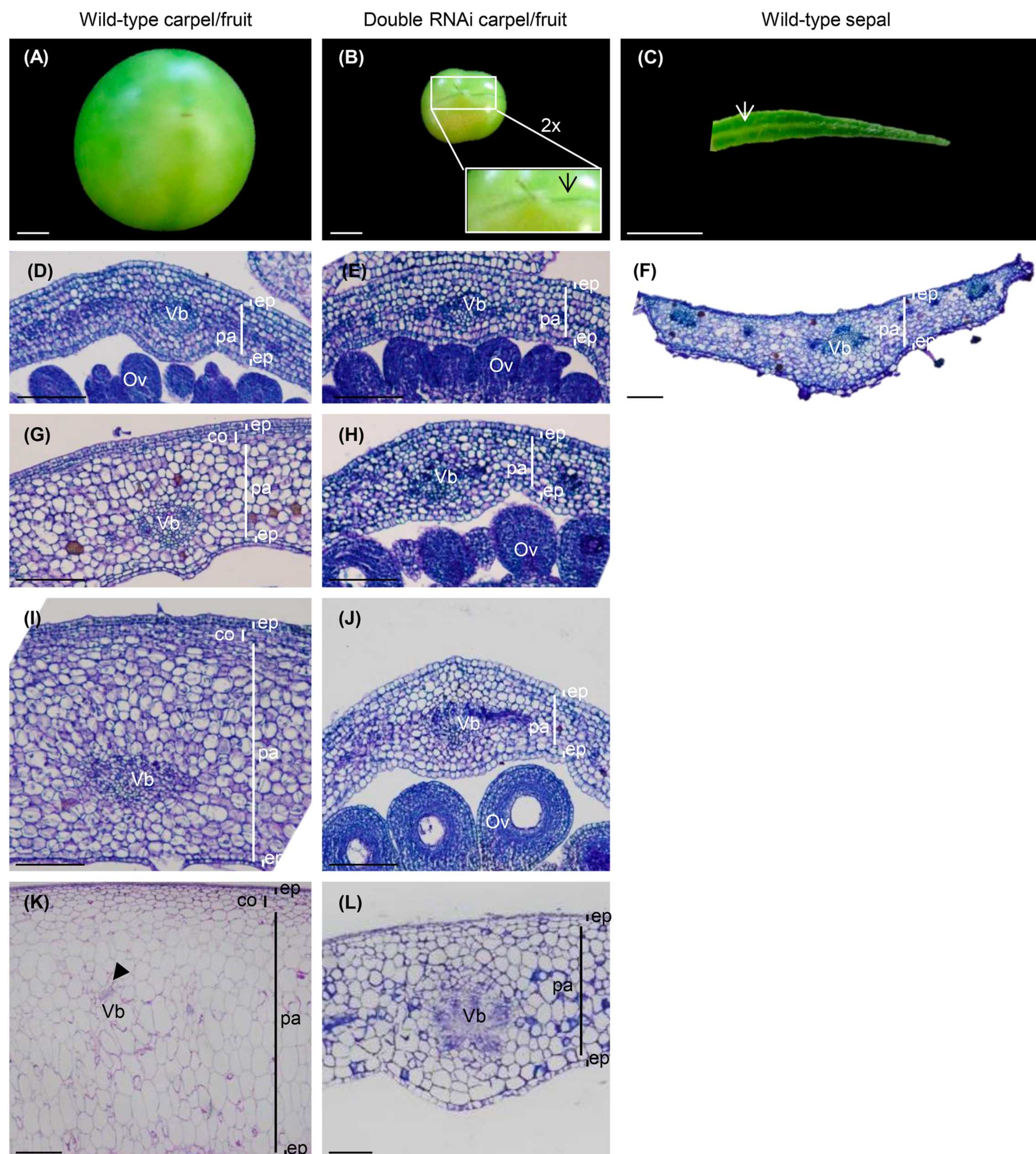


Fig. 5 Histological features of tomato sepals and fruits of double *TAG1*–*TAGL1* RNAi lines. **a**–**l** External appearance of WT (**a**) and double RNAi (**b**) fruits at immature *green* stage, and of a WT sepal (**c**). *Arrows* indicate an external *dark green line* along the middle of the fruit surface (**b**) similar to the central primary vein of wild type sepal (**c**). Toluidine *blue* staining of transversal sections of sepals developed by WT flowers at anthesis day (AD) (**f**), as compared with carpels of floral buds (**d**–**e**), AD flowers (**g**–**h**), flowers 10 days after anthesis (**i**–**j**) and mature *green* fruits (**k**–**l**) developed by WT (**d**, **g**, **i**, **k**) and double RNAi (**e**, **h**, **j**, **l**) plants. *Scale bars* represent 1 cm in (**a**–**c**), 100 µm in (**d**–**j**), 1 mm in (**k**), 500 µm in (**l**). Vb, vascular bundles; *Ov* ovules; *Ep* epidermis, *Co* collenchyma, *Pa* parenchyma

et al. 1994). However, Pan et al. (2010) recently demonstrated that carpel development and fruit ripening were not affected when *TAG1* is specifically silenced through an interference RNA construct, indicating that *TAG1* did not perform a relevant function during fruit ripening. Such differences suggest that besides *TAG1*, the expression of other tomato *AG*-like genes, most likely *TAGL1*, may also be misregulated in the antisense *TAG1* lines reported by Pnueli et al. (1994), as Pan et al. (2010) argued in their work. Our results agree with the observations of these latter authors in that we found that ethylene biosynthesis was not significantly affected in *TAG1* RNAi fruits and they even showed a slightly higher hormone content than WT fruits at the BR+10 stage (Table 1). Accordingly, the average value of stiffness and pigmentation in *TAG1* RNAi fruits were similar to WT and the expression levels of ripening genes such as

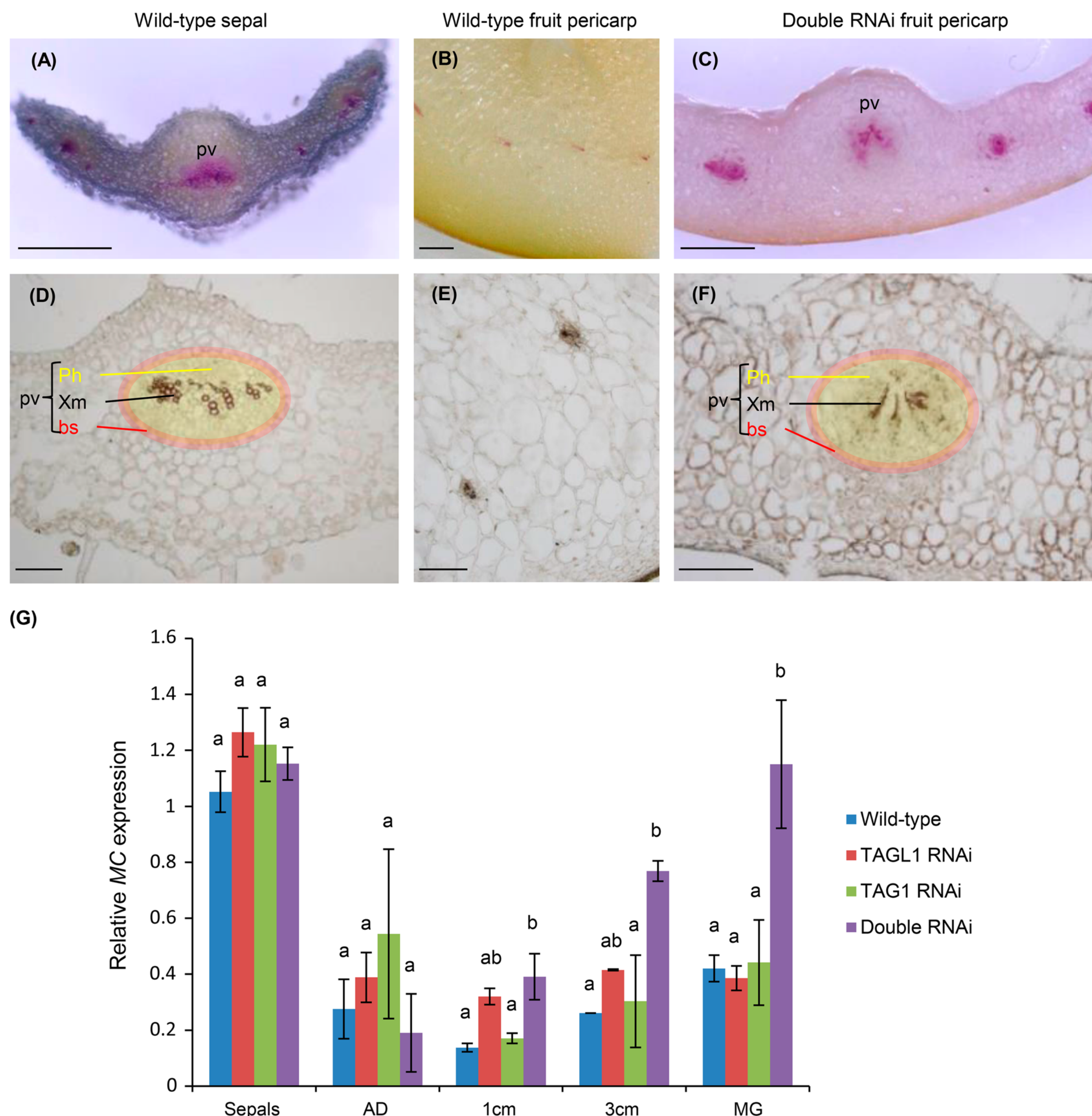


Fig. 6 Sepal-like tissue features of fruit pericarp promoted by dual repression of *TAG1* and *TAGL1* genes. **a–f** Phloroglucinol staining of lignin in thick (**a–c**) and in 8 µm (**d–f**) transversal sections of WT sepals (anthesis day stage, AD) (**a**, **d**), and fruit pericarp from WT (**b**, **e**) and double RNAi (**c**, **f**) plants, the *two letter* at breaker stage. *Scale bars* represent 1 mm in (**a–c**), 100 µm in (**d**), and 500 µm in (**e**, **f**). *pv* primary vein; *Xm* xylem; *Ph* phloem; *bs* bundle sheaths. **g** Relative expression of *MC* gene in sepals and flowers at anthesis day (AD), 1 cm-wide fruits (1 cm), 3 cm-wide fruits (3 cm), and fruits at mature *green* (MG) stages from WT, *TAG1* RNAi, *TAGL1* RNAi and double RNAi plants. *Data* are means of three biological replicates ± standard error of the mean. Values followed by the *same letter* (**a**, **b**) are not statistically significant ($P < 0.01$)

TDR4, *RIPENING-INHIBITOR* (*RIN*), *NON-RIPENING* (*NOR*), *COLOURLESS NON-RIPENING* (*CNR*), *NEVER RIPE*, *ACC OXIDASE 1*, *ACC SYNTHASE 2* (*ACS2*), *ACS4*, *E4*, *POLYGALACTURONASE* (*PG*), *PECTIN METHYL ESTERASE* (*PME*) and *PHYTOENE SYNTHASE* (*PSY*) were not significantly altered (Fig. 9m; Table 1).

Recent reports have highlighted the crucial role of *TAGL1* as a master regulator of fruit ripening (Itkin et al. 2009; Vrebalov et al. 2009; Gimenez et al. 2010). Accordingly, our results showed that repression of *TAGL1* induced significant ripening changes, mainly decreasing the ethylene content in the fruits, which is consistent with the yellow-orange colour and higher stiffness of *TAGL1* RNAi fruits (Fig. 9c; Table 1). In addition, cuticles from *TAGL1* RNAi fruit showed a significant reduction of thickness (Fig. 9k), most likely due to decreased biosynthetic activity of epidermal cells (Gimenez et al. 2015). In accordance, gene expression analyses revealed significantly reduced expression of the ethylene biosynthesis *ACS2* gene, and low transcript levels of genes involved in lycopene biosynthesis (*PSY*) and cell wall degradation (*PME*, *PG* and *E4*) (Fig. 9m).

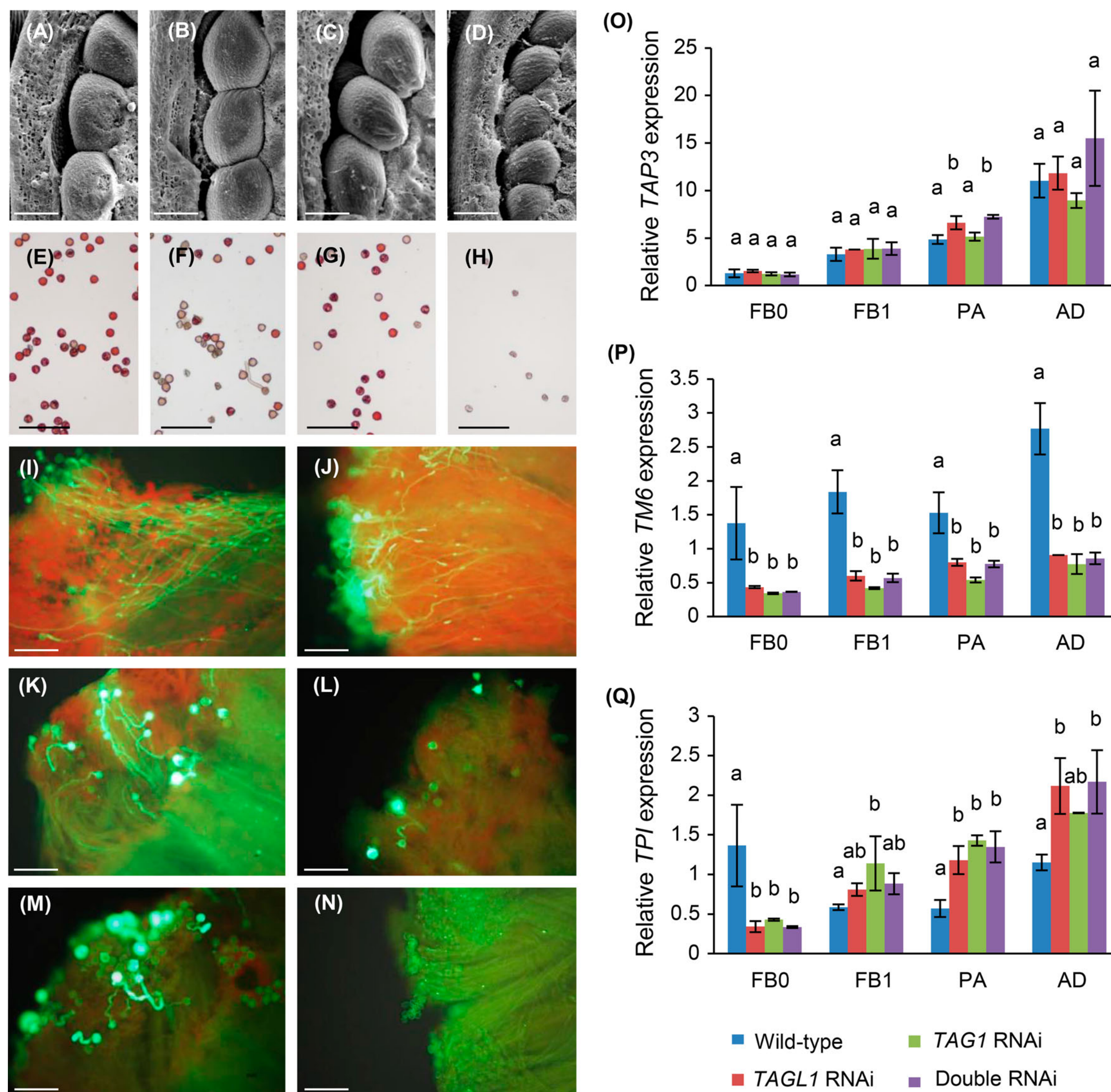


Fig. 7 Pollen viability in *TAG1*, *TAGL1* and double *TAG1–TAGL1* silencing lines. **a–h** Morphological features of ovules (**a–d**) and in vitro assays of pollen viability (**e–h**) from WT (**a**, **e**), *TAG1* RNAi (**b**, **f**), *TAGL1* RNAi (**c**, **g**) and double RNAi (**d**, **h**) flowers at anthesis day stage. **i–n** In vivo assays of pollen viability performed in self-pollinated flowers of WT (**i**) and *TAGL1* RNAi (**j**) plants, and in flowers from the backcrosses *TAG1* RNAi × WT (**k**), WT × *TAG1* RNAi (**l**), double RNAi × WT (**m**) and WT × double RNAi (**n**). *Scale bars* represent 100 µm in (**a–n**). **o–q** Relative expression of *TAP3* (**o**), *TM6* (**p**) and *TPI* (**q**) genes in flower buds at two developmental stages (FB0 and FB1), and flowers at pre-anthesis (PA) and anthesis day (AD) stages from WT, *TAG1* RNAi, *TAGL1* RNAi and double RNAi plants. *Data* are means of three biological replicates ± standard error of the mean. Values followed by the *same letter* (**a**, **b**, **c**) are not statistically significant ($P < 0.01$)

The difference between fleshy fruit ripening in single RNAi lines here reported suggests a functional divergence between *TAG1* and *TAGL1* MADS-box genes. To corroborate this hypothesis, we further characterized the phenotypic effects on fruit ripening of silencing both *TAG1* and *TAGL1* (Fig. 9d). Apart from the abnormalities affecting fruit development described above, double RNAi fruits exhibited comparable characteristic as *TAGL1* RNAi fruit. They were unable to ripen and exhibited a yellow-orange colour, higher stiffness, and thinner cuticle, as well as lower ethylene content than WT fruits (Fig. 9d, h, l; Table 1). The expression levels of ripening genes such as *ACS2*, *E4*, *PG*, *PME* and *PSY* were also inhibited in double mutant pericarps (Fig. 9m). All these ripening features, although more extreme, were comparable to those observed in *TAGL1* RNAi fruits, but they were never found in *TAG1* RNAi fruits (Fig. 9b, f, j).

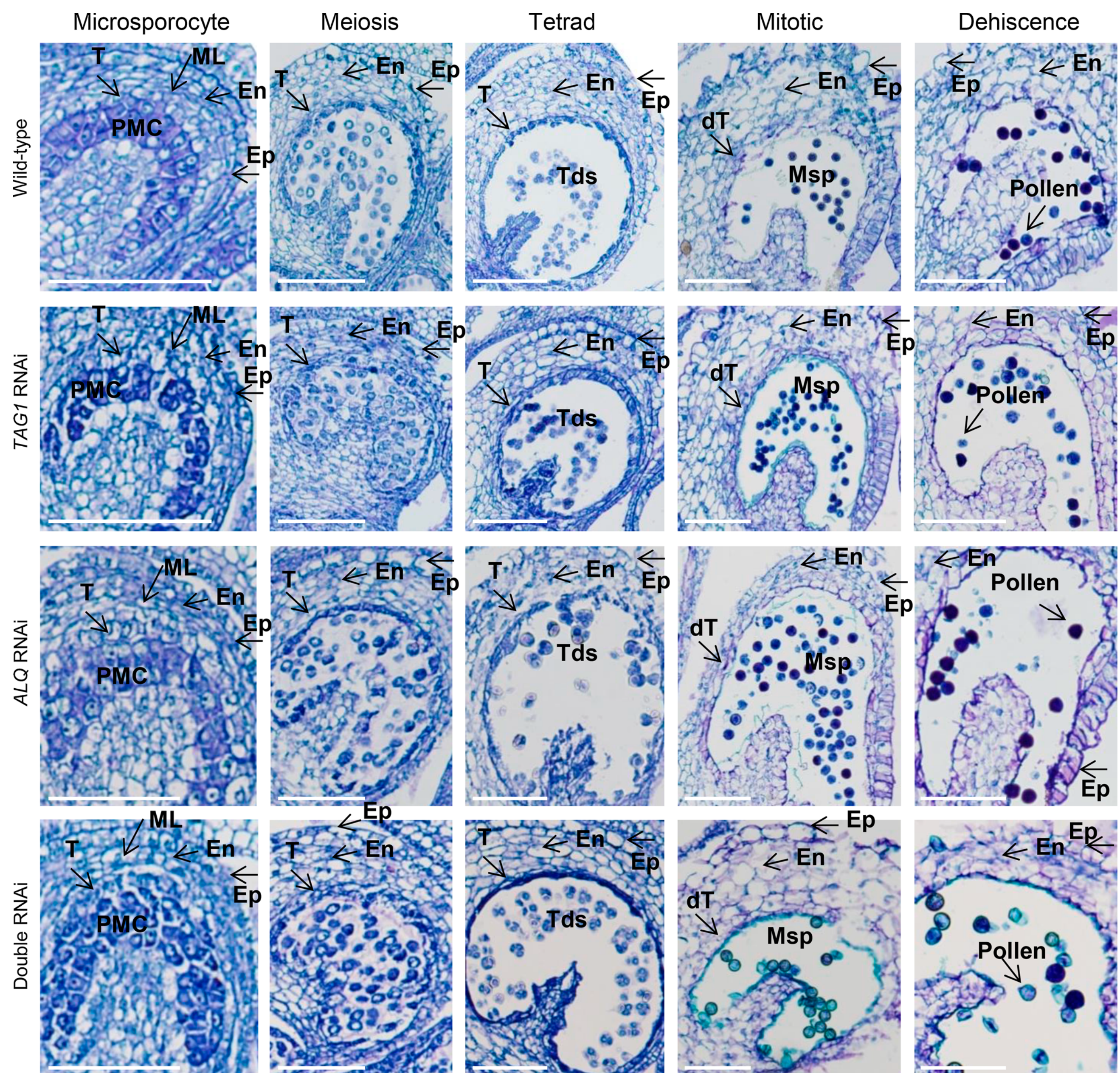
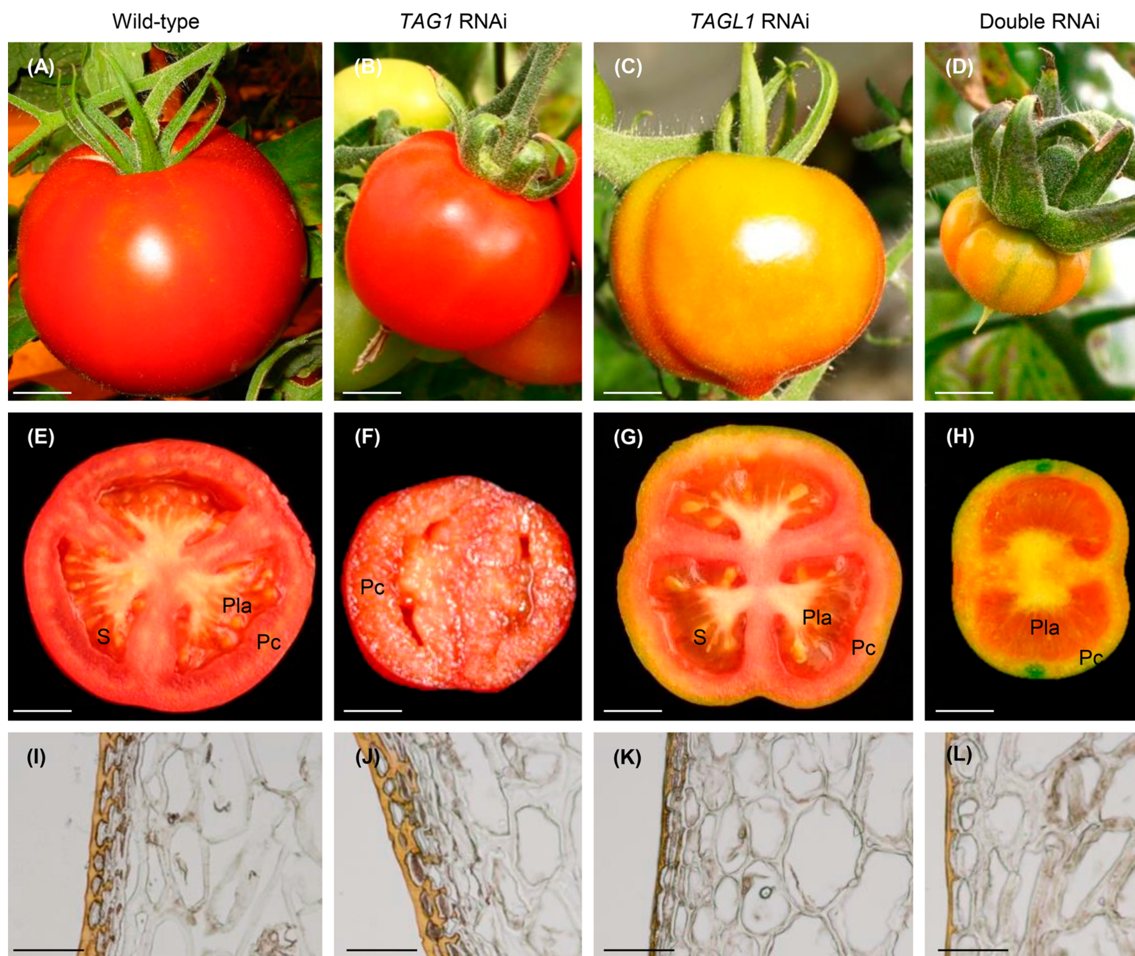


Fig. 8 Anther and pollen development of *TAG1* RNAi, *TAGL1* RNAi and double RNAi flowers. Several developmental stages were considered: microsporocyte, meiosis, tetrad, mitotic and dehiscence phase. *Scale bars* represent 100 µm. *T* tapetum; *ML* middel cell layer; *En* endothecium; *Ep* epidermis; *PMC* pollen mother cell; *Tds* tetrads; *dT* degenerated tapetum; *Msp* microspore

Discussion

TAG1 and *TAGL1* cooperate in the genetic control of flower development

TAG1 and *TAGL1* MADS-box genes belong, respectively, to the euAG and PLE lineages resulting from the duplication of the tomato AG clade (Kramer et al. 2004; Vrebalov et al. 2009). While *TAG1* has been considered a C class gene involved in the specification of tomato stamen and carpel identities (Pnueli et al. 1994), recent reports have demonstrated the crucial role of *TAGL1* during flower and fruit development and fruit ripening in this model species (Itkin et al. 2009; Vrebalov et al. 2009; Gimenez et al. 2010). *TAG1* and *TAGL1* showed similar expression patterns during flower development as their transcripts preferentially accumulate in stamens and carpels (Fig. 1; Pnueli et al. 1994; Gimenez et al. 2010), suggesting that both genes are required for floral organogenesis in tomato (Gimenez et al. 2010). Along with this, constitutive expression of *TAGL1*



(M)	*TAG1* RNAi			*TAGL1* RNAi			Double RNAi		
	AD	MG	BR+10	AD	MG	BR+10	AD	MG	BR+10
TDR4	~	~	~	~	~	~	~	~	~
ACS2	~	~	~	~	~	↓↓↓	~	~	↓↓
ACS4	~	~	~	~	~	~	~	~	~
ACO1	~	~	~	~	~	~	~	~	~
NR	~	~	~	~	~	~	~	~	~
NOR	~	~	~	~	~	~	~	~	~
RIN	~	~	~	~	~	~	~	~	~
CNR	~	~	~	~	↑	↑	~	↑	~
PSY	~	~	~	~	~	↓	~	~	↓
PME	~	~	~	~	↓	↓	~	↓↓	↓↓
PG	~	~	~	~	~	↓	~	~	↓↓↓
E4	~	~	~	~	~	↓	~	~	↓

Fig. 9 Ripening characteristics of *TAG1*, *TAGL1* and double *TAG1*–*TAGL1* silencing fruits. **a**–**l** External morphology (**a**–**d**), equatorial sections (**e**–**h**) and Sudan III staining of cuticle (**i**–**l**) of tomato fruits at BR+8 stage from WT (**a**, **e**, **i**), *TAG1* RNAi (**b**, **f**, **j**), *TAGL1* RNAi (**c**, **g**, **k**) plants and double RNAi plants (**d**, **h**, **l**). *Scale bars* represent 1 cm in (**a**–**h**) and 50 µm in (**i**–**l**). *Pc* pericarp; *Pla* placenta; *S* seeds. **m** Schematic representation of gene expression analyses performed in *TAG1* RNAi, *TAGL1* RNAi and double RNAi fruits, as compared to WT fruits. Upward and downward *arrows* indicate up- and down-regulation, respectively. Changes of gene expression respect to *WT* were indicated by one (twofold to tenfold), two (tenfold to 50-fold) or three (higher than 50-fold) *arrows*. Similar expression *levels* were indicated by ~ *symbol*. *AD* anthesis day; *MG* mature *green*; *RR red* ripe

promoted developmental conversion of sepals into succulent carpel-like organs and petals into staminoid organs (Vrebalov et al. 2009; Gimenez et al. 2010), these homeotic changes being similar to those reported in tomato plants overexpressing *TAG1* (Pnueli et al. 1994). However, homeotic changes affecting floral organ identity were not observed in transgenic lines where *TAGL1* is significantly silenced (Fig. 2; Gimenez et al. 2010). These results support that *TAG1* and *TAGL1* could act redundantly in specifying tomato stamen and carpel identities.

TAG1 RNAi plants characterized in this study also showed normal development of reproductive floral organs, an unexpected result given that Pnueli et al. (1994) reported homeotic abnormalities in the third (stamens) and fourth (carpels) floral whorls of *TAG1* antisense plants, and Pan et al. (2010) showed identity changes in stamens of *TAG1* RNAi plants. There are several explanations for these seemingly contradictory results. First, other tomato *AG*-like genes besides *TAG1* could have been suppressed in the *TAG1* antisense lines reported by Pnueli et al. (1994). Second, differences in the tomato genetic background could also influence reproductive development. Indeed, while cv. Moneymaker used in this work does not bear known mutations, several developmental mutations have been reported in the cv. Microtom (Meissner et al. 1997) used by Pan et al. (2010). However, we think that the most plausible explanation relies on the incomplete level of inhibition of *TAG1* expression in the RNAi lines generated in our work. This hypothesis is further supported by the similarity between the phenotypes we observed and those promoted by weak mutant alleles of *AG* and *PLE* genes in *Arabidopsis* and *Antirrhinum*, respectively (Davies et al. 1999; Causier et al. 2005). Most likely a full knock-out of *TAG1* and *TAGL1* genes would promote more severe floral organ transformations than those found in double silencing plants. Our results also support that a threshold transcript level of *TAG1* and *TAGL1* may be enough to promote stamen and carpel development. This gene expression scenario would facilitate a compensatory mechanism involving *TAG1* and *TAGL1* since the latter can likely compensate for the loss of C function when the former is partially silenced. In agreement with this hypothesis, down-regulation of both genes led to some cell abnormalities that weakly modified the organ identity of stamens and carpels of double RNAi plants.

Given that B- and C-class MADS-box transcription factors interact to regulate stamen development, down-regulation of *TM6* and up-regulation of *TPI* in double *TAG1–TAGL1* RNAi plants suggest the participation of both B-class genes in stamen abnormalities. However, additional factors controlled by *TAG1* and *TAGL1* should be required to promote stamen development as similar modifications in the expression levels of B-class genes were detected in both single RNAi lines, and such transcriptional changes were not associated with developmental defects of stamens.

In summary, results here reported indicate that *TAG1* and *TAGL1* act redundantly and that a balanced expression pattern of these two MADS-box genes could be required for stamen and carpel development. Such a balanced mechanism has previously been proposed during flower development of tomato, petunia and *N. benthamiana* (Gimenez et al. 2010; Fourquin and Ferrándiz 2012; Heijmans et al. 2012), and could also operate in fruit ripening (Klee and Giovannoni 2011).

TAG1 and *TAGL1* play redundant roles to suppress sepal developmental program during fruit formation

We have shown that dual silencing of *TAG1* and *TAGL1* in tomato transgenic plants substantially prevents fruit development so that only extremely small fruit organs are formed as a result of a slow and reduced growth. Further characterization of double RNAi plants also showed alterations to the fruit developmental process. As result, tomato fruit display some features typical of sepals, the most remarkable ones being decreased cell division and tissue differentiation, which makes that the cell layer number and distribution of double mutant fruits were comparable to WT sepals. Such developmental changes lead to a significant reduction in pericarp growth, a lack of seed formation, and the development of vascular tissues accompanied by lignin biosynthesis in double RNA fruit. These characteristics are observed in sepal development and are the opposite of what occurs during fruit development. Taken together, these data indicate that *TAG1* and *TAGL1* cooperate to suppress the sepal developmental program, thereby promoting carpel and fruit development and maintaining proper organ identity.

The appearance of distinctive features of sepal in double RNAi fruit is most likely due to the increased expression of *MC*, which reaches the messenger level characteristic of sepals, suggesting that a transcriptional control of *MC* by *TAG1* and *TAGL1* is required for the proper development of tomato fruit. It is known that MC interacts with TAG1 and TAGL1; moreover MC and TAGL1 have been reported to form protein complexes with TAG1 through the SEPALLATA (SEP) member TM5 (Leseberg et al. 2008). These data corroborate a role for all three of these MADS-box factors in the regulation of carpel and fruit development. MADS-box genes of PLE lineage have been linked to fruit development and ripening program in species such as *Arabidopsis*, grapevine, peach, *N. Benthamiana* and tomato (Boss et al. 2001; Pinyopich et al. 2003; Tadiello et al. 2009; Gimenez et al. 2010; Fourquin and Ferrándiz 2012), despite the fact that the *PLE* gene was initially described as a floral identity gene in *Antirrhinum* (Bradley et al. 1993). In summary, our results dissecting the functional redundancy of *TAG1* and *TAGL1* during flower and

fruit development suggest that *TAGL1* has retained stamen and carpel identity functions, which are characteristic of *PLE*-like genes from *Antirrhinum* and eu*AG* genes from *Arabidopsis*, apart from maintaining its *SHP*-like function in the fruit ripening program. This dual function for *PLE* genes like *TAGL1* may be characteristic of the *Solanaceae* family, as it has only been reported in *N. benthamiana* (Fourquin and Ferrándiz 2012) and tomato thus far.

TAG1 and *TAGL1* are redundantly involved in pollen development

The lack of seed development is one of the major developmental defects observed in both the *TAG1* RNAi and double *TAG1–TAGL1* RNAi lines. Histological analyses and pollen viability assays indicated that seedless fruit development is likely caused by abnormalities in pollen formation and maturation, and that silencing of both *TAG1* and *TAGL1* has a synergistic effect on pollen formation as double RNAi plants were completely unable to develop viable pollen. In addition, both *TAG1* and *TAGL1* are expressed in stamens (Fig. 1; Pnueli et al. 1994; Gimenez et al. 2010), which together indicate that these two genes have redundant functions in pollen formation like also occurs with their homologues in *Arabidopsis*. In *Arabidopsis*, *AG* appears to induce microsporogenesis through the activation of the *SPOROCYTELESS* (*SPL*) gene (Ito et al. 2004). In addition, constitutive expression of *Arabidopsis SHP2*, a *TAGL1* homologue, was sufficient to rescue stamen development in *ag* mutants although *SHP2* was not expressed in stamens, suggesting that *SHP* genes have retained the stamen related *AG* activity (Pinyopich et al. 2003). Microsporogenesis is not altered in double RNAi lines (Fig. 8), suggesting that some other factors regulating microgametogenesis process should be affected in these lines. Most likely, such factors might collaborate with TAG1 and TAGL1 to promote pollen formation in tomato in a similar way than SPL in Arabidopsis.

Changes in pollen formation and viability found in *TAG1* RNAi and double RNAi lines could be mediated by expression changes of B-function genes *TM6* and *TPI*. However, changes in *TM6* and *TPI* expression levels were also observed in *TAGL1* RNAi plants despite the fact that alterations in the pollen viability were not observed in this line. These results indicated that additional factors regulated by both MADS-box factors should participate in pollen development. In *Arabidopsis*, other *AGAMOUS*-like genes (*AGL*) such as *AGL18*, *AGL29*, *AGL30*, *AGL65*, *AGL66*, *AGL94*, *AGL104* have been involved in pollen development (Pina et al. 2005; Verelst et al. 2007a, b; Adamczyk and Fernandez 2009). However, the functional role of these MADS-box genes has not been studied in tomato so far. In conclusion, results here reported provide evidence for the implication *TAG1* and *TAGL1* in pollen formation of tomato as an integrated part of the reproductive developmental program of this model plant.

Functional diversification of *TAG1* and *TAGL1* is required for the genetic control of fruit development and ripening

Phenotypic characterization of single and double RNAi lines have shown that both *TAGL1* and *TAG1* genes are involved in fruit development, although they affect different aspects of this complex process. While *TAGL1* promotes fleshy pericarp development through the control of cell division and lignin biosynthesis, *TAG1* is involved in seed and placenta development. Moreover, silencing of *TAG1* showed an epistatic effect on *TAGL1* inhibition regarding seed formation, whereas *TAGL1* repression was epistatic to *TAG1* silencing with respect to placenta development. Therefore, our results provide new evidence that *TAG1* and *TAGL1* have diverged in their functions to control different features of fruit development. In *N. benthamiana*, although fruit formation is fully blocked when *NbAG*, the orthologous gene to *Arabidopsis AG* and tomato *TAG1* (Fourquin and Ferrándiz 2012), is silenced, the repression of *NbSHP* (orthologous to *SHP* and *TAGL1*) did not affect fruit formation in this dry-fruited species, even though it prevented fruit dehiscence. Taken together, these results suggest that functional diversification of euAG and PLE lineage genes has followed different pathways in dry and fleshy fruited species of the *Solanaceous* family and that the *SHP*-like genes of both species have retained their functions in late stages of fruit development, i.e. dehiscence and ripening.

TAGL1 gene has been reported as a major regulator of fruit ripening through the control of the ethylene pathway and the interaction with other ripening transcriptional factors as *RIN*, *NOR* and *CNR* (reviewed in Seymour et al. 2013). Our study strongly supports the functional role of *TAGL1* as regulator of several developmental processes related to fruit formation and ripening such as cuticle generation, pericarp development, and lignin biosynthesis, in agreement with previous reports (Vrebalov et al. 2009; Gimenez et al. 2010; Gimenez et al. 2015). Regarding the function of *TAG1*, tomato fruits developed by *TAG1* RNAi lines previously reported by Pan et al. (2010) and those characterized here did not show defects in fruit ripening. They showed normal ethylene production, similar colour and stiffness features to wild-type fruits, as well as a correct cuticle formation, and therefore, these data do not support a role for *TAG1* in the ripening process. In addition, an epistatic effect of *TAGL1* silencing over *TAG1* repression was found in such a way that double RNAi fruits showed similar ripening features to single *TAGL1* RNAi ones. Taken together, these results indicate that *TAGL1* but not *TAG1* plays essential functions in the fruit ripening

process, and provide novel insights about the functional diversification of these MADS-box factors.

Materials and methods

Plant material

Tomato seeds (*Solanum lycopersicum* L. cv. Moneymaker) were provided by C.M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu/). Plants were grown under natural greenhouse conditions using standard crop management practices.

Generation of single and double RNAi tomato lines

The couples of primers RNAiTAGL1F (5́-TCTAGACTCGAGTACCCAATCTTTGCTATATCGCC-3́) and RNAiTAGL1R (5́-ATCGATGGTACCAACTGAGAAGACGACTCATCGAC-3́), and RNAiTAGF (5′-TCTAGACTCGAGTAATCCACAAAAGAAGACTG-3′) and RNAiTAGR (5′-ATCGATGGTACCACACCAAGCAAAAAAAATA-3′) were used to amplify a 298 bp fragment from the *TAGL1* 5́-noncoding region and a 170 bp fragment of the *TAG1* 3′-untranslated region respectively. Such fragments were cloned following the same experimental procedure described by Gimenez et al. (2010) to generate the binary plasmids and tomato interference RNA silencing lines (*TAG1* RNAi and *TAGL1* RNAi).

Sixty-seven independent *TAG1* RNAi lines and seventy-seven independent *TAGL1* RNAi lines were obtained in the cv. Moneymaker; they were subsequently verified for the presence of the transgen. Expression levels of *TAG1* and *TAGL1* were determined by real-time quantitative PCR (RT-qPCR), using gene specific primers indicated in Online Resource 1.

With the aim to obtain double RNAi lines, *TAG1* RNAi and *TAGL1* RNAi lines showing the most severe phenotype and a significant silencing level were selected and crossed, i.e. 46a and 12b lines, respectively. Presence of transgenes in double RNAi lines were verified by standard PCR techniques, and simultaneous silencing of *TAGL1* and *TAG1* gene expression was confirmed by RT-qPCR assays.

Expression analyses of other MADS-box genes, such as *TM5* and *TM29*, were carried out in *TAG1* RNAi, *TAGL1* RNAi and double RNAi lines in order to verify that silencing by the interference RNA method was specific for *TAG1* and *TAGL1* genes (Online Resource 2).

RNA preparation and gene expression analyses

Total RNA was extracted from 100 mg of flowers and fruits from WT, *TAG1* RNAi, *TAGL1* RNAi and double RNAi plants at several stages of reproductive development: 0–3 mm floral buds (FB0), 4–7 mm floral buds (FB1), pre-anthesis flowers (PA, 7–10 mm), flowers at 2 days before anthesis (AD-2), flowers at anthesis day (AD, opening day flower), 1 cm-wide fruits (1 cm), 3 cm-wide fruits (3 cm), and fruits at mature green (MG, green fruits that have reached their maximum size), breaker (BR, green fruits that begin to change their shade to orange-yellow) and 10 days after breaker (BR+10, red ripe fruits for immediate consumption) stages. RNA preparation and gene expression studies were performed from three biological replicates and two technical copies according to procedures described by Gimenez et al. (2010). Primer combinations used to detect gene-specific amplicons are indicated in Online Resource 1. The *Ubiquitine3* gene (Hoffman et al. 1991) was used as control and the absence of genomic DNA contamination was checked using a *TAGL1* promoter specific amplicon (*TAGL1pro*) as negative control. In situ hybridization experiments were carried out in floral buds at stage 8, according to Brukhin et al. (2003), as previously described by Gimenez et al. (2010).

Scanning-electron microscopy (SEM)

SEM studies were performed as previously described by Lozano et al. (1998). Flowers at AD and 5 days before AD stages were fixed in FAEG, dehydrated, critical point dried in a drier Bal Tec (Liechtenstein) CPD 030, and gold-coated in a Sputter Coater (Bal-Tec SCD005). Then, samples were visualized with a Hitachi (Tokyo, Japan) S-3500 N scanning electron microscope at 10 kV.

Ethylene production

Ethylene production from 8 red ripe fruits of each genotype was estimated using a gas chromatograph (Varian 3900, Palo Alto, CA, USA) fitted with a Porapak Q column and a flame ionization detector, and the protocol previously described by Gimenez et al. (2010).

Phenotype and structural analyses of tomato flowers and fruits

Fifteen to twenty fruits were collected from the second and third inflorescence and used to determine weight, size, pericarp thickness, seed number and firmness. Fruit firmness was analyzed with Digital Firmness Tester (Durofel DFT 100) using a 5.64 mm diameter tip.

For structural analyses, flowers, pericarps and sepals were fixed in FAE, dehydrated, embedded in paraffin and cut using a Leica RM2035 microtome. 8 µm transversal sections were stained for 2 min in a 1 % Toluidine Blue in distilled water solution to analyze cellular distribution using an optical microscope (Nikon, Optiphot-2). Cuticle and lignin staining were performed with Sudan III and

phloroglucinol solutions, respectively, as previously described by Gimenez et al. (2010).

Pollen viability assays

In vitro pollen viability assays were performed by means of stain of pollen grains from 10 control and transgenic flowers with 0.5 % 2, 3, 5-triphenil tetrazolium chloride (TTC) (w/v) in 0.5 M sucrose for 2 h at 50 °C in a humid box in darkness and then visualized with an OPTIPHOT-2 (Nikon) optical microscopy. At least two hundred pollen grains were scored taking into account their color intensity and external morphology.

In vivo pollen viability was also evaluated. For this purpose, fifteen flowers self-pollinated and reciprocal crossed were recollected 2 days after pollination, fixed in FAE (Formaldehyde: Acetic acid: 70 % ethanol/1:2:17) for at least 24 h, washed in tap water over night at 4 °C, softened with NaOH 0.8 N during 6 h and washed again in tap water over night at 4 °C, to stain the pollen tubes with 0.1 % aniline blue (w/v) in K3PO4 0.1 N for 2 h in darkness and to visualize the fluorescence with an Optiphot-2 (Nikon) optical microscopy associated to HB-10101AF Mercury Lamp (Nikon).

Statistics

Mean comparison (Fisheŕs Least Significant Difference test, LSD) was used to determine significant differences in gene expression levels and agronomic traits. Analyses were performed using the Statgraphics Centurion XVI software package and data presented as mean ± standard error.

Acknowledgments The authors also thank research facilities provided by the Campus de Excelencia Internacional CeiA3. We thank Dr. F. J. Yuste-Lisbona for critical reading of the manuscript.

Funding This work was supported by the Spanish Ministry of Economy and Competitiveness (Grant Numbers AGL2012-40150-C03-01, AGL2012-40150-C03-02 and AGL2015-64991-C3-1-R); and the European Commission through the JAE-Doc Program of the Spanish National Research Council (CSIC) (Grant Number AGL2012-40150-C03-01 to B.P.).

Author contributions E. G. conducted the experiments, assisted in data interpretation and drafted the manuscript. L. C. collaborated in the experimental work. B. P. and V. M. generated transgenic plants and collaborated in genetic analyses. I. L. P. contributed to a critical review of the manuscript. T. A. assisted in data analysis and reviewed the manuscript. R. L. planned the research work, assisted in data interpretation, and edited the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have not conflict of interest.

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