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RESEARCH PAPER

Functional diversification of *AGAMOUS* lineage genes in regulating tomato flower and fruit development

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Abstract

***AGAMOUS* clade genes encode MADS box transcription factors that have been shown to play critical roles in many aspects of flower and fruit development in angiosperms. Tomato possesses two representatives of this lineage, *TOMATO AGAMOUS* (*TAG1*) and *TOMATO AGAMOUS-LIKE1* (*TAGL1*), allowing for an analysis of diversification of function after gene duplication. Using RNAi (RNA interference) silencing, transgenic tomato lines that specifically down-regulate either *TAGL1* or *TAG1* transcript accumulation have been produced. *TAGL1* RNAi lines show no defects in stamen or carpel identity, but show defects in fruit ripening. In contrast *TAG1* RNAi lines show defects in stamen and carpel development. In addition *TAG1* RNAi lines produce red ripe fruit, although they are defective in determinacy and produce ectopic internal fruit structures. *e2814*, an EMS- (ethyl methane sulphonate) induced mutation that is temperature sensitive and produces fruit phenotypes similar to that of *TAG1* RNAi lines, was also characterized. Neither *TAG1* nor *TAGL1* expression is disrupted in the *e2814* mutant, suggesting that the gene corresponding to the *e2814* mutant represents a distinct locus that is likely to be functionally downstream of *TAG1* and *TAGL1*. Based on these analyses, possible modes by which these gene duplicates have diversified in terms of their functions and regulatory roles are discussed.**

Key words: *AGAMOUS*, flower development, fruit development, MADS box, tomato.

Introduction

Numerous duplication events have occurred in the genomes of the lineages leading to present-day angiosperms, due in part to multiple occurrences of polyploidization (Wendel, 2000; Blanc and Wolfe, 2004). MADS box transcription factors have attracted particular interest due to their presence in high numbers in the core eudicots and their known roles in a great variety of plant developmental processes (Ng and Yanofsky, 2000). The duplication and subsequent diversification in function of MADS box genes may have played an important role in the origin and diversification of the angiosperms (Ng and Yanofsky, 2000; Irish, 2003; Soltis *et al.*, 2007).

One sublineage of MADS box genes, the *AG* lineage, has been shown to have a diversity of roles in flower and fruit development across a number of angiosperm species. In gymnosperms, *AG* genes are expressed in microsporophylls, megasporophylls, and ovules (Tandre *et al.*, 1995; Rutledge *et al.*, 1998; Winter *et al.*, 1999; Jager *et al.*, 2003). Therefore, it has been suggested that the ancestral function of *AG* genes is in specifying male and female reproductive organs (Theissen *et al.*, 2000; Kramer *et al.*, 2004). In angiosperms, a duplication in the *AG* clade resulted in the *euAG* and *PLE* lineages within the core eudicots (Kramer *et al.*, 2004). Functional analyses of *AG* clade genes in different core eudicot species have revealed interesting examples of functional conservation, diversification, and subfunctionalization (Bradley *et al.*, 1993; Angenent *et al.*, 1995; Davies *et al.*, 1999; Kapoor *et al.*, 2002; Nitasaka,

2003). In *Antirrhinum majus*, the *euAG* lineage gene is *FARINELLI* (*FAR*), and *PLENA* (*PLE*) corresponds to the *PLE* lineage gene (Causier *et al.*, 2005). Loss-of-function analyses have shown that *Antirrhinum PLE* is necessary for stamen and carpel development, while *FAR* appears to be responsible only for aspects of pollen development in the stamens (Carpenter and Coen, 1990; Bradley *et al.*, 1993; Davies *et al.*, 1999). In *Arabidopsis*, a different parsing of these reproductive functions has occurred with the *Arabidopsis euAG* lineage gene, *AGAMOUS* (*AG*), specifying stamen and carpel identities, as well as floral determinacy (Yanofsky *et al.*, 1990; Favaro *et al.*, 2003). A recent duplication has resulted in two paralagous *PLE* lineage genes in *Arabidopsis*, *SHATTERPROOF1* and *SHATTERPROOF2* (*SHP1* and *SHP2*), which are redundantly required for dehiscence zone formation in the silique, as well as aspects of ovule development (Liljegren *et al.*, 2000; Pinyopich *et al.*, 2003). These data indicate that the *SHP* genes have assumed novel roles in specifying development specific to a derived fruit type. Petunia, a euasterid like *Antirrhinum*, nonetheless has *AG* gene functions that are more similar to those of *Arabidopsis*. The Petunia *euAG* gene *PMADS3* displays loss- and gain-of-function phenotypes similar to those of *Arabidopsis AG* (Tsuchimoto *et al.*, 1993; Kater *et al.*, 1998; Kapoor *et al.*, 2002, 2005). Though no loss-of-function phenotype has been characterized for the Petunia *PLE* gene *FBP6*, overexpression studies have found that unlike *PMADS3*, *FBP6* overexpression produces smaller petals, but does not produce homeotic transformations of sepals and petals (Kater *et al.*, 1998).

Currently, there are no available mutants of *AG* clade genes in tomato, although *AG* lineage genes have been identified and their expression patterns have been characterized to some extent (Busi *et al.*, 2003; Hileman *et al.*, 2006). *TAG1* belongs to the *euAG* clade [Fig. 1 (Vrebalov *et al.*, 2009)] and has been functionally characterized using antisense technology (Pnueli *et al.*, 1994). These antisense analyses suggested that loss of *TAG1* function was associated with homeotic transformations of stamens and carpels; however, these phenotypes could have reflected the coordinate loss of function of several *AG*-related genes in tomato. Functional analyses of *TAGL1* have recently been carried out using RNA interference (RNAi), or by repressing its function using a dominant chimeric repressor construct (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). These analyses have demonstrated that *TAGL1* has a unique role in regulating several aspects of ripening, particularly carotenoid accumulation, fleshy fruit expansion, and ethylene production. Furthermore, *TAGL1* function cannot completely substitute for *SHATTERPROOF* function in *Arabidopsis*, indicating that these genes have diverged in their biochemical activities (Vrebalov *et al.*, 2009).

Because the functional analyses of *TAGL1* and *TAG1* have been carried out using transgenic lines that were generated in different backgrounds and using different strategies, it has been difficult to compare directly the phenotypes produced by loss of function of the *AG* lineage

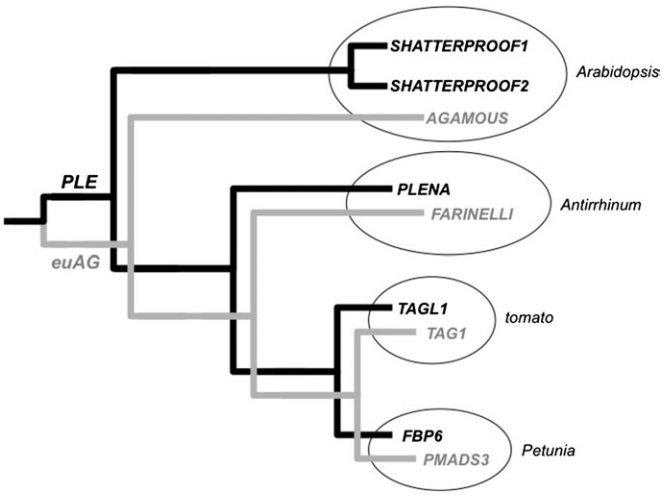

Fig. 1. *AGAMOUS* clade genes in four model species. A major duplication event occurred prior to the emergence of the core eudicots that gave rise to two clades of *AGAMOUS*-like MADS box genes. *PLE* and *euAG* lineages are shown in black and grey, respectively.

paralogues in tomato. In this study, loss-of-function-analyses of *TAG1* and *TAGL1* in the same genetic background (cv MicroTom) were carried out using RNAi. It was found that *TAGL1* plays a novel role in regulating tomato fruit ripening and has a qualitatively distinct function from that of *TAG1*. As in earlier studies, it was found that *TAG1* plays roles in tomato in specifying normal stamen and carpel development. In addition, though, it was also found that *TAG1* RNAi lines produce fourth whorl fruits with defects in determinacy. A temperature-sensitive EMS (ethyl methane sulfonate) mutant has also been identified in tomato that has qualitatively distinct stamen defects and 'fruit inside fruit' development. This mutant, however, appears to correspond to a distinct locus that probably acts downstream of *TAG1* and *TAGL1*. Together, these analyses demonstrate the extent of functional diversification between two closely related genes in a fleshy fruited species and the tremendous plasticity in the parsing of function in MADS box genes in the core eudicots, as well as illuminating aspects of the genetic pathway controlling fruit development in tomato.

Materials and methods

Plant materials and growth conditions

Mutant line *e2814* was provided by the Zamir Lab (http://zamir.sgn.cornell.edu/mutants/). Plants were grown in a greenhouse at Marsh Gardens (Yale University) under 16 h day and 8 h night conditions with auxiliary sodium lamps. Tomato (*Solanum lycopersicum* cv MicroTom) tissue culture experiments were carried out at 24 °C with a 16 h light and 8 h dark cycle. Transgenic tomato plants were grown at 22 °C with a 16 h light and 8 h dark cycle.

Transformation constructs and plant transformations

For tomato transformation experiments, RNAi constructs were generated using the Gateway System (Invitrogen, Carlsbad, CA, USA). A 222 bp region of the *TAGL1* 3′-untranslated region was

amplified using the TAGL1FB1 primer containing an *att*B1 site (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTGAA-ATTTGGGGTCAAGG-3′) and the TAGLIRB2 primer containing an *att*B2 site (5′-GGGGACCACTTTGTACAAGAAAGC-TGGGTGCATATTATTTATATAAGGC-3′). A longer 495 bp region of the *TAGL1* C-terminal domain and 3′-untranslated region was amplified using the GL1FB1A primer containing an *att*B1 site (5′-GGGGACAAGTTTGTACAAAAAAGCAGG-CTGAGCTGCAGAACGCCAACAT-3′) and the TAGL1RB2 primer.

A 262 bp region of the *TAG1* 3′-untranslated region was amplified using the TAG1FB1 primer containing an *att*B1 site (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAATGTGCT-TGAGAGATTGTC-3′) and the TAG1RB2 primer containing an *att*B2 site (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT-GGACAGAAAAACAGTTGTGAA-3′).

A longer 430 bp region of the *TAG1* C-terminal domain and 3′-untranslated region was amplified using the G1FB1A primer containing an *att*B1 site (5′-GGGGACAAGTTTGTACAAA-AA-AGCAGGCTGAGCTGCAGAACGCCAACAT-3′) and the TAG1RB2 primer.

DNA products were amplified using the following program: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 1 min, followed by 72 °C for 7 min.

These products were cloned into a pH7GWIWG2 (II) or pB7GWIWG2 (II) destination vector (Karimi *et al.*, 2002; http ://www.psb.ugent.be/gateway/index.php).

Transformation of tomato (*S. lycopersicum* cv MicroTom) wild-type cotyledon explants was performed as previously described (McCormick, 1991). The presence of the transgene was verified in the T_0 generation by PCR using three sets of primers. HYG1-F (5′-GTTCGGTCGGCATCTACTCTATTC-3′) and HYG1-R (5′-TCGGCTCCAAC-AATGTCCTGAC-3′) amplified the *hygromycin* (*HYG*) resistance gene. BAR-F (5′-GCTGCCAG-AAACC-CAGGTCA-3′) and BAR-R (5′-CGGACATGCCGGCGGTC-TGC-3′) amplified the *BAR* resistance gene. The 35S2 primer (5′-CCTCTATATAAGGAAGTTCATTTCA-3′) and the gene-specific reverse primers with an *att*B2 site (TAG1RB2 or TAGL1RB2) amplified the region encompassing the end of the *35S* promoter and the transgene. The PCR program for *HYG* was 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 7 min. The PCR program for *BAR* was 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 7 min. The PCR program for 35S2 and gene-specific product was 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 1 min, followed by 72 °C for 7 min.

Carotenoid extraction and HPLC

Carotenoid extraction and HPLC were performed according to a previously published protocol (Alba *et al.*, 2005). Pooled green fruit [36 days post anthesis (dpa)] and red fruit (45 dpa) from transgenic and control lines were used. All samples were run with two technical and two biological replicates.

Quantitative RT-PCR

RNA from 5 g of green fruit (36 dpa) and red fruit (45 dpa) from control and transgenic lines was extracted using a previously published protocol (Griffiths *et al.*, 1999). Quantitative real-time PCR was performed using three biological and three technical replicates for each sample using Taqman One-Step RT-PCR Master Mix Reagents (Applied Biosystems, Foster City, CA, USA) in a 20 µl total sample volume [10 µl of 2× Master Mix; 0.5 µl of 40× Multiscribe™, and RNase Inhibitor Mix; 900 nM of each primer; 250 nM Taqman MGB probe with VIC reporter dye; 3 µl of total RNA (150 ng total); and 4 µl diethylpyrocarbonate (DEPC)-H_2O]. A standard curve was included on each plate for the specific gene being analysed using wild-type RNA. For each gene analysis, template-free and negative reverse transcriptase controls were included. The real-time PCR was performed on an ABI PRISM™ 7900HT Sequence Detection System using the following reaction conditions: reverse transcription at 48 °C for 30 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The ABI PRISM™ SDS version 2.1 software (Applied Biosystems) was used to calculate gene-specific threshold cycles (Cts) including the endogenous reference (18S) for every sample. Cts were calculated and relative quantitations using a standard curve method were used to calculate mRNA levels. Relative transcript levels were calculated using 18S controls. For each gene tested, the following primers and probes were used: IPP, 5′-TGATGGGAACAAGCCGATGT-3′ (forward), 5′-TGAACCT-CCGCAAGAATTGTAA-3′ (reverse), 5′-CTACTGCTTCAGCT-TC-3′ (probe); GGPS2, 5′-GTCCACTGGCATGGCTGCTG-3′ (forward), 5′-ATCAACAGCATTTGGTCCACCC-3′ (reverse), 5′-GTCAGTTCCTTGACCTTG-3′ (probe); PSY1, 5′-AACATA-TGCTAATGACTCCCGAGAGA-3′ (forward), 5′-ATGCGTTT-GGGCCATCAA-3′ (reverse), 5′-TATGGTGCAGAAGAACA-3′ (probe); PSY2, 5′-GTCGCTGGTACAGTAGGATTGATG-3′ (forward), 5′-TCTCTGTCGTTGCCTTTGATTC-3′ (reverse), 5′-ATGGGCATTGCACC-3′ (probe); PDS, 5′-AGATTGTTATTG-CTGGTGCAGG-3′ (forward), 5′-TGTGACCAGCATCTGCC-AA-3′ (reverse), 5′-CTGTAGACAAACCACCCAA-3′ (probe); ZDS, 5′-ATCCTCTGATGGAAGCATGTATGTT-3′ (forward), 5′-GCATCAGCTTTTACAATTTTCTTCTG-3′ (reverse), 5′-TG-GGCTTGCCATGTCAAAGGCC-3′ (probe); CRTISO, 5′-CAG-GACAAGGTGTTATAGCTGTA-3′ (forward), 5′-GAGCACT-GTCCAGCACATCTGAT-3′ (reverse), 5′-CTAAGTCAGCTG-CAACAC-3′ (probe); LCY-B, 5′-TCGTCCTGGCTTGCGTA-TAGA-3′ (forward), 5′-TGTTCATCTTCTTCAATGCTCTTCA-3′ (reverse), 5′-CATGGTGGCTCGTTTAA-3′ (probe); CYC-B, 5′-GGCTCAATTCGACGTGATCA-3′ (forward), 5′-AGAGTG-GTGAAGGGTCAACACA-3′ (reverse), 5′-CGGAGCTGGCCC-TGCTGGG-3′ (probe); CRTR-B1, 5′-GAACGACGTTTTCGC-CATAAC-3′ (forward), 5′-TGAGGCCTTTATGGAAGAAA-CC-3′ (reverse), 5′-AACGCTGTTCCAGCAATAGCCCTCCT-3′ (probe); and CRTR-B2, 5′-TGCCTTTTTCTGAAATCT-TAGCTACA-3′ (forward), 5′-CTCGCCCAGTACTCCATTCC-3′ (reverse), 5′-TCTCTCGTTTGGCGCTGCCGT-3′ (probe).

RT-PCR

Total RNA from tomato tissue was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. A 2.5 µg aliquot was used for cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA was diluted 1:10 and 1–2 ul was used for PCR. The following primers were used: TAG1-F (5′-AGCTCTTGCTGGAATGAAAC-3′), TAG1-R (5′-AAGCT-CATGATAGTTTGATG-3′), TAGL1-F (5′-GCATTGGGCAG-TTTAAGCCC-3′), TAGL1-R (5′-TCGCGACGAGAGTAATG-AGG-3′), LeAP3-F (5′-GAGAAAATGCAAGAGCAGC-3′), LeAP3-R (5′-CAAAAGTAGTAATATCAGAGCC-3′), LePI-F (5′-CAATCAACTTACCCATAAAG-3′), LePI-R (5′-GATTAA-TTAGTGTTTCTAGC-3′), TM6-F (5′-CGAGAAAATGCAA-GAAAACTTG-3′), TM6-R (5′-AGATCACGAGAACCAAAT-CC-3′), ACT1 (5′-GATGGATCCTCCAATCCAGACACTG-TA-3′), and ACT2 (5′-GTATTGTGTTGGACTCTGGTGATG-GTGT-3′).

The PCR program for *TAGL1* and *ACTIN* was 94 °C for 5 min, 28 cycles of 94 °C for 30 s, 59 °C for 45 s, 72 °C for 1 min., followed by 72 °C for 10 min. The PCR program for *TAG1* was 94 °C for 5 min, 29 cycles of 94 °C for 30 s, 59 °C for 45 s, 72 °C for 1 min, followed by 72 °C for 10 min. The PCR program for *LeAP3*, *LePI*, and *TM6* was 95 °C for 10 min, 31 cycles of 95 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 10 min. Gel images were scanned and band intensities were normalized to *ACTIN* and quantified using NIH Image J (http://rsb.info.nih.gov/ij/).

Scanning electron microscopy

Plant tissue was fixed overnight in FAA (3.7% formaldehyde, 5% glacial acetic acid, 50% ethanol), then dehydrated to 100% ethanol. Samples were dried using a critical point dryer, sputter coated in gold, and analysed on a Zeiss ISI-SS40 scanning electron microscope.

Results

TAG1 *and* TAGL1 *have overlapping but distinct expression patterns*

Previous studies have reported that *TAG1* and *TAGL1* are both expressed principally in flowers and in developing fruits (Busi *et al.*, 2003; Hileman *et al.*, 2006). To characterize this pattern of expression more explicitly, RT-PCR analyses were carried out using *TAGL1* and *TAG1* gene-specific primers (Fig. 2). *TAGL1* expression was undetectable in roots, seedlings, leaves, and mature seeds. In flowers, *TAGL1* transcripts were detected at low levels in petals, with stronger expression in stamens and carpels; expression was undetectable in sepals. *TAGL1* was expressed in both pericarp and internal tissues of young green and mature green fruit in which the fruit are fully expanded. Expression in both the pericarp and internal tissues was diminished by the red fruit stage. Although *TAGL1* expression was observed in developing ovules at earlier stages, mature seeds showed no expression. Expression patterns in flowers and fruit were similar for *TAG1*, with highest expression levels also seen in inflorescences, stamens, and carpels, with lower but detectable levels in later fruit stages. Unlike *TAGL1*, we observed detectable levels of *TAG1* expression in leaves and sepals (Fig. 2).

TAG1 *RNAi lines show defects in stamen identity and floral determinacy*

To explore *TAG1* function in tomato cv MicroTom, an RNAi construct was generated utilizing a 262 bp region from the *TAG1* 3′-untranslated region, and this fragment was inserted into a Gateway RNAi vector. This construct was introduced into cv MicroTom using *Agrobacterium*-mediated T-DNA transfer. Seven independent *TAG1* RNAi lines were recovered and verified for transgene integration by the presence of *BAR* or *HYG* resistance genes in the T_0 generation. These lines showed a reduction in the levels of *TAG1* transcripts, with three lines showing a significant reduction in *TAG1* transcript abundance (Fig. 3A). These three lines showed a variety of floral defects (Fig. 4). It was found that these lines displayed slight defects in stamens, resulting in the production of smaller amounts of pollen compared with control lines. However, unlike previous reports (Pnueli *et al.*, 1994), no transformation of fourth whorl carpels into sepalloid structures was observed. Instead, these RNAi lines still produced red fruit, but displayed varying degrees of a loss of determinacy (Fig. 4A, B). In some cases, a dramatic 'fruit inside fruit' phenotype was observed, in which ectopic fruit structures continued to develop in an indeterminate fashion (Fig. 4D). These lines did not produce seeds, and crosses with wild-type plants were also unsuccessful. Therefore, all characterizations of the phenotype were done in the T_0 generation.

To determine if the *TAG1* RNAi (262 bp construct) phenotypes reflected only a partial loss of function, RNAi lines were also generated using a longer region (430 bp) encompassing both the 3' coding region and 3′-untranslated region of the *TAG1* gene, since longer double-stranded RNA transcripts may produce a stronger phenotype. Two transgenic lines were generated in the MicroTom cultivar

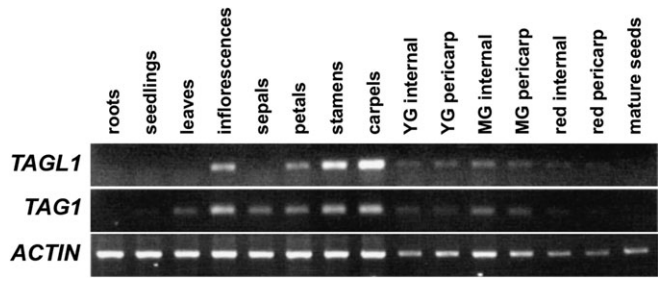

Fig. 2. *TAGL1* and *TAG1* expression levels in wild-type tissue. *TAG1* and *TAGL1* expression levels were detected by RT-PCR using dissected tissues from different stages as indicated. For fruit, pericarp was separated from the rest of the fruit tissue (internal) for analysis. YG, young green; MG, mature green fruit.

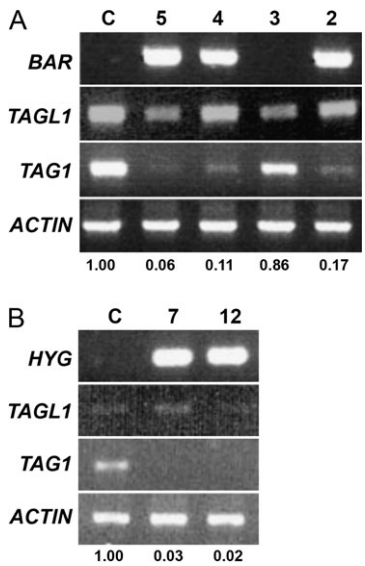

Fig. 3. *TAG1* RNAi lines show reduction in *TAG1* expression levels. Semi-quantitative RT-PCR on buds from control and *TAG1* RNAi lines. *TAG1* levels were normalized to actin and are shown relative to the control sample. Expression of *TAGL1* was also examined in all lines as indicated. (A) RNAi lines made using a shorter (262 bp) fragment for silencing. Line 3 was scored as negative for transformation. Lines 2, 4, and 5 show a significant reduction in *TAG1* RNA levels. (B) RNAi lines made using a longer (430 bp) fragment for silencing. Lines 7 and 12 show almost complete lack of *TAG1* transcripts. BAR, BASTA resistance marker; HYG, hygromycin resistance marker, indicating the presence of the transgene.

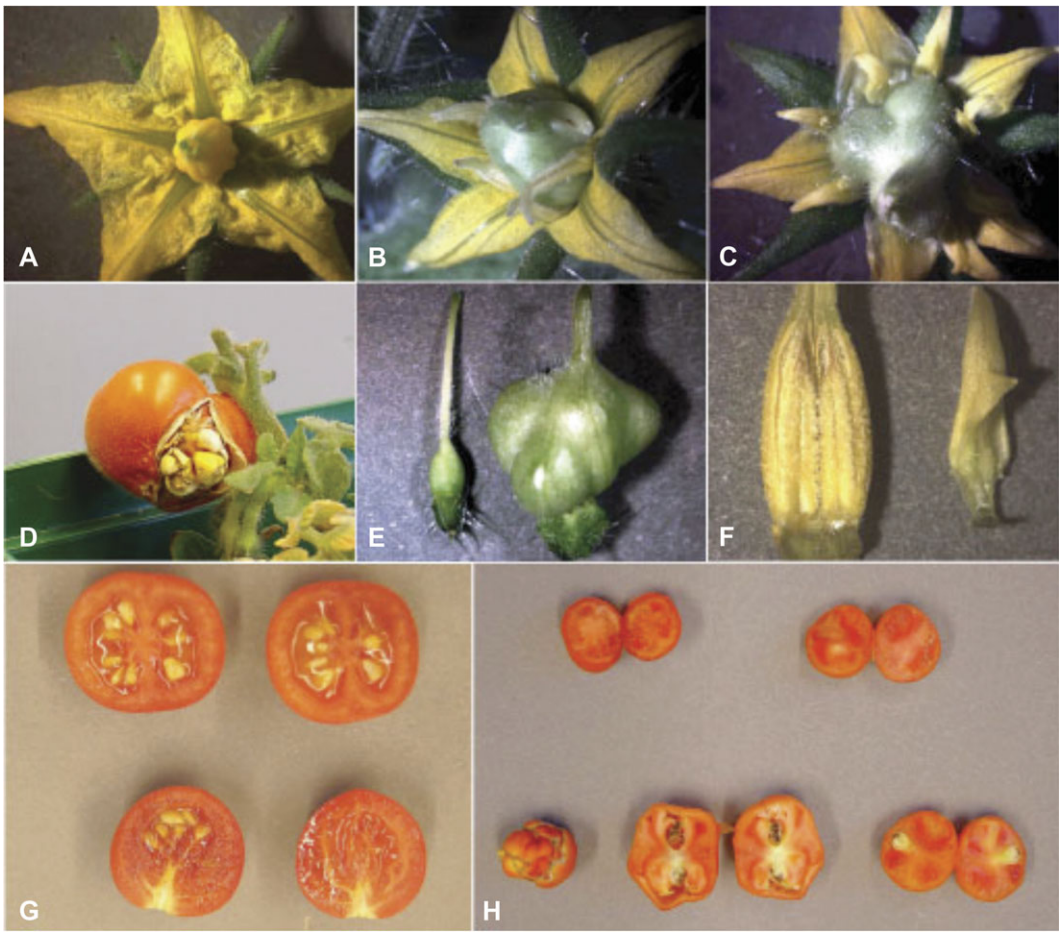

Fig. 4. RNAi silencing of *TAG1* produces defects in stamens and affects floral determinacy. (A) Control flower ~3 dpa. (B) *TAG1* RNAi line 4 flower made using a shorter (262 bp) fragment for silencing. (C) TAG1 RNAi line 7 flower made using a longer (430 bp) fragment for silencing. (D) *TAG1* RNAi line 2 with the 'fruit inside fruit' phenotype. (E) Control (left) and *TAG1* RNAi line 7 (right) carpels. (F) Control (left) and *TAG1* RNAi line 7 (right) stamens showing petalloid tissue. (G) Control red, ripe fruit. (H) Range of phenotypes of *TAG1* RNAi line 7 fruit: (top) ectopic exocarp tissue, (bottom left) 'fruit inside fruit' (bottom centre and right) internal flower structures.

and verified for the presence of the transgene in the T_0 generation. These lines showed almost complete loss of endogenous *TAG1* transcripts (Fig. 3B). Like the 262 bp construct lines, these *TAG1* RNAi lines were also sterile, so all phenotypic characterizations were done in the T_0 generation. In these 430 bp construct lines a severe phenotype was observed in flowers (Fig. 4). These flowers produced stamens with petalloid tissue (Fig. 4F). In addition, a strong loss of determinacy was observed at anthesis (Fig. 4E), resulting in a dramatic 'fruit inside fruit' phenotype (Fig. 4G, H). In common with the 262 bp *TAG1* RNAi construct, the 430 bp *TAG1* RNAi construct still resulted in the formation of red fruit for both transgenic lines, and did not display any transformation of carpels into sepalloid structures.

Studies were conducted to examine whether *TAGL1* expression was affected in the *TAG1* RNAi lines (Fig. 3). Little difference was seen in the levels of *TAGL1* transcripts in the *TAG1* RNAi lines, suggesting that *TAG1* does not regulate the expression of *TAGL1*. These observations also indicated that *TAGL1* is not targeted by the *TAG1* RNAi construct. Since *TAG1* and *TAGL1* are the most closely related MADS box genes in the tomato genome (Vrebalov *et al.*, 2009), these observations support the gene-specific targeting of *TAG1* by the two RNAi constructs.

TAGL1 *functions in regulating fruit ripening*

To assess *TAGL1* function in tomato cv MicroTom, two RNAi constructs designed to reduce *TAGL1* transcript levels were generated. One construct was designed to target a 222 bp region encompassing the 3′-untranslated region of *TAGL1*; a second construct was also generated using a longer region (495 bp) that targeted both the C-terminal domain and the 3′-untranslated region of *TAGL1*. Both constructs were predicted to be gene specific in that they correspond to a variable region with low similarity to other MADS box genes. These constructs were introduced into wild-type tomato (cv MicroTom) plants via *Agrobacterium*-mediated transformation and independently derived lines were verified for the presence of the transgene. Ten lines were generated with the 222 bp RNAi construct and one line with the 495 bp RNAi construct that were characterized for their effects on down-regulation of endogenous

TAGL1 expression in homozygous T_1 plants (Fig. 5). There was wide variation in the extent of down-regulation of *TAGL1*, but all lines appeared to be gene specific in their effects in that the expression of *TAG1* was not noticeably affected (Fig. 5).

RNAi lines 1, 3, 9, 12, 18, 26 (all targeting the 222 bp region) and line 4A (targeting the 495 bp region) all showed an ~50% reduction or greater in the expression of *TAGL1* (Fig. 5). In these seven lines a striking fruit phenotype was observed where all fruits produced had a distinctive orange colour, compared with the normal bright red fruits produced in wild-type and control lines (Fig. 6F–I, and data not shown). Also, *TAGL1* RNAi fruits at the mature green stage (~36 dpa) were also darker green than control fruits (Fig. 6B–E). The RNAi lines showed no other defects in vegetative, flower, or fruit development, set seeds normally, and produced viable progeny. It was also observed that the differences in colour were not due to a delay in ripening, since experimental lines produced orange fruit at the same number of days post-anthesis as control lines and stopped changing colour at ~45 dpa (data not shown). The fruits from *TAGL1* RNAi lines never appeared bright red, but remained orange to orange-red. This phenotype is similar to that produced by RNAi-induced repression of *TAGL1* in cv Ailsa Craig (Vrebalov *et al.*, 2009), as well as in experiments in which *TAGL1* expression was down-regulated using a chimeric repressor (Itkin *et al.*, 2009). However, no noticeable change in pericarp thickness was observed as has been documented for down-regulation of *TAGL1* in cv Ailsa Craig (Vrebalov *et al.*, 2009). This could reflect the smaller fruit size and overall thinner pericarp that is characteristic of cv MicroTom which could potentially obscure a *TAGL1* RNAi pericarp phenotype.

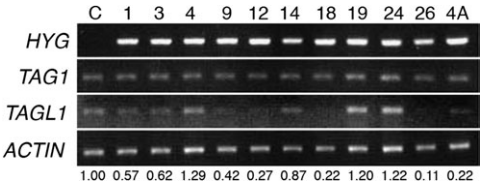

Fig. 5. *TAGL1* expression levels in *TAGL1* RNAi lines. Semi-quantitative RT-PCR on green fruit from control (C) and *TAGL1* RNAi lines. *TAGL1* levels were normalized to actin and are shown relative to the control sample. Lines 1–26 were produced using the 222 bp fragment for silencing; line 4A was produced using the longer (495 bp) fragment for silencing. Expression of *TAG1* was also examined by RT-PCR in all lines. HYG, hygromycin resistance marker indicating the presence of the transgene.

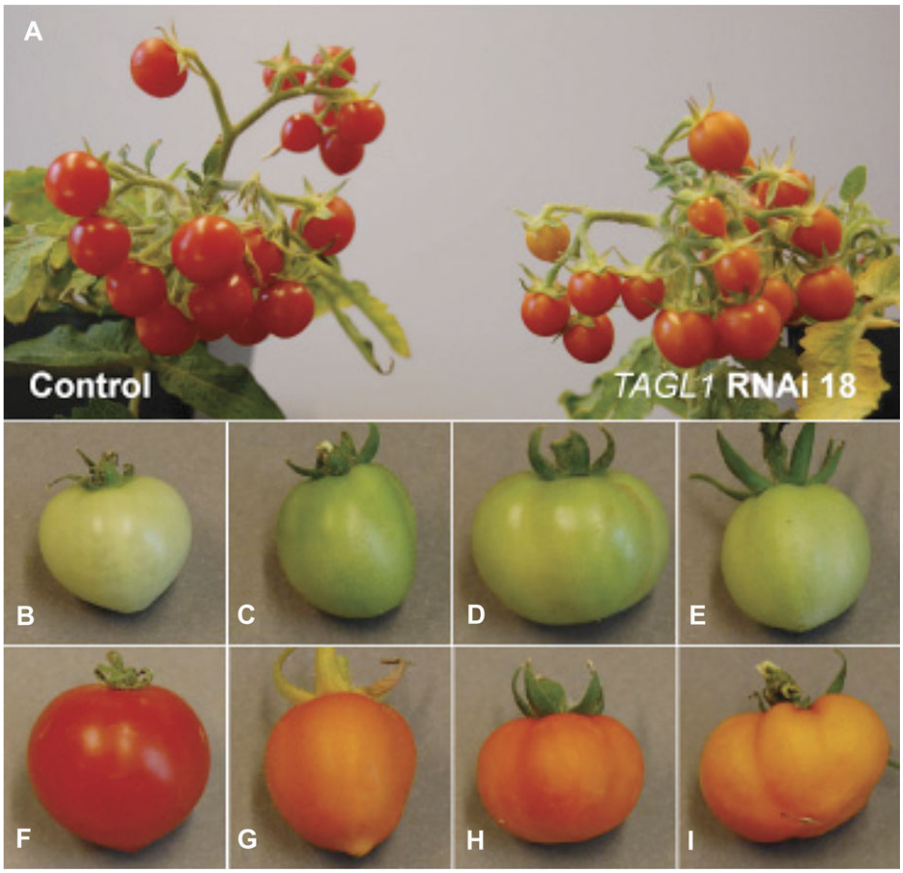

Fig. 6. RNAi silencing of *TAGL1* affects fruit colour. (A) Overview of control (left) and RNAi line *TAGL1-18* (right) plants showing differences in fruit colour. (B–E) Fruit 36 dpa; (F–I) fruit 45dpa. (B) and (F) Control line mature green (36 dpa) and red (45 dpa) fruits. (C) and (G) RNAi line *TAGL1-4A*. Note the pointy fruit in (G). (D) and (H) RNAi line *TAGL1-9*. (E) and (I) RNAi line *TAGL1-18*.

TAGL1 *regulates carotenoid biosynthetic gene expression and carotenoid accumulation*

The dramatic alteration in fruit pigmentation in the RNAi lines suggested that *TAGL1* controls the expression of the carotenoid biosynthetic gene pathway. Using HPLC, the amounts of phytoene, phytofluene, lycopene, lutein, α-carotene, β-carotene, chlorophyll *a*, and chlorophyll *b* were measured in mature green (~36 dpa) and ripe fruit (~45 dpa) from three RNAi lines and one control line. Specific alterations were found in the levels of chlorophyll *b*, as well as in the levels of β-carotene, lutein, and lycopene in the RNAi lines as compared with controls (Fig. 7). In all RNAi lines, increased levels of β-carotene and of lutein were found in both mature green and ripe fruit. Beta-carotene is orange-red while lutein is yellow-orange, and alteration in the relative levels of these carotenoids presumably is responsible for the orange colour phenotype observed in the RNAi lines. Lycopene levels appeared to be somewhat lower in the RNAi lines. In a parallel set of analyses, Vrebalov *et al.* (2009) also observed an increase in levels of β-carotene and lutein, and a reduction in the levels of lycopene in RNAi induced down-regulation of *TAGL1* in tomato cv Ailsa Craig, supporting the observations of specific alterations in the levels of these carotenoids in *TAGL1*-repressed fruit. It was also observed that chlorophyll *b* levels were higher in green fruit from all three RNAi lines as well (Fig. 7), which could explain the darker green phenotype of mature green stage *TAGL1* RNAi fruit.

Using quantitative RT-PCR, gene expression levels of a number of carotenoid biosynthetic genes were also analysed, including *IPP*, encoding isopentenyl diphosphate; *PSY1*, phytoene synthase 1; *PSY2*, phytoene synthase 2; *PDS*, phytoene desaturase; *CrtISO*, carotenoid isomerase; *ZDS*, ζ-carotene desaturase; *LCY-B*, lycopene β-cyclase; *CRTR-B1*, β-ring carotene hydroxylase; and *CrtR-b2*, β-ring carotene hydroxylase (chromoplast specific). Significant changes were found in the expression of several of these genes in the RNAi lines, as well as developmental differences in some cases in terms of how such genes were expressed. In particular, it was found that the chromoplast-specific lycopene β-cyclase (*CYC-B*) gene was expressed at significantly higher levels in green fruits in *TAGL1* RNAi lines compared with control lines (Fig. 8). This would account for the orange colour and higher levels of β-carotene seen in mature *TAGL1* RNAi fruits, as has been observed in mutants that overexpress *CYC-B* (Ronen *et al.*, 2000). These observations are also consistent with those reported in Vrebalov *et al.* (2009) in which levels of β-carotene as well as lutein were higher in *TAGL1*-suppressed tomato cv Ailsa Craig fruit. Levels of *IPP* expression were also somewhat higher in green fruit from RNAi lines. Carotenoid isomerase (*CrtISO*) gene expression levels were reduced in red fruit of *TAGL1* RNAi lines, as were the levels of *CRTR-b2* transcripts (Fig. 8). As *CrtlISO* and *CRTR-b2* are expressed predominantly in chromoplasts as opposed to chloroplasts (Liu *et al.*, 2003; Galpaz *et al.*, 2006), their down-regulation in *TAGL1* RNAi lines also probably contributes to the orange phenotype of the resulting fruit.

The e2814 *mutant resembles the* TAG1 *RNAi fruit phenotype and shows stamen defects*

Currently, no stable mutants of *TAG1* or *TAGL1* have been identified in tomato. In an effort to try to identify such a mutant, the available tomato mutant collection generated through EMS and fast-neutron mutagenesis (http://zamir.sgn.cornell.edu/mutants) was utilized. There are currently 3417 phenotypically characterized mutants available in the inbred M82 background. One mutant, *e2814*, was listed in the database as a recessive mutant showing partial sterility

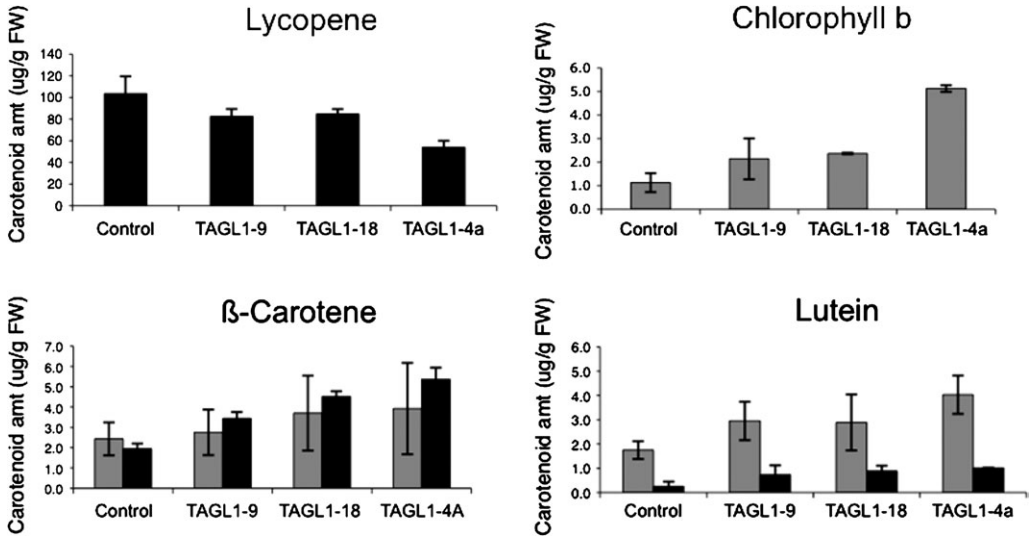

Fig. 7. Chlorophyll and carotenoid levels in *TAGL1* RNAi lines. Chlorophyll *b* and carotenoid levels were determined using mature green (36 dpa; grey bars) and ripe (45 dpa; black bars) fruit from three different *TAGL1* RNAi lines as well as controls, by HPLC using mean HPLC peak areas (n=2). Standard errors are shown.

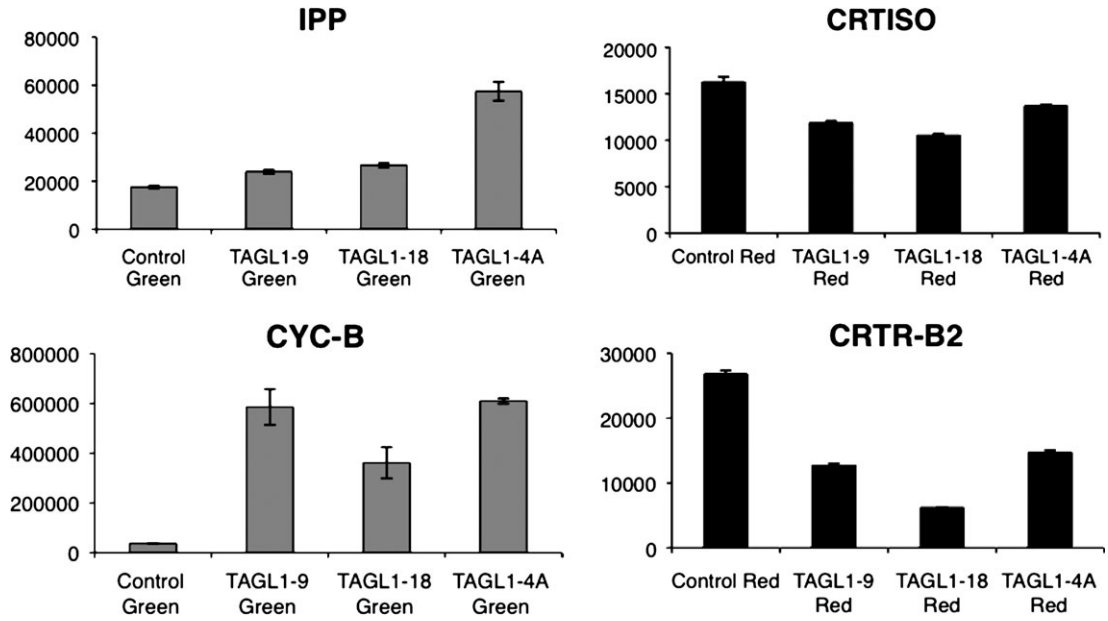

Fig. 8. Transcript levels of carotenoid biosynthesis genes in *TAGL1* RNAi lines. Relative transcript levels were determined by quantitative RT-PCR using gene-specific primers on green (grey bars) and red (black bars) fruit. 18S RNA was used as an internal control to normalize the relative level of each transcript. Standard errors of three replicates are shown. *IPP*, encoding isopentenyl diphosphate; *CrtISO*, carotenoid isomerase; *CYC-B*, lycopene β-cyclase (chromoplast specific); and *CrtR-b2*, β-ring carotene hydroxylase (chromoplast specific).

and a 'fruit inside fruit' phenotype. Homozygous *e2814* mutant plants were examined and it was noted that the 'fruit inside fruit' phenotype was only observed when plants were exposed to higher temperatures (approximately 30-37 degrees C) in the spring and summer. Plants grown in cooler temperatures in the autumn produced wild-type flowers and fruits and produced viable seeds, indicating that e2814 is temperature sensitive.

It was found that the 'fruit inside fruit' phenotype closely resembled the *TAG1* RNAi phenotype observed in more severely affected fruits (Fig. 9). In some cases, a more dramatic 'fruit inside fruit' phenotype in the *e2814* mutants was observed, where an almost intact second fruit was growing within a larger fruit (Fig. 9L). In all fruits examined, the fourth whorl always produced red fruit and in no cases was transformation of carpels into green sepal-like structures seen. There were also no seeds found in fruits with this severe phenotype.

Floral phenotypes were also observed in plants grown at higher temperatures. Unlike the conversion of stamens to petals or petalloid structures as seen in *TAG1* RNAi-induced loss-of-function phenotypes, *e2814* flowers showed conversion of stamens into carpel-like structures (Fig. 9B). In some cases, stamens were converted into green, twisted style-like tissue (Fig. 9C). Closer examination of the mutant third and fourth whorl structures revealed green or yellow style-like structures of varying thickness in place of third whorl stamens. Also the fourth whorl style of *e2814* mutant flowers appeared twisted and larger than in the wild type (Fig. 9D). There was also fusion of third whorl structures to the fourth whorl (Fig. 9E). Scanning electron microscopy of the fused third whorl structures showed that the epidermal cells somewhat resemble wild-type fourth whorl epidermal cells (Fig. 9F–H).

e2814 *probably represents a mutation in a novel gene*

To test the hypothesis that the lesion in *e2814* may correspond to a mutation in *TAG1*, RNA was isolated from wild-type and *e2814* floral buds, the RNA was reverse transcribed to cDNA, and the full-length coding region of *TAG1* from these cDNAs was sequenced. No differences between wild-type and the *e2814* cDNA were found at the nucleotide level (data not shown). Sequencing of the full-length coding region of cDNA corresponding to the *TAGL1* gene also revealed no differences at the nucleotide level (data not shown).

e2814 *does not affect expression of MADS box genes involved in floral development*

Although the sequence of the full-length coding region of *TAG1* was unchanged in the *e2814* mutant line, changes in transcript levels of *TAG1* were also investigated. Since the phenotype appears to be temperature sensitive, flowers were collected from mutant lines that showed a distinct floral phenotype and from the same lines that did not show a phenotype. Using cDNA made from these tissue samples and wild-type tissue samples grown in the same conditions, the levels of *TAG1* transcripts were checked using RT-PCR. No changes in transcript levels were observed in any of the mutant samples compared with the wild type (Fig. 10). *TAGL1* expression levels were also unchanged (Fig. 10). Since *e2814* flowers have defects in stamen development,

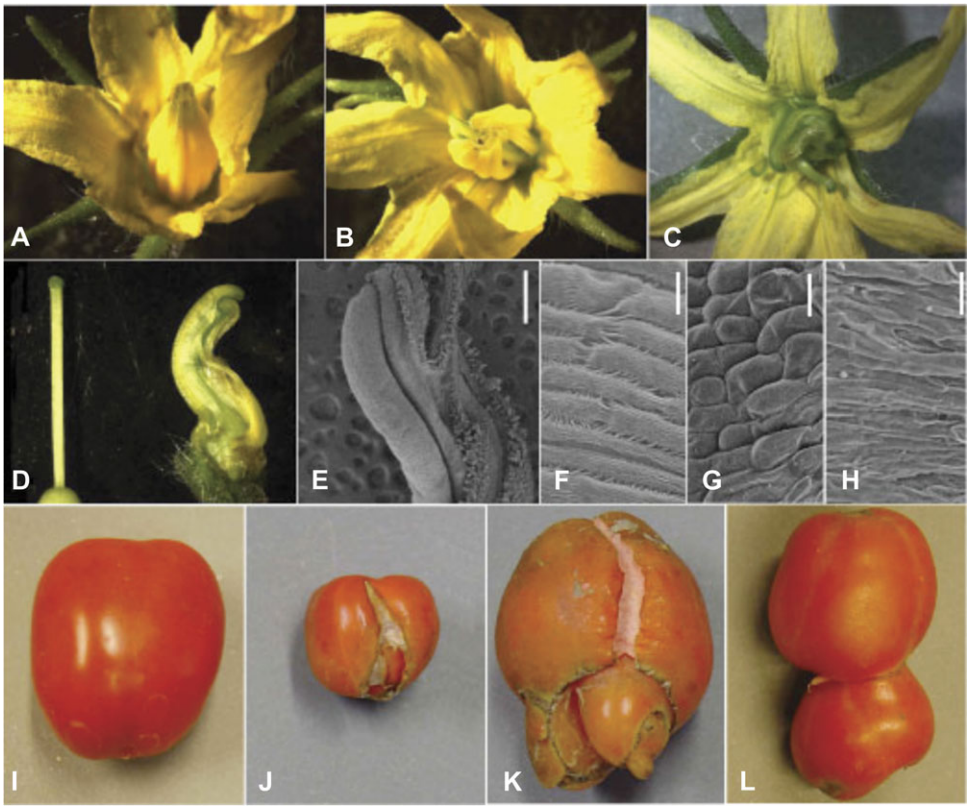

Fig. 9. Phenotypes of the *e2814* mutant. (A) Wild-type cv M82 flower. (B) *e2814* mutant flower showing green, narrow stamens. (C) *e2814* mutant flower shows conversion of all stamens to green, carpelloid structures. (D) Wild-type M82 carpel (left) and *e2814* carpel with fused stamens (right). (E) Scanning electron microscopy image of an *e2814* carpel and a fused, carpelloid stamen. (F) Epidermal cells of a wild-type M82 carpel. (G) Epidermal cells of a wild-type M82 stamen. (H) Epidermal cells of carpelloid, fused stamens from (E). (I) Wild-type M82 ripe fruit. (J) *TAG1-9* RNAi fruit. (K) *e2814* mutant fruit resembles the *TAG1* RNAi fruit phenotype. (L) *e2814* mutant fruit with severe 'fruit inside fruit' phenotype. Bars=1040 mm (E), 13 mm (F and H), 35 mm (G).

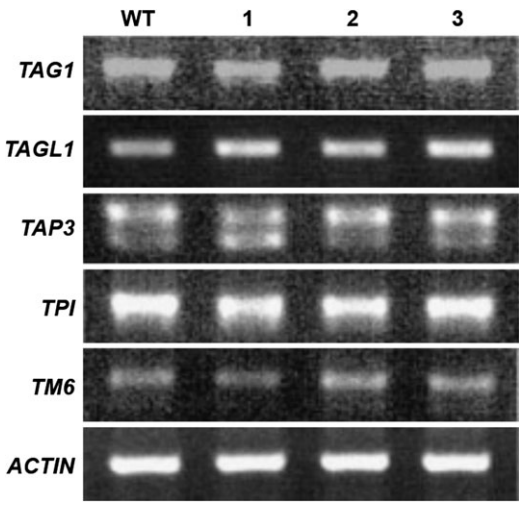

Fig. 10. *e2814* lines show no change in the expression of several MADS box genes. RT-PCR on flowers from M82 wild-type (WT); *e2814* flowers showing a wild-type phenotype (1), and stamen and carpel phenotypes (2, 3).

transcript levels of B class genes in tomato were also examined (de Martino *et al.*, 2006). Semi-quantitative RT-PCR of *TAP3*, *TPI*, and *TM6* also showed no difference in expression levels in the *e2814* mutant (Fig. 10).

Discussion

TAG1 *and* TAGL1 *have distinct roles in fruit development*

A duplication event early in the diversification of the core eudicots has led to two *AGAMOUS* clades, the *euAG* and the *PLE* lineages (Kramer *et al.*, 2004). Functional analyses of *euAG* and *PLE* genes in *Arabidopsis* and *Antirrhinum* have demonstrated that these paralogues have diversified in a distinct manner in each species (Yanofsky *et al.*, 1990; Davies *et al.*, 1999; Liljegren *et al.*, 2000; Causier *et al.*, 2005). The roles of the tomato members of these lineages, *TAG1* and *TAGL1*, were examined and it was shown that they have unique and distinct functions in fruit development.

The RNAi studies of *TAG1* demonstrate that loss of function of this gene in tomato cv MicroTom results in a loss of determinacy in the fourth whorl, resulting in a 'fruit inside fruit' phenotype, as well as defects in stamen identity. This phenotype differs from previously published results in which *TAG1* expression was down-regulated using antisense technology and produced transformation of fourth whorl organs into sepal-like organs (Pnueli *et al.*, 1994). There are two possible explanations for this discrepancy. One

possibility is that the phenotype from the *TAG1* RNAi lines represents a weak phenotype and low levels of *TAG1* expression are still present and can still act to specify fourth whorl carpel identity. In *Arabidopsis*, studies of *AG* partial loss-of-function situations have shown that carpel identity, stamen identity, and determinacy can be separated genetically (Mizukami and Ma, 1992; Sieburth *et al.*, 1995). These studies support the idea that floral meristem determinacy requires the highest level of *AG* expression, and even slight reduction in *AG* levels through antisense, RNAi, or other disruptive mutations can cause defects in fourth whorl determinacy. However, in the present analyses, overt homeotic changes in carpel identity were never observed, even in the strongest loss-of-function *TAG1* RNAi lines. The *TAG1* RNAi lines produced in this study showed a range of phenotypes, even within the different fruits of the same transgenic line. In general, stronger phenotypes (particularly more severe 'fruit inside fruit' phenotypes) were observed in fruits derived from older flowers. However, in all cases, even in the fruits with dramatic 'fruit inside fruit' phenotypes, the fruits remained red, unlike the earlier antisense study. In contrast, Pnueli *et al.* (1994) produced a total of 14 antisense lines with aberrant flowers. Two lines exhibited the most extreme phenotypes, but all 14 lines displayed a consistent fourth whorl phenotype with carpels transformed to sepals, accompanied by loss of determinacy.

An alternative explanation to resolve the differences between these studies is that the homeotic transformations observed in the antisense analysis reflect a synthetic phenotype in which multiple *AG* lineage genes were coordinately down-regulated due to the strategy being used. In the present study, the RNAi construct was designed for silencing to only target *TAG1*, taking care to avoid stretches of 20–22 bp that matched *TAGL1*, the most closely related MADS box gene. As such, presumably this analysis of *TAG1* RNAi-induced gene-specific silencing more accurately reflects the loss of function of *TAG1* alone.

Although it is clear that, upon duplication, there has been subsequent diversification of function in many of the *AG* lineage genes, several studies have shown that, despite assuming new functions, some *AG* lineage genes may still retain some aspects of a presumed ancestral carpel identity function (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003). In tomato, it was found that reduction of expression of *TAGL1* by RNAi produces defects in fruit ripening. Down-regulation of *TAGL1* has been associated with defects in carpel fleshiness as well (Vrebalov *et al.*, 2009), supporting the idea of a conserved role in fourth whorl development. The *TAGL1* RNAi-induced defects in tomato fruit ripening are associated with a reduction in chlorophyll *b* degradation, and alterations in carotenoid levels. This is consistent with other analyses that have demonstrated that *TAGL1* function is required for normal accumulation of carotenoids, particularly lycopene, chlorophyll breakdown, and gene expression changes associated with ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). Although *TAGL1* is clearly playing specialized roles in controlling aspects of fruit ripening, it may still retain cryptic carpel identity function. In the case of *TAG1* RNAi-induced repression carried out in this study, *TAGL1* expression is unaffected and may still be specifying carpel identity. Coordinate down-regulation of *TAG1*, *TAGL1*, and potentially other closely related MADS box genes would be needed to test whether redundancy can explain the specification of carpel identity in tomato.

A new mutant in fruit development distinct from TAG1 *and* TAGL1

One of the difficulties in elucidating the function of *TAG1* or *TAGL1* in tomato is the lack of a stable mutant. Though antisense strategies, RNAi, and virus-induced gene silencing (VIGS) have provided many insights, these techniques have many drawbacks. Most notably, VIGS produces transient phenotypes and RNAi lines often produce weaker phenotypes after a few generations (Burch-Smith *et al.*, 2004). Both VIGS and RNAi have the added problem of variable silencing, even within the same transgenic line. To that end, a stable mutant affecting carpel development, *e2814*, was identified in the hope that it would correspond to a mutation in an *AG* clade gene.

The *e2814* mutation is temperature sensitive, and at the restrictive temperature (~30–37 °C), displays a 'fruit inside fruit' phenotype and partial sterility. The temperature sensitivity of a mutant is often associated with alterations in protein conformation that disrupt biological activity. Several temperature-sensitive mutations in MADS box genes have been identified (Bowman *et al.*, 1989; Schwarz-Sommer *et al.*, 1992; Zachgo *et al.*, 1995); one of these, a mutation in the *Antirrhinum DEFICIENS* gene, has a deletion in a lysine residue in the K domain that probably disrupts protein–protein interactions at the restrictive temperature (Zachgo *et al.*, 1995). However, sequencing of the *TAG1* and *TAGL1* coding regions from the *e2814* mutant did not identify any such point mutations, suggesting that *e2814* corresponds to a different locus.

Analyses of *Arabidopsis AG* have shown that it functions in part to regulate *SPOROCYTELESS/NOZZLE* and *DAD1* directly, two genes that play roles in microsporogenesis and late stamen maturation (Ito *et al.*, 2004, 2007). Along with regulation of jasmonic acid biosynthesis through control of *DAD1*, *AG* has also been shown to regulate the expression of *GA4*, an enzyme that catalyses the biosynthesis of gibberellin (Gomez-Mena *et al.*, 2005). *TAGL1*, in addition to regulating carotenoid biosynthetic gene expression, also controls ethylene evolution during tomato fruit ripening (Itkin *et al.*, 2009; Vrebalov *et al.*, 2009). Based on the present expression analyses, the locus corresponding to *e2814* is probably downstream of both *TAG1* and *TAGL1* in fruit development, but also appears to have a *TAG1*- and *TAGL1*-independent role in regulating stamen differentiation. This may be occurring in part through feedback controls through various hormonal response pathways. One possibility is that *e2814* corresponds to one of the large number of ripening-associated

transcripts that have recently been identified as being regulated by *TAGL1* (Itkin *et al.*, 2009). Identification of the gene corresponding to the *e2814* mutant should be valuable in elucidating the network of genes involved in stamen and fruit development in tomato and facilitate better comparisons between how these regulatory networks may have evolved in different plant species.

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