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Aberrant forms of ARF8 stimulate parthenocarpy.

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Expression of aberrant forms of *AUXIN RESPONSE FACTOR8* stimulates parthenocarpy in Arabidopsis and tomato

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Abbreviations: ARF, Auxin Response Factor; At, Arabidopsis thaliana; Sl, Solanum lycopersicum
ABSTRACT

Fruit initiation in *Arabidopsis thaliana* (*At*) is generally repressed until fertilization occurs. However, mutations in *AUXIN RESPONSE FACTORS 8* (*ARF8*) uncouple fruit initiation from fertilization, resulting in the formation of seedless, parthenocarpic fruit. Here we induced parthenocarpy in wild-type *Arabidopsis* by introducing either the mutant genomic (g) *Atarf8-4* sequence or gAtARF8:GUS translational fusion constructs by plant transformation. Silencing of endogenous *AtARF8* transcription was not observed indicating that the introduced, aberrant ARF8 transcripts were compromising the function of endogenous ARF8 and/or associated factors involved in suppressing fruit initiation. To analyze the role of ARF8 in tomato (*Solanum lycopersicum; Sl*), we initially emasculated 23 tomato cultivars to test for background parthenocarpy. Surprisingly, all had a predisposition to initiate fertilization independent fruit growth. Expression of gAtarf8-4 in transgenic tomato (cultivar ‘Monalbo’) resulted in a significant increase in the number and size of parthenocarpic fruit. Isolation of tomato *ARF8* cDNA indicated significant sequence conservation with *AtARF8*. *SlARF8* may therefore control tomato fruit initiation in a similar manner as *AtARF8* does in Arabidopsis. Two *SlARF8* cDNAs differing in size by 5 bp were found, both arising from the same gene. The smaller cDNA is a splice variant and is also present in Arabidopsis. We propose that low endogenous levels of the splice variant products might interfere with efficient formation/function of a complex repressing fruit initiation, thereby providing an explanation for the observed ovary expansion in tomato and also Arabidopsis after emasculation. Increasing the levels of aberrant *Atarf8-4* transcripts may further destabilize formation/function of the complex in a dosage dependent manner enhancing tomato parthenocarpic fruit initiation frequency and size and mimicking the parthenocarpic dehiscent silique phenotype found in homozygous *Atarf8-4* mutants. Collectively these data suggest that similar mechanisms, involving auxin signaling exist to inhibit parthenocarpic fruit set in tomato and Arabidopsis.
INTRODUCTION

Signaling processes, initiated by pollination and fertilization are normally required to initiate seed and fruit development (Raghavan, 2003). Subsequent fruit growth depends, in part, on the coordinated action of growth substances produced in the ovary and seed after pollination and/or fertilization (Gillaspy et al., 1993; Garcia-Martinez and Hedden, 1997). Various phytohormones, including auxin, cytokinin and gibberellins, have been implicated in seed and fruit growth and development (Nitsch, 1952; Coombe, 1960; Nitsch, 1970; Garcia-Martinez and Hedden, 1997; Fos et al., 2000; 2001).

The effect of pollination and fertilization in stimulating fruit growth can be mimicked by hormone applications (Goodwin, 1978; Gillaspy et al., 1993; Vivian-Smith and Koltunow, 1999) or by expression of auxin biosynthesis genes in ovaries and ovules (Rotino et al., 1997; Ficcadenti et al., 1999; Carmi et al., 2003; Mezzetti et al., 2004) which lead to the formation of fertilization-independent or parthenocarpic (seedless) fruit. Elevated or altered levels of phytohormones have also been observed during fruit growth in naturally occurring parthenocarpic plants (Mapelli et al., 1978; George et al., 1984; Talon et al., 1990; 1992).

Recent studies in tomato and Arabidopsis have revealed that the auxin signaling pathway is involved in controlling the early events of fruit initiation. Two components of the auxin signaling pathway, Auxin Response Factor8 (ARF8) from Arabidopsis (Vivian-Smith et al., 2001; Goetz et al., 2006) and the Aux/IAA protein IAA9 from tomato (Wang et al., 2005), have been implicated in repressing fruit initiation in the absence of the fertilization cue. Antisense repression of IAA9 in tomato and recessive mutations in Arabidopsis ARF8 uncoupled fruit initiation from pollination and fertilization and gave rise to parthenocarpic fruit (Wang et al., 2005; Goetz et al., 2006). Aux/IAA proteins can bind to ARF proteins to activate or inhibit the transcription of auxin responsive genes (Ulmasov et al., 1999; Hardtke et al., 2004; Tatematsu et al., 2004). It has been proposed that both Arabidopsis and tomato possess ARF8- and IAA9-like orthologues that interact and, together with potentially other as yet unknown proteins, form a protein complex that prevents fruit set prior to fertilization (Goetz et al., 2006; Swain and Koltunow, 2006).
Parthenocarpy in mutant and wild-type Arabidopsis has been examined by ‘emascula-
tion’ which involves removing all of the floral organs surrounding the carpel before the anthers open and shed pollen onto the stigma (Vivian-Smith and Koltunow, 1999). Emasculation of wild-type Arabidopsis flowers in Landsberg erecta (Ler), Columbia (Col) and Wassiljewska (WS) ecotypes results in a slight increase in carpel length and girth from cellular expansion but a dehiscence zone indicative of a differentiated fruit does not form (Fig. 1; Vivian-Smith and Koltunow 1999). Recessive mutations arising from lesions in the coding region of AtARF8, such as those found in Atarf8-1 and Atarf8-4 mutant alleles, induce parthenocarpic silique development whereby carpel growth continues further than the initial growth that is observed in emasculated wild-type plants and differentiation of a seedless, dehiscent silique occurs. Atarf8-1 is a T-DNA insertion mutant and Atarf8-4 contains a mutation in the putative translation initiation codon. Both alleles produce transcripts that have the potential to encode truncated ARF8 proteins and it has been suggested that this might explain why full complementation of the mutant phenotypes is not achieved when wild-type genomic ARF8 sequences are introduced (Tian et al., 2004; Goetz et al., 2006). Analysis of the expression of a genomic wild-type AtARF8:GUS translational fusion in transgenic Ler + AtARF8:GUS plants showed that parthenocarpic phenotypes can be induced in some transgenic lines, suggesting that the altered ARF8 protein can have dominant-negative effects. Furthermore, the expression of the transgenically introduced AtARF8:GUS translational fusion was temporally and spatially altered in the arf8-4 mutant background relative to the expression pattern in the wild-type plants. This may relate to possible interactions between the gene products or to indirect effects on gene expression relating to the mutant arf8 background (Goetz et al., 2006). These results led us to propose that a putative complex repressing fruit initiation in Arabidopsis could be destabilized in at least two ways. Mutations in ARF8 may prevent the production of a functional protein so that the complex cannot form. Alternatively, aberrant ARF8 transcripts or proteins might be formed that disrupt complex formation or function via some form of dosage dependent competitive interference and thus stimulate parthenocarpy (Goetz et al., 2006).

In this paper, we directly examined whether the introduction of aberrant forms of ARF8 can induce parthenocarpic fruit initiation in Arabidopsis and tomato. These data
together with the isolation and analysis of tomato ARF8 sequences suggest that ARF8 is also involved in the regulation of fruit initiation in tomato.
RESULTS

Induction of parthenocarpy in wild-type *Arabidopsis thaliana* by g*Atarf8-4*

To test if products from the *Atarf8-4* gene can induce parthenocarpy in Arabidopsis, the *Atarf8-4* gene sequence was introduced into wild-type Ler plants by plant transformation. A total of ten independent, homozygous lines containing one to three copies of the *Atarf8-4* gene were recovered and analyzed. Carpel elongation after emasculation was observed in nine out of the ten lines and formation of the dehiscence zone occurred in the three out of those ten lines which also exhibited greatest elongation (Table I). These three lines also displayed other typical *Atarf8-4* mutant phenotypes because the siliques were broad shouldered in appearance (Fig. 1), reduced seed set was evident after fertilization in the basal part of the siliques and the carpels frequently protruded from unopened flower buds (data not shown). The carpels from the remaining seven lines elongated slightly after emasculation but otherwise did not show additional phenotypes related to *Atarf8-4*.

The point mutation in *Atarf8-4* creates a cleaved amplified polymorphic sequence (CAPS) marker (Vivian-Smith et al., 2001) and expression of the introduced mutant gene was confirmed by using the CAPS marker in reverse transcription-polymerase chain reaction (RT-PCR) assays and also by quantitative real time PCR (qRT-PCR). A mixture of mutant *Atarf8-4* and wild-type *AtARF8* transcripts was observed using the CAPS marker in all transformants (data not shown) and levels of total *AtARF8* transcript were also found to be higher in transgenic plants compared to wild-type plants (Table I), indicating that co-suppression or RNA silencing of the endogenous *AtARF8* gene were not the cause of the induced parthenocarpic phenotype. These data indicate that expression of *arf8-4* mutant genes seem to induce variable siliques elongation and dehiscence zone formation responses. Although it is not possible to determine the exact ratios of expression levels between the mutant and wild-type mRNAs from these experiments, the observed variation in the extent of parthenocarpy is probably due to differences in expression levels of the introduced *Atarf8-4* copies. Variable responses, as observed in the transgenic lines here, are expected if competitive interference occurs between the introduced aberrant *Atarf8-4* products and the endogenous wild-type ARF8.
Expression of a gAtarf8-4:GUS translational fusion construct induces parthenocarpy

The genomic Atarf8-4 mutant sequence was translationally fused to GUS and introduced to Ler and arf8-4 Arabidopsis backgrounds by plant transformation in order to examine if a protein product is made. A total of eight independent, homozygous lines were generated in the Ler background and four independent, homozygous lines in the arf8-4 background and all the lines expressed GUS (Fig. 2). Since the GUS gene does not contain its own translation initiation codon, the detection of GUS expression indicates that translation of the fusion protein is initiated from an alternative start codon somewhere within the mutant Atarf8-4 gene. Consequently a protein fragment can be produced from the mutant allele despite the point mutation of the putative translation initiation codon of AtARF8 in Atarf8-4. The exact nature of this mutant protein is however unclear and remains to be determined in future experiments.

Interestingly, the spatial and temporal expression patterns of the translational gAtarf8-4:GUS construct in both the Ler and arf8-4 backgrounds during flower and seed initiation were identical to those previously published for gAtARF8:GUS in the Ler and arf8-4 backgrounds (compare Fig. 2 with Goetz et al., 2006). As for gAtARF8:GUS, expression of gAtarf8-4:GUS showed reduced expression levels in arf8-4 plants in some tissues (e.g. petals and anther filaments) and a temporal change in expression was observed, especially in the carpel walls, the septum, the funiculi and the ovules, where GUS activity was detected earlier and persisted in the mutant background. A detailed description of the expression patterns is provided in Goetz et al. (2006). Thus, as for the gAtARF8:GUS translational construct, expression of the gAtarf8-4:GUS translational fusion is spatially and temporally altered in the arf8-4 background compared to the expression of the same constructs in the Ler background (Fig. 2), indicating that the regulation of their expression is impaired in arf8-4. Therefore the arf8-4 mutant genetic background influences the spatial and temporal expression pattern of the introduced genes.

We previously observed that a dehiscence zone formed in some of the emasculated gAtARF8:GUS plants examined at that time (Goetz et al., 2006). In the current study we emasculated a total of seven independent, homozygous lines
transformed with the gAtARF8:GUS construct and five independent, homozygous lines transformed with gAtarf8-4:GUS construct to examine their efficiency in inducing parthenocarpy. All lines showed the formation of a dehiscence zone, an indicator for fruit differentiation, and silique elongation was observed in five out of seven Ler + gAtARF8:GUS lines and in four out of five Ler + gAtarf8-4:GUS lines with the greater silique elongation observed in the gAtARF8:GUS lines (Fig. 1 and Fig. 3). Levels of endogenous AtARF8 mRNA did not detectably decrease in transgenic plants (data not shown) therefore RNA silencing is unlikely to be the cause of the phenotype.

These data show that expression of the translational gAtARF8:GUS and gAtarf8-4:GUS fusion constructs induce dehiscence zone formation but variable silique elongation responses. These data together with those obtained from plants transformed with the Atarf8-4 mutant gene indicate that the introduced aberrant constructs are able to destabilize the function of endogenous ARF8 and/or interacting factors that restrict fertilization dependent fruit initiation in some way, leading to the induction of parthenocarpic phenotypes.

**Parthenocarpy occurs in many commercial tomato cultivars**

Arabidopsis produces a dry, dehiscent fruit. To examine the role of ARF8 during the initiation of fleshy fruit growth we selected tomato for further experiments. We emasculated a total of 23 different cultivars to examine their potential for parthenocarpic fruit initiation (see Materials and Methods). All of the cultivars tested exhibited some ovary growth after emasculation and most were able to initiate seedless fruit formation to varying degrees where the parthenocarpic fruits colored up and ripened. Data concerning eight indicative cultivars is provided in Table II.

The extent of fruit set observed varied between the different tomato cultivars in terms of the percentage of emasculated buds that initiated fruit development, final fruit size and in particular the time lag between emasculation and fruit initiation. Naturally smaller fruited cultivars were most efficient in setting parthenocarpic fruit (Fig. 4A and Table II). In general, the size of ripe parthenocarpic fruit was smaller than fertilization-induced fruit size (Table II), although we did not carry out an exhaustive analysis in this regard. In cultivars with poor fruit initiation, many emasculated flowers abscised at the
abscission zone in the pedicel. If the emasculated flowers were retained some ovary expansion and fruit development was generally observed with very slow growth or early arrest in the varieties with poor fruit initiation (Fig. 4B). In some cultivars the parthenocarpic fruit had a different shape (Fig. 4C) and pulp formed better in the seedless fruit of some cultivars compared to others. Emasculated flowers of cultivar ‘Monalbo’ took between 63 and 143 days to form red, ripe parthenocarpic fruit with an average development time of 90 days (± 18.4 days). When multiple flowers on a single truss were emasculated on the same day, different rates of fruit initiation and growth was detected on nine individual trusses observed. Figure 4D shows an example of such a truss from cultivar ‘Sweet Cherry Gold’ where a ripe fruit is senescing and different sized fruits have developed to varying degrees. Collectively, these data demonstrate that in most tomato cultivars examined after emasculation some limited ovary growth up to full fruit development occurs.

The cultivar ‘Monalbo’ was chosen for subsequent experiments. It has been selfed for many generations and is frequently used experimentally. It has moderate parthenocarpic ability and there was a substantial time difference between the initiation of fertilization-induced fruits at eight days after pollination compared to parthenocarpic fruit development at around 35 days post emasculation. Fertilization-induced fruits in ‘Monalbo’ were larger than the smaller seedless fruit obtained after emasculation (Fig. 5A, B; Table II).

gAtarf8-4 sequences enhance parthenocarpy in the tomato cultivar ‘Monalbo’

We transgenically introduced the gAtarf8-4 mutant allele as a genomic construct into the tomato cultivar ‘Monalbo’ to examine if this would influence parthenocarpic fruit initiation in tomato. We chose the Atarf8-4 allele because it stimulated fertilization-independent fruit initiation in Arabidopsis in the experiments described above via a non-gene-silencing mechanism. Phylogenetic studies suggested a close relationship between ARFs from different plant species, implying that they derived from a common ancestor (Wang et al., 2007). Therefore we hypothesized that using gAtarf8-4 should enable functional competition with tomato ARF8 sequences and also provide the opportunity to distinguish and detect transcripts formed from introduced and endogenous genes. Three
independent primary transgenic ‘Monalbo’ lines containing one, three and five copies of the *Atarf8-4* gene were selected for further analysis. Expression analysis using qRT-PCR showed that *Atarf8-4* levels in the transgenic plants increased proportionately with the number of introduced copies of the *Atarf8-4* gene in the tomato plants (Table III).

Esmasculation of untransformed ‘Monalbo’ plants showed that the majority of parthenocarpic fruit developing on the control plants were small (Fig. 5A), while wild-type fruits that developed after fertilization were much larger (Fig. 5B). All three of the primary transgenic plants showed enhanced parthenocarpy with respect to an increase in the number of emasculated flowers that initiated fruit development, and in terms of final fruit size compared with the emasculated, untransformed control plants (Table III; Fig. 5C). The extent of this enhanced parthenocarpic capability correlated with an increase in the steady state pool of introduced mutant *Atarf8-4* transcripts (Table III).

Enhanced parthenocarpic fruit set and growth was maintained in segregating plants containing one to five copies of the *Atarf8-4* gene obtained from self-pollinated primary transgensics. Retention of emasculated flowers and subsequent parthenocarpic fruit initiation increased up to 79% in the transgenic lines compared to 50% in wild-type plants (Table IV). Larger fruits were generally observed in plants containing more copies of the introduced *Atarf8-4* gene. Many of the parthenocarpic fruit from transgenic lines were similar in size and weight to seeded fruit from untransformed control plants and a greater percentage of fruit grew much larger than the average small sized seedless fruit formed after the emasculation of untransformed ‘Monalbo’ flowers (Table IV; Fig. 5A-C). The addition of the g*Atarf8-4* mutant allele has resulted in an enhanced pathenocarpic phenotype in terms of increased parthenocarpic fruit set and also size, thus we conclude that fruit initiation in tomato is controlled in a similar manner as found previously in Arabidopsis and that a *Solanum lycopersicum ARF8* (*SlARF8*) orthologue has a role in controlling fruit initiation in tomato.

**Larger parthenocarpic fruit contain seed-like structures (pseudoembryos)**

Tomato fruit from the control and transgenic plants were also examined for seed, pulp and flesh content. Emasculated flowers of wild-type ‘Monalbo’ predominantly formed small fruits with small locules and little pulp (Fig. 5D). After fertilization
‘Monalbo’ formed large fruits with large locules that were filled with pulp, in which the seeds were embedded (Fig. 5E). Fruits that developed after emasculation in the transgenic plants had pulp filled locules, a thinner mesocarp and enlarged central columella (Fig. 5F).

When assessed for seed content, the small parthenocarpic fruits from both emasculated control and transgenic flowers predominantly contained enlarged ovules (Fig. 5G), compared to the fully developed, large, hairy seeds found in fertilization-induced fruit (Fig. 5H). In the larger parthenocarpic fruit from emasculated control ‘Monalbo’ and transgenic plants (generally 20 grams and above) many of the enlarged ovules had further differentiated into well developed seed-like structures often called pseudoembryos (Fig. 5J). The size and level of differentiation of these seed-like structures varied, but was generally more advanced the larger the parthenocarpic fruit were in which they formed. Fully differentiated pseudoembryos were about one eighth of fertilization-induced seed size (compare Fig. 5H and 5J). Seed coat differentiation had occurred producing soft, seed-like structures with thickened lignified radial walls and protruding hairs. Sections of the fertilization-induced tomato seeds contained embryo and endosperm (Fig. 5I), whereas the well developed pseudoembryos from the large parthenocarpic transgenic fruit lacked both embryo and endosperm and the collapsed embryo sac was surrounded by deeply staining multi-layered endothelium (Fig. 5K). A correlation between the frequency and extent of pseudoembryo development with fruit growth and final fruit size has been observed previously (Kataoka et al., 2003). In our study we noticed a similar association between the development of large pseudoembryos, final fruit size and copy number of the introduced Atarf8-4 gene in ‘Monalbo’. We also found pseudoembryos in parthenocarpic fruit following emasculation of other tomato cultivars. These data suggest that SlARF8 may also play a role in regulating seed initiation and/or differentiation in tomato.

**Two distinct ARF8 mRNAs are found in *Solanum lycopersicum* ‘Monalbo’ and Arabidopsis**

The tomato orthologue of ARF8 was isolated from ‘Monalbo’ to investigate potential links to parthenocarpy given that mutations in Arabidopsis ARF8 induce
parthenocarpic fruit initiation and that expression of gAtarf8-4 was able to enhance parthenocarpy in transgenic tomatoes. Screening of tomato EST databases from flowers and fruits identified three overlapping unigene sequences with high homology to AtARF8. Based on these sequences, PCR primers were designed and RT-PCR, and 5’ and 3’ RACE (rapid amplification of cDNA ends) were used to amplify and clone the full length mRNA clones of Solanum lycopersicum ARF8 (SlARF8).

Amplifications and cloning using these PCR primers led to the isolation of two nearly identical cDNA sequences. The longer ARF8 clone of 2872bp (SlARF8 (full)) had 74% sequence identity with AtARF8 at the DNA level (Supplementary Fig. 1A). The similarity between the predicted protein sequence from tomato and AtARF8 was 76%, while the predicted protein identity was 66% (Table V; Supplementary Fig. 1B) reflecting the observed conservation of ARFs across various plant species (Table V). The next closest related AtARF protein sequence was AtARF6 with 65% similarity and 55% identity, suggesting that the isolated sequence was a strong candidate for SlARF8.

The second SlARF8 cDNA sequence (SlARF8 (-5bp)) was identical to SlARF8 (full) except for a 5bp deletion within the DNA binding domain of the predicted protein (compare SlARF8 (-5bp) and SlARF8 (full); Fig. 6A). This 5bp deletion is predicted to introduce an early stop codon immediately after the deletion within the DNA binding domain (Boxed in Fig. 6A) potentially resulting in a truncated ARF8 protein containing 185 amino acids. We considered that the shorter cDNA might arise from alterations in splicing at an exon/exon boundary of the same gene from initial comparison of both SlARF8 cDNA sequences with AtARF8 mRNA and the AtARF8 genomic sequence. Alternatively, given the tomato genome is not yet sequenced, it could have originated from mutations or sequence deletions during recent gene duplication events. DNA gel blot analysis using a 359 bp fragment comprising part of the Q-rich middle region and the beginning of the C-terminal protein interaction domain of SlARF8 (Fig. 6B) as a probe to ‘Monalbo’ genomic DNA showed the presence of multiple bands (data not shown). Given the high sequence similarity between ARFs, especially in the DNA binding domain and the C-terminal protein interaction domain, a cross reaction with other ARFs in the ‘Monalbo’ genome is to be expected.
To directly examine the origin of the shorter transcript, we used PCR to isolate an 1856 bp genomic fragment of the *SlARF8* gene spanning exons IV to VIII and including the region around the 5 bp deletion (Fig. 6B). A total of 5 cloned PCR products were examined and found to be identical in sequence. This tends to argue against multiple genes giving rise to the two *SlARF8* mRNAs as intron sequences are rarely conserved to this extent even in recently duplicated genes and duplicate genes have been found to exhibit dramatically accelerated rates of protein evolution (Castillo-Davis et al., 2004).

Comparison of the two *SlARF8* mRNA sequences with the *SlARF8* genomic sequence, the intron/exon structure of *AtARF8* and consensus splice donor and acceptor sites confirmed that the shorter mRNA is likely to be a product of alternative splicing. Intron/exon structure comparison of the *AtARF8* gene, the *SlARF8* gene fragment and the *ARF8* gene from *Brassica rapa* (Br) showed that the positions and sizes of introns IV to VIII identified in the *SlARF8* genomic fragment are very similar to those in the *AtARF8* and *BrARF8* genes, except that intron VI is significantly larger in *SlARF8* at 1083 bp compared with 281 bp in *AtARF8* and 132 bp in *BrARF8* (Supplementary Fig. 1C). A splice donor site (GC) marking the end of exon VI exists at 479 bp in the isolated *SlARF8* genomic sequence (arrow in Fig. 6C). This indicates that there is an exon/exon boundary after 553 bp of the mRNA, immediately 5’ to the 5 bp deletion found in *SlARF8* (-5bp).

The consensus sequence for splice acceptor sites is (T/C)AG (Simpson and Filipowicz, 1996) and analysis of the *SlARF8* genomic fragment showed that there is one splice acceptor site that appears to be used to generate the longer (full length) *SlARF8* mRNA (Fig. 6C; TAG, splice acceptor site 1). However, an alternative splice acceptor site (CAG) is also present 5 bp downstream from that used to generate the large mRNA at position 1564-1566 (Fig. 6C; splice acceptor site 2). We speculate that the presence of the shorter mRNA sequence results from an alternative splicing event in the same *ARF8* gene when this downstream acceptor site is used.

To test this hypothesis we developed a dCAPS marker to examine the presence or absence of the two *SlARF8* mRNA variants in ‘Monalbo’ and some of the other tomato cultivars known to form parthenocarpic fruit after emasculation. Using the dCAPS marker with control *SlARF8* plasmid DNA resulted in a single band as expected. When mRNA from ‘Monalbo’ flowers collected around anthesis was used, the dCAPS marker
showed two bands, indicating both SLARF8 (full) and SLARF8 (-5bp) mRNA variants were present (Fig. 6D). Twelve additional tomato cultivars were tested with the dCAPS marker. Both mRNA variants were found in flowers of all cultivars collected at anthesis as indicated by the presence of two bands (Fig. 6D).

We also tested the expression of SLARF8 in flowers of the primary transgenic lines transformed with the gAtarf8-4 construct. There was no detectable decrease in the levels of endogenous SLARF8 (full) and SLARF8 (-5bp) variant transcripts, indicating that co-suppression or silencing of the endogenous SLARF8 mRNAs did not occur in these plants (Fig. 6E). In addition, various ‘Monalbo’ tissues were sampled and tested and all samples contained the two different SLARF8 mRNA variants (Fig. 5E). The factors that give rise to the alternatively spliced mRNAs in tomato are thus not floral-specific.

These observations in tomato prompted us to examine whether an alternative splice acceptor sequence is also present in the ARF8 gene sequence of Arabidopsis (Ler Col and Ws ecotypes). We found that the alternative splice acceptor site (CAG) is present at the same position in Arabidopsis (see position 553-555 of AtARF8 sequence in Fig. 6A) as previously identified in tomato. To determine if it is recognized by the splice machinery of Arabidopsis to produce an alternatively spliced AtARF8 mRNA variant, we performed the dCAPS marker test for the 5 bp deletion of the alternative splice variant with Arabidopsis sequence specific primers using cDNAs from ecotypes Ler, Col and Ws. The results show two bands were present for all three ecotypes tested (Fig. 6E), indicating the presence of the splice variant. As there is a single ARF8 gene in the Arabidopsis genome, both transcripts clearly arise from the same gene.

These data indicate that in both Arabidopsis and tomato low levels of endogenous, aberrant ARF8 transcripts exist in the form of the shorter splice variant mRNA which is predicted to give rise to a truncated protein. In most tomato cultivars we examined, we observed some limited ovary growth up to full fruit development following emasculation. Similarly, some carpel elongation occurs in emasculated Arabidopsis ecotypes (Vivian-Smith and Koltunow, 1999). This is in contrast to other plants in which unfertilized flowers usually abscise and indicates that the controls restricting fruit development in Arabidopsis and selected tomato cultivars in the absence of fertilization might not be as tightly regulated. It is tempting to speculate that the presence of the ARF8 splice variant
in both tomato and Arabidopsis is possibly contributing to a capacity for this low level of initial growth. This needs further investigation. We have observed that the alternative splice acceptor sequence is also present in the gARF8 sequences from *Brassica rapa* and rice (data not shown) and further analysis of the gene sequences and expression patterns of *ARF8* in these and other plants will be necessary to determine if an alternative splice variant of *ARF8* exists in these species and what biological effects it might have on fruit initiation.
DISCUSSION

Transgenic introduction of aberrant ARF8 transcripts induces parthenocarpy in Arabidopsis

Recent work in Arabidopsis has shown that ARF8 might be part of a regulatory complex that negatively regulates fruit initiation. Lesions in AtARF8 lead to parthenocarpic fruit formation (Goetz et al., 2006). Here we have shown that the introduction of aberrant ARF8 sequences to wild-type Arabidopsis plants in the form of a genomic Atarf8-4 sequence or mutant and wild-type genomic translational GUS fusions can stimulate parthenocarpy in Arabidopsis via a non-gene silencing mechanism. Thus, we suggest that the introduction of aberrant molecules can destabilize the formation of a proposed inhibitory complex controlling the transition from flower to fruit development, allowing fruit initiation in the absence of fertilization. Mechanisms by which this might occur could employ dosage effects involving the competitive interference of aberrant proteins, inhibiting the N-terminal DNA binding domain of ARF8 from binding to the correct promoter sequences in auxin responsive genes and/or preventing the C-terminal protein interaction domain to bind to Aux/IAA proteins, or to dimerize with other proteins that may make up a repressive complex.

The extent of parthenocarpy induced in Arabidopsis varied depending on the construct used and individual lines analyzed, which is most likely due to differences in expression levels and effectiveness of the products of the various constructs. Variable responses like this are expected if competitive interference occurs between the introduced aberrant ARF8 protein and the regulatory complex. The stronger effects with the gAtARF8:GUS construct over the gAtarf8-4 and gAtarf8-4:GUS constructs seem to support this. Due to the previously identified point mutation in the translation initiation codon (Goetz et al., 2006), any translation from the Atarf8-4 mutant transcripts would have to start from an alternative start codon and is therefore likely to be less efficient than translation from the wild-type AtARF8 transcript. While equal or higher gene expression has been shown for all constructs via PCR methods and expression of the GUS fusion proteins was verified by analysis of the GUS staining patterns (Goetz et al., 2006), we were unable to confirm the presence and sizes of proteins formed in gAtarf8-4, gAtarf8-
4:GUS and gAtARF8:GUS plants by Western blot analysis due to the lack of effective and specific antibodies.

There were also differences between the translational GUS constructs and the gAtarf8-4 allele itself in terms of efficiency of parthenocarpic induction in Arabidopsis. Clear parthenocarpic phenotypes were more readily observed in lines containing the translational GUS constructs. Although the elongation response was variable with all constructs, dehiscence zone formation was only variable in lines transformed with the gAtarf8-4 construct, while it was present in all lines transformed with the translational GUS constructs. We suggest that one reason for this is that elongation and dehiscence zone formation are two separate pathways that might be controlled through the same complex, but activated in different ways.

Since the GUS protein is attached to the C-terminal protein-protein interaction domain of Atarf8-4 and AtARF8 (CTD, Fig. 6B), the greater effectiveness of the translational GUS constructs in inducing the dehiscence zone could be due to the presence of the GUS protein in that position. ARFs can homodimerize and also heterodimerize with other proteins including Aux/IAA proteins via the CTD (Liscum and Reed, 2002; Hardtke et al., 2004; Tatematsu et al., 2004) and these interactions may be important in regulating the formation of the dehiscence zone. In transgenic Arabidopsis plants containing the translational fusions, the presence of the GUS protein may interfere with the normal protein interaction activity and/or binding of the DNA binding domain (DBD; Fig. 6B) to promoters of early auxin responsive genes. These possibilities require further analysis at the protein level.

Evidence for dosage related ARF8 function in flower development is provided in a study analyzing combinations of Atarf6 and Atarf8 mutants. Nagpal et al. (2005) examined floral phenotypes of Atarf6-2 and Atarf8-3 single mutants, Atarf6-2/Atarf6-2 Atarf8-3/AtARF8 and Atarf6-2/AtARF6 Atarf8-3/Atarf8-3 sesquimutants and Atarf6-2 Atarf8-3 double mutants. Their results indicated that ARF8 and the closely related ARF6 can act partially redundantly and that gene dosage quantitatively affected the observed phenotypes.

Collectively the data presented show that in addition to the genetic induction of parthenocarpy, as demonstrated in our previous work (Goetz et al., 2006), parthenocarpy
can also be stimulated by expressing aberrant products that affect the auxin response pathway. Thus, interference with the proposed inhibitory complex in Arabidopsis either by genetic ablation of either protein or by introducing inhibitory or competitive effects through non-functional or inactive versions of transcripts or proteins, allows parthenocarpic fruit development by permitting fruit initiation to occur in the absence of fertilization.

**Genetics, expression and induction of parthenocarpy in tomato**

Parthenocarpy is a desirable trait in horticultural crops, as it enables fruit set and growth to be independent of pollination, fertilization and seed development. Therefore it may circumvent the environmental constraints on fruit production and ensure yield stability. Marketable parthenocarpy in tomato would provide the possibility to produce seedless fruits with high consumer appeal and could also be a valuable trait for industrial tomatoes because parthenocarpic fruit can have a higher percentage of soluble solids, improving yield and flavor of paste and reducing processing costs.

Fruit set in tomato is temperature dependent and commercial fruit quantities are obtained when night temperatures are maintained between 15-21°C. Problems in pollen formation limit fruit set if night temperature is lower than 13°C or when the day temperature is higher than 38°C (Baksh et al., 1978; Rylski, 1979; Lin et al., 1983a; George et al., 1984; Ho and Hewitt, 1986; Vardy et al., 1989a; Lukyanenko, 1991; Adams et al., 2001). Breeding programs have been aimed at generating tomato cultivars that set seedy fruit under normal pollinating conditions and commercially marketable but seedless fruit under pollen formation-limiting conditions. At least four recessive parthenocarpic or *pat* loci have been identified in tomato by growing plants mainly at cool temperatures and scoring for seedless fruit formation. There are differences in the quantification of parthenocarpy in genetic studies but in addition to the *pat* loci other “minor parthenocarpy loci” which influence pat expression in tester lines used in the crosses have also been identified (Vardy et al., 1989a, 1989b). The molecular identity of all of these loci is unknown.

Emasculation has rarely been used to score parthenocarpic fruit growth in tomato which is understandable in the plant breeding context where the intention is to provide
the grower with varieties that are facultative for parthenocarpy in that they set fruit under normal growth conditions and also temperatures that limit pollen formation. In our study plants were emasculated and subsequently monitored for long periods of time. Our analysis showed that many commercially available tomato cultivars and also experimentally used lines that have been selfed for many generations show some degree of natural parthenocarpy following emasculation ranging from ovary expansion to varying sized seedless fruits. Some of these small and misshapen fruits are likely to be similar to those referred to as “pseudo-fruits”, “puffs” or “nuts” in the literature (Foster and Tatman, 1937; Vardy et al., 1989a; Pandolfini et al., 2002; Carmi et al., 2003). We have previously demonstrated that parthenocarpy in Arabidopsis arf8 mutants is not expressed if floral whorls surrounding the carpel are not removed, as signals from these tissues inhibit the expression of parthenocarpy (Vivian-Smith et al., 2001). Our preliminary studies indicate that the pollen is the primary source of this inhibition (Goetz and Koltunow, unpublished observations). It is not certain if similar floral whorl signals suppress parthenocarpy in tomato. But one explanation for the relatively high frequency of parthenocarpic growth we observed may be that by emasculating the tomato flowers we have removed floral whorl suppression signals allowing the expression of parthenocarpy.

Here we have established that ARF8 sequences are highly conserved between Arabidopsis and tomato at the mRNA and predicted protein level. When we introduced the mutant genomic Atarf8-4 sequence to ‘Monalbo’ a significant increase in parthenocarpic fruit initiation, fruit size and weight was observed following emasculation above the background levels in control plants supporting a role for ARF8 in regulating fruit initiation in tomato. Moreover, silencing of SlAux/IAA9 has been demonstrated to induce parthenocarpic fruit initiation in tomato (Wang et al., 2005). Together, the involvement of ARF and Aux/IAA proteins implicates the auxin response pathway in the repression of tomato fruit initiation in the absence of fertilization. It is tempting to speculate that SlARF8 and SlAux/IAA9 interact and the analysis of this together with the analysis of the expression of these two proteins in pat plants may provide informative clues concerning the regulation of parthenocarpy in tomato.
Aberrant endogenous ARF8 transcripts together with existing data provide a model for fruit initiation in tomato and Arabidopsis

When we isolated SlARF8 cDNA sequences from tomato, we found a second variant of the mRNA that is most likely formed as a result of alternative splicing. Analysis of ARF8 expression in wild-type Arabidopsis ecotypes Ler, Col and Ws confirmed the presence of the same alternative splice form and both plants show ovary expansion and growth following floral emasculation. This is in contrast to other plants where unfertilized flowers abscise. The use of other splice sites resulting in the alternative splicing of plant genes has been reported previously and heat stress or temperature dependent splicing has been described as the cause in several of these cases (Burr et al., 1996; Sablowski and Meyerowitz, 1998; Lazar and Goodman, 2000; McKibbin et al., 2002; Colot et al., 2005; Reddy, 2007). It is not clear however, what cues lead to the generation of the ARF8 splice variant in Arabidopsis and tomato.

Our data suggests that ARF8 has a role in modulating fruit initiation in both Arabidopsis and tomato. Given the large amount of biochemical data available about the auxin signal transduction pathway (Ulmasov et al., 1999a; 1999b; Gray et al., 2001; Rogg and Bartel, 2001; Liscum and Reed, 2002; Tiwari et al., 2003; Hardtke et al., 2004; Kepinski and Leyser, 2004; Tatematsu et al., 2004; Kepinski and Leyser, 2005), we have devised a model which proposes that ARF8 and Aux/IAA9 orthologues interact in these plants (potentially with other proteins) to form a regulatory complex (Fig. 7). This complex can bind to the promoters of a range of primary auxin responsive genes that play an essential role in repressing fruit initiation in the absence of fertilization. The presence of the ARF8 splice variant transcripts and possible products could act to partially destabilize this complex. In Arabidopsis (and possibly tomato) this destabilization may be revealed as some fruit growth following emasculation and coincident removal of floral whorl inhibitory signals. Assuming fertilization induces an auxin burst, which has been documented in orchids (O’Neill, 1997) and shown to target Aux/IAA proteins to the proteasome degradation pathway (Ulmasov et al., 1999; Gray et al., 2001; Tiwari et al., 2001; Zensner et al., 2001), this would completely remove the transcriptional block allowing the expression of genes promoting fruit initiation. The involvement of auxin in fruit initiation is supported by studies showing that exogenous application of auxin
(King, 1947; Asahira et al., 1967; Lin et al., 1983b) and the expression of auxin biosynthesis genes in transgenic tomato ovaries (Ficcadenti et al., 1999; Carmi et al., 2003) induce commercially acceptable parthenocarpic fruit and elevated auxin levels have been found in a range of parthenocarpic fruits (Mapelli et al., 1978; George et al., 1984; Talon et al., 1990; 1992). Experimental confirmation of the interacting partners of ARF8 and Aux/IAA9 and an examination of any potential sources of auxin during fertilization-dependent fruit set in both Arabidopsis and tomato are required to substantiate the model. The role of the splice variant in complex destabilization might be further tested by introducing genomic sequences that produce only the splice variant transcript into Arabidopsis and tomato.

Obviously additional signals and activators are required following removal of the block in fruit initiation to promote fruit development and growth. In fertilization-induced fruits, seeds have long been known to produce growth promoting hormones. Work in garden peas suggests that seeds produce a modified auxin which stimulates GA production in the surrounding carpel tissues (Ozga et al., 2002; Ozga and Reinecke, 2003). Parthenocarpic fruits are likely to have other sources of growth hormones, but a link between auxin and GA in Arabidopsis is supported by the dependence of Atarf8 parthenocarpy on GA activity (Vivian-Smith et al., 2001) and increased GA levels are associated with growth of parthenocarpic tomato fruit (Fos et al., 2000; 2001). In tomato, the pat-2 and pat-3/pat-4 mutations, for example, have been found to independently accumulate high concentrations of GAs in unpollinated tomato ovaries via different pathways. It was therefore suggested that natural parthenocarpy induced by pat-2 and pat-3/pat-4 is due to increased synthesis of active GAs (e.g. GA1) in unpollinated ovaries as a result (Fos et al., 2000; Fos et al., 2001).

The pseudoembryos observed in parthenocarpic tomato may contribute to early growth of fruits as a substitute for developing seeds, either by producing phytohormones or by creating a sink for hormones to be transported into the growing fruits (Nitsch, 1952; Varga and Bruinsma, 1976; George et al., 1984; Kataoka et al., 2003). Well developed pseudoembryos have been observed in parthenocarpic tomato lines controlled by the pat-2 allele (Kataoka et al., 2003), auxin-induced parthenocarpic fruits (King, 1947; Asahira et al., 1967; Lin et al., 1983b), transgenic tomatoes containing auxin biosynthesis genes
(Carmi et al., 2003) and larger seedless fruits of the ‘Monalbo’ lines containing the \textit{Atarf8-4} gene in addition to parthenocarpic fruits formed following emasculation of a range of varieties in this study. They clearly correlate with the increased size of parthenocarpic fruit.

Groot et al. (1987) suggested that GAs are important for seed growth and development in tomato. The application of uniconazole, an inhibitor of gibberrellin biosynthesis, to parthenocarpic tomato lines containing the pat gene and also auxin induced parthenocarpic fruits three days after anthesis, abolished fruit growth and pseudoembryo development. Gibberellin treatment two days after uniconazole application restored fruit and pseudoembryo growth (Kataoka et al., 2003). This provides further support for the linkage of GA biosynthesis to fruit and pseudoembryo growth in parthenocarpic tomato once the block to fruit development is removed.

In our study, the development of ovules into pseudoembryos in parthenocarpic tomato fruit contrasted with the observed senescence of Arabidopsis ovules around five days after emasculation in both wild-type and \textit{arf8-4} plants. Arabidopsis ovules similarly senesced when parthenocarpy was induced by application of various growth hormones (Vivian-Smith, 2001). This suggests that there may be different dependencies on ovule and ovary communication events in tomato and Arabidopsis during fruit development and this requires further investigation.

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MATERIALS AND METHODS

Arabidopsis growth and scoring parthenocarpy

*Arabidopsis thaliana* seeds (cv Ler, Col, Ws) were surface-sterilized and grown as described previously (Vivian-Smith and Koltunow, 1999). Parthenocarpy was assessed by flower emasculation (Vivian-Smith and Koltunow, 1999; Vivian-Smith et al., 2001). Only siliques above flower position 20 were used, collected and photographed and their lengths were determined using the Scion Image Beta 4.02 program (http://www.scioncorp.com/frames/fr_scion_products.htm). Plants producing siliques that significantly and reliably elongated more than the corresponding wild-type plants and that formed a dehiscence zone were scored as parthenocarpic.

Cloning of *arf8-4* and translational GUS constructs

The cloning of the *arf8-4* mutant gene in the pGEM-T Easy Vector (Promega) was described earlier (Goetz et al., 2006). It was subcloned into the pBIN19 vector (Bevan, 1984) and transformed into *Agrobacterium tumefaciens* strain AGL1. Ler plants were transformed with the construct via the floral dip method (Clough and Bent, 1998). The cloning of the translational *ARF8:GUS* and *arf8-4:GUS* constructs and the plant transformations have been detailed earlier (Goetz et al., 2006). The *arf8-4* mutation creates a CAPS marker. Amplification with the primers ARF8-143F (5’-AGG AGA TGG AGA AAG ACG AG-3’) and ARF8+48R (5’-CTC TCC TTC ATG ACC CTG TTG-3’) and subsequent digest with *Hsp92 II* (Promega, NSW) resulted in bands of 142bp + 41bp + 8bp from *Ler* wild-type plants, whereas 183bp + 8bp bands were present in *arf8-4* plants.

RNA preparation, RT-PCR and qRT-PCR

Total RNA was extracted from Arabidopsis and tomato plant tissues using the RNeasy Plant Mini kit (Qiagen, Vic) according to manufacturer’s instructions. One µg of total RNA treated with an on-column RNase-free DNase protocol (Qiagen, Vic), was used as template for cDNA synthesis with the ThermoScript™ RT PCR System (Invitrogen, Vic).
Quantitative real time PCR (qRT-PCR) was performed as described before (Goetz et al., 2006).

**Growth of tomato and cultivars used**

The tomato cultivars used were either obtained as young plants from nurseries and repotted or purchased as seed and grown as described below. Roma, LA1563, LA1714, LA3130 and the male sterile lines LA1222 and UC82b were obtained from the C.M Rick Tomato Genetics Resource Centre (University of California). ‘Monalbo’ and ‘ChicoIII’ were kindly donated by Andrea Mazzucato (University of Viterbo). ‘Moneymaker’, ‘Beefsteak’, ‘BigBoy’, ‘Roma’ and ‘Margold’ were obtained from Eden Seeds (Lower Beechmont, Qld). ‘Mighty Red’, ‘Roma’, ‘Cocktail Supreme’, ‘Health Kick’, ‘Red Mamma’, ‘Sweet Cherry Gold’, ‘Grosse Lisse’, ‘Top Dog’ and ‘Patio Prize’ were obtained from local nurseries and ‘Cherry Ripe’, ‘Greek’ and ‘LM’ were obtained from a local private garden. Flowers from each cultivar were emasculated, tagged and checked after 3-4 weeks for signs of fruit growth. Most cultivars showed signs of fruit development after the indicated time.

Seeds of tomato (*Solanum lycopersicon* ‘Monalbo’ (LA2818 in Charles Rick collection UC Davis) were sown in a peat-based seed and modular compost and were germinated in a glasshouse compartment. Temperatures in the glasshouse varied between 10-19°C minimum temperature at night and 24-34°C maximum temperature during the day. After 3 weeks, seedlings were pricked out into 1l pots containing peat-based potting compost. At first flowering, plants were transplanted into 10l pots containing peat-based potting compost and plants were supported by canes.

The *arf8-4* allele from Arabidopsis was used for transformation of the cultivar ‘Monalbo’. Tomato transformations were done essentially as described by Fillatti et al. (1987) and transgenic plants were selected by germinating sterilized seeds on selective medium (1/2 MS medium, 3% sucrose and 100mg l⁻¹ kanamycin). Flowers of transgenic lines were emasculated and tagged one to two days before anthesis, and developing parthenocarpic fruits were harvested, weighed and analyzed when fruits were red and ripe.
Isolation of ARF8 mRNA from Solanum esculentum

EST databases from Solanum esculentum were screened for sequences with high homology to AtARF8. The three UniGene sequences SGN-U228441, SGN-U238776 and SGN-U227556 were identified and analyzed further. Alignments of the three UniGenes showed that SGN-U227556 and SGN-U238776 overlap and that after a 70 bp gap SGN-U238776 and SGN-U228441 also overlap with high identity. Based on the consensus sequences, the primers SIARF8_SF1 to SIARF8_SF12 (Supplemental Table I) were designed to amplify and clone this sequence and perform 5'- and 3'-RACE experiments to amplify and clone the full length mRNA sequence of SIARF8.

5' and 3' untranslated regions were isolated with the 5'-RACE System for Amplification of cDNA Ends, version 2.0 (Invitrogen) and the 3'-RACE System for Amplification of cDNA Ends (Invitrogen), respectively. The gene-specific primers used were SIARF8_SF11 and SIARF8_SF12 (Supplemental Table I) for 5'-RACE and SIARF8_SF6 and SIARF8_SF7 (Supplemental Table I) for 3'-RACE.

dCAPS marker for ARF8 variants

The two SIARF8 variants can be distinguished with a dCAPS marker (Neff et al., 2002). Amplification with the primers SIARF8_SF11 and SIARF8_AvrII (Supplemental Table I) from one µl of cDNA from tomato and subsequent digest with XmaJI (Fermentas) resulted in a band of 199bp for the SIARF8 (full) variant and a band of 177bp for the SIARF8 (-5bp) variant. An alternative dCAPS marker using the primers SIARF8_SF11 and SIARF8_SacII and digestion with Cfr42I (Fermentas) can also be used and resulted in the same bands as described above. For detection of the two AtARF8 variants in the Arabidopsis ecotypes, the primers AtARF8_SF11 and AtARF8_SacII (Supplemental Table I) and digestion of the PCR product with Cfr42I (Fermentas) were used. This resulted in the same sized bands as described above.

Embedding and sectioning of seeds and pseudoembryos

Seeds, ovule traces and pseudoembryos were collected from tomato fruits and fixed in 3.1% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.4. Fixed tissues were dehydrated in an acetone series and infiltrated with acetone:Spurrs resin (1/1) overnight.
at room temperature in a vacuum chamber. They were then transferred to 100% Spurrs resin overnight in a vacuum chamber, embedded in fresh Spurrs resin and polymerized at 65°C overnight. Specimen were cut into 2µm thin sections and then stained with 0.1% toluidine blue in 0.02% sodium carbonate to determine cell identity.

Microscopy and photography of fruits and seeds
Photographs of whole and cut fruits were taken with a Nikon Coolpix 995 digital camera. Whole-mount seeds and seed sections were viewed with Stemi2000C or Axioskop microscopes (Carl Zeiss, Jena, Germany). Digital images were captured using a Spot II camera (Diagnostic Instruments Inc., MI). Image processing and reproduction were performed with Auto Montage Essentials (Syncroscopy, Frederick, MD) and Photoshop 7.0 (Adobe Systems, San Jose, CA).

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT5G37020 (AtARF8) and EF667342 (SlARF8).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. ARF8 sequence comparisons
(A) Comparison of ARF8 cDNA sequences from Arabidopsis (AtARF8) and tomato (SlARF8). Black shading indicates identical residues.
(B) Comparison of predicted protein sequences of ARF8 from Arabidopsis (AtARF8) and tomato (SlARF8). Black shading indicates identical residues.
(C) Diagram of the intron/exon structure of ARF8 genes. Grey boxes indicate 5’ and 3’ UTRs, black boxes represent exons and white boxes introns. The tables contain the lengths of the respective introns and exons in base pairs. At, Arabidopsis thaliana; Sl, Solanum lycopersicum; Br, Brassica rapa
Supplemental Table I Primer names and sequences used to amplify and clone *SLARF8* and for amplification of the *SLARF8* dCAPS markers.

| Primer name         | Primer sequence                                      |
|---------------------|------------------------------------------------------|
| SIARF8_SF1          | 5’-CCAGCCTGATCTTTGAGGGTCTTC-3’                       |
| SIARF8_SF2          | 5’-GGAGGAGAAGGCTGAAAGTCTCAATCTTC-3’                  |
| SIARF8_SF3          | 5’-TCGAAAAAGTAGTCAAAGGCTCAATCTTC-3’                  |
| SIARF8_SF4          | 5’-GAACCGAGGTATGTCCAATGACC-3’                        |
| SIARF8_SF5          | 5’-CTGAAATCCAGAAGCCCTTAGT-3’                         |
| SIARF8_SF6          | 5’-GAGGTCATTTGACATCAGTCGGTC-3’                       |
| SIARF8_SF7          | 5’-GCAGCCTTGTATTGGACAGGAGA-3’                        |
| SIARF8_SF8          | 5’-CCCAGAAATCAATCTCTCTTTTTTGCTC-3’                   |
| SIARF8_SF9          | 5’-AGCCCTTAGAGGAAAAAGAGATGGATCAC-3’                  |
| SIARF8_SF10         | 5’-TATGGGAGATTGAGCCCTTTGACATC-3’                     |
| SIARF8_SF11         | 5’-CGCTAGACAGAAGCATGATAC-3’                          |
| SIARF8_SF12         | 5’-GTGTCCCTTTTGGTTGCTGCTAC-3’                        |
| SIARF8_AvrII        | 5’-TGG AAA TTC AGG CAT ATT TTC CTA G-3’               |
| SIARF8_SacII        | 5’-ATT CAG GCA TAT TTT CCG CG-3’                     |
| AtARF8_SF11         | 5’-TAT CAC TAG ATA AAA CAG ATG ATG G-3’               |
| AtARF8_SacII        | 5’-TGG AAG TTT AGG CAT ATC TTC CGC G-3’               |

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FIGURE LEGENDS

**Figure 1.** Pistils from emasculated flowers.
Comparison of a pistil from Ler at anthesis and unpollinated pistils from Ler, arf8-4, Ler+arf8-4 and Ler+ARF8:GUS plants seven days post emasculation. Bar = 4mm

**Figure 2.** Expression of the translational arf8-4:GUS fusion protein.
(A) and (B) GUS staining patterns in Ler flowers and siliques transformed with arf8-4:GUS at anthesis (stage 13) (A) and after fertilization (stage 15) (B).
(C) and (D) GUS staining patterns in arf8-4 flowers and siliques transformed with arf8-4:GUS at anthesis (stage 13) (C) and after fertilization (stage 15) (D).
Goetz et al. (2006) contains a detailed description of the expression patterns during flower and fruit development in Ler and arf8-4 mutants containing the gAtARF8:GUS translational fusion for comparison with the figures presented here. The numbers at the bottom left indicate the stages of flower development (Smyth et al., 1990). a, anther; gy, gynoecium; pd, pedicel; pe, petals; se, sepals; sp, septum; si, silique; st, stigma. Bars = 1mm.

**Figure 3.** Comparison of pistil lengths of Ler, arf8-4 and Ler+garf8-4:GUS and Ler+gARF8:GUS lines seven days post emasculation.
The table below the graph indicates if the dehiscence zone formed (+) or not (-) and gives the total number of ARF8 and arf8-4 genes present in the lines. Bars show pistil lengths ± standard deviations for the individual lines.

**Figure 4.** Expression of natural parthenocarpy in several tomato cultivars.
(A) Parthenocarpic fruit development in the cultivar ‘Patio Prize’.
(B) Slow growth and early arrest of parthenocarpic fruit development (right) in the cultivar ‘Roma’.
(C) Different shape of parthenocarpic fruits (right) compared to fertilized, seeded fruits (left) in the cultivar ‘Health Kick’.
(D) Different rates of parthenocarpic fruit growth in the cultivar ‘Sweet Cherry Gold’. Flowers emasculated on the same day produce fruits ranging from early arrest of parthenocarpic fruit development (arrow) to mature, ripe fruits on the same truss. Bars = 5cm in (A) and 1cm in (B) to (D).

**Figure 5.** Fruits and seeds of wild-type and transgenic tomato plants.

(A) Typical small, parthenocarpic fruit developing from ‘Monalbo’ wild-type plant after emasculation.

(B) Large fruit developing from ‘Monalbo’ wild-type plant after fertilization.

(C) Large, parthenocarpic fruit developing from Monalbo+*arf8-4* #7 plant after emasculation.

(D) Small, emasculated ‘Monalbo’ wild-type fruit cut in half. A little orange pulp is visible in the locule.

(E) Large, fertilized ‘Monalbo’ wild-type fruit cut in half. The locule is filled with pulp and the seed are clearly visible.

(F) Large, emasculated Monalbo + *arf8-4* #7 fruit cut in half. The central columella (c) is enlarged and the locule is filled with pulp. Some pseudoembryos can be seen buried deep in the pulp.

(G) Ovule traces from a typical small, parthenocarpic ‘Monalbo’ wild-type fruit.

(H) Seed from a fertilized ‘Monalbo’ wild-type fruit and

(I) Cross-section through the seed, showing the developing embryo inside.

(J) Pseudoembryo from a parthenocarpic fruit from Monalbo + *garf8-4* #7 and

(K) Cross-section through the pseudoembryo, showing a collapsed embryo sac without an embryo.

Bars = 1cm in (A) to (F) and 1mm in (G) to (K). ces, collapsed embryo sac; em, embryo; en, endosperm; sh, seed hair; t, testa.

**Figure 6.** Cloning and identification of two distinct *SlARF8* mRNAs.

(A) Sequence comparison between the two distinct *SlARF8* mRNA variants and the *AtARF8* mRNA. The arrowhead indicates the position of the exon/exon boundary between exons VI and VII, just in front of the 5 bp deletion in the *SlARF8* (-5bp) mRNA.
variant. The arrow and white box mark the early stop codon introduced in \textit{SIARF8} (-5bp) by the 5 bp deletion. The numbers are the running counts of the bases in the respective mRNA sequences, starting at the translation initiation codon.

(B) Graphic representation of the ARF8 exon/intron structure and the protein domain structure. The arrowhead indicates the position of the 5 bp deletion in the \textit{SIARF8} (-5bp) mRNA. The grey bar shows the position and length of the \textit{SIARF8} gene fragment cloned and sequenced.

(C) Sequence alignment of the \textit{SIARF8} gene fragment and \textit{SIARF8} mRNA to identify splice donor and acceptor sites. The splice donor site (gc) and the two potential splice acceptor sites (tag and cag) are underlined and the splice sites are indicated by arrows. The numbers are the running count of the bases in the \textit{SIARF8} gene fragment sequence. The alignment contains a large gap in the sequence of intron VI.

(D) Analysis of the tomato \textit{ARF8} dCAPS marker. DNA from a \textit{SIARF8} mRNA plasmid was used as control. cDNAs from flowers of ‘Monalbo’ and 12 additional tomato cultivars collected at anthesis were tested for the presence of the two \textit{SIARF8} mRNA variants.

(E) Analysis of cDNAs from various ‘Monalbo’ tissues collected at different developmental stages for the presence of the two \textit{SIARF8} mRNA variants. Expression of \textit{SIARF8} in anthesis flower cDNAs from ‘Monalbo’ wild-type and ‘Monalbo’ + gAtarf8-4 lines was also determined with the help of the tomato \textit{ARF8} dCAPS marker. The Arabidopsis ecotypes Ler, Col and Ws were also tested for the presence of the \textit{ARF8} (-5bp) variant using the dCAPS marker with Arabidopsis sequence specific primers.

\textbf{Figure 7.} A model for the role of ARF8 and Aux/IAA9 proteins in the control of fruit initiation and growth.

ARF8 and Aux/IAA9 proteins, together with potentially other, as yet unknown proteins (= ?), form a regulatory complex that can either directly block transcription of target (fruit initiation) genes, or act indirectly by preventing ARF8 from functioning as a transcriptional activator. After pollination and fertilization occur, auxin acts by binding to its receptor, TIR1, promoting degradation of Aux/IAA9 proteins via the SCF$^{TIR1}$ ubiquitin ligase complex. In the absence of Aux/IAA9, ARF8 together with additional
signals and activators (= A) stimulate expression of early auxin responsive genes, initiating fruit growth and development. Destabilization of the regulatory complex or reduction of its functionality by aberrant ARF8 transcripts and possible products can lead to a reduction or loss of the inhibition of transcription of the fruit initiation genes resulting in parthenocarpic fruit growth.
| Line              | Silique lengths<sup>a</sup> | Copy #<sup>b</sup> | Dehiscence<sup>c</sup> | mRNA expression ratio<sup>d</sup> |
|------------------|-----------------------------|-------------------|------------------------|---------------------------------|
| Ler              | 2.9mm ± 0.2                 | 1                 | -                      | 1.0                             |
| arf8-4           | 4.9mm ± 0.2                 | 1                 | +                      | 1.7 ± 0.2                       |
| Ler + arf8-4 #1  | 3.4mm ± 0.2                 | 3                 | -                      | n.d.                            |
| Ler + arf8-4 #2  | 3.0mm ± 0.2                 | 4                 | -                      | 4.1 ± 0.3                       |
| Ler + arf8-4 #3  | 3.6mm ± 0.1                 | 2                 | -                      | 2.6 ± 0.0                       |
| Ler + arf8-4 #4  | 4.6mm ± 0.3                 | 3                 | +                      | 3.0 ± 0.2                       |
| Ler + arf8-4 #5  | 3.8mm ± 0.2                 | 3                 | -                      | 4.2 ± 0.4                       |
| Ler + arf8-4 #6  | 5.9mm ± 0.2                 | 2                 | +                      | 2.8 ± 0.0                       |
| Ler + arf8-4 #7  | 3.6mm ± 0.2                 | 2                 | -                      | n.d.                            |
| Ler + arf8-4 #8  | 3.5mm ± 0.2                 | 2                 | -                      | n.d.                            |
| Ler + arf8-4 #9  | 3.8mm ± 0.2                 | 3                 | -                      | n.d.                            |
| Ler + arf8-4 #10 | 6.1mm ± 0.1                 | 3                 | +                      | 3.5 ± 0.3                       |

Table I Analysis of silique elongation, ARF8/arf8-4 copy number, dehiscence and ARF8 expression ratios in lines transformed with the arf8-4 gene.

<sup>a</sup> Pistil lengths measured seven days after emasculation (± standard deviation; minimum of 40 flowers were emasculated and measured for each line).

<sup>b</sup> Copy numbers of endogenous ARF8 or arf8-4 and introduced arf8-4 genes as determined by Southern blot analysis.

<sup>c</sup> - = no dehiscence zone formation; + = dehiscence zone formation.

<sup>d</sup> The expression ratio of ARF8 mRNA was determined from flowers at anthesis. The ratio for each line is determined in relation to the expression in the Ler wild-type line, which was set to 1.0 as the reference point.
|                | No. of flowers emasculated | No. of fruits developed | % fruit set      | seeds | pulp | diameter | weight        |
|----------------|---------------------------|-------------------------|------------------|-------|------|----------|---------------|
| Chico III      | 66                        | 44                      | 66.7%             | no    | no   | 3.0 cm ± 0.4 cm | 15.72 g ± 5.04 g |
| Chico III (pollinated) | -                         | 21                      | -                | yes   | yes  | 3.5 cm ± 0.4 cm | 25.91 g ± 5.98 g  |
| Cocktail Supreme | 45                        | 37                      | 82.2%             | no    | some | 2.4 cm ± 0.1 cm | 5.68 g ± 0.98 g   |
| Health Kick    | 48                        | 24                      | 50.0%             | no    | little | 3.7 cm ± 0.7 cm | 16.28 g ± 6.90 g  |
| Monalbo        | 335                       | 163                     | 48.7%             | no    | some | 2.5 cm ± 0.8 cm | 11.14 g ± 11.04 g |
| Monalbo (pollinated) | -                        | 12                      | -                | yes   | yes  | 5.4 cm ± 0.5 cm | 77.81 g ± 19.68 g |
| Patio Prize    | 28                        | 24                      | 85.7%             | no    | some | 3.5 cm ± 0.4 cm | 17.20 g ± 4.66 g  |
| Red Mamma      | 139                       | 94                      | 67.6%             | no    | little | 2.6 cm ± 0.3 cm | 8.57 g ± 1.85 g   |
| Sweet Cherry Gold | 182                    | 77                      | 42.3%             | no    | yes  | 1.4 cm ± 0.3 cm | 1.26 g ± 0.49 g   |
| Top Dog        | 65                        | 13                      | 20.0%             | no    | some | 4.8 cm ± 0.4 cm | 37.13 g ± 9.84 g  |

**Table II** Fruit development after emasculation in various tomato cultivars.
| T1 generation (emasculated) | No. of fruits developed | Average fruit size and weight | Percentage parthenocarpic fruit development | No. of Atarf8-4 genes | Relative mRNA expression ratio<sup>a</sup> |
|----------------------------|-------------------------|-----------------------------|-------------------------------------------|-----------------------|----------------------------------------|
|                             | small (<3cm; <14g)      | medium (<4cm; <30g)         | large (>4cm; >30g)                       |                       |                                        |
| Monalbo wild-type           | 73% (n=64)              | 20% (n=18)                 | 7% (n=6)                                 | 2.6 cm ± 0.8 cm       | 51%                                   |
|                             |                         |                             |                                           | 11.06g ± 10.19 g     | 0                                      | -                                      |
| Monalbo + garf8-4 # 81      | 65% (n=21)              | 16% (n=5)                  | 19% (n=6)                                | 2.9 cm ± 0.9 cm       | 64%                                   |
|                             |                         |                             |                                           | 13.54g ± 10.61 g     | 1                                      | 1.0                                    |
| Monalbo + garf8-4 #6        | 29% (n=7)               | 38% (n=9)                  | 33% (n=8)                                | 3.3 cm ± 0.7 cm       | 51%                                   |
|                             |                         |                             |                                           | 19.27g ± 8.80 g      | 3                                      | 20.1 (±0.8)                            |
| Monalbo + garf8-4 #7        | 5% (n=2)                | 12% (n=5)                  | 83% (n=35)                               | 4.3 cm ± 0.6 cm       | 72%                                   |
|                             |                         |                             |                                           | 34.01g ± 12.25 g     | 5                                      | 102.2 (±23.6)                         |

**Table III** Analysis of T1 generation tomatoes transformed with the *Atarf8-4* gene

<sup>a</sup> Expression level of *Atarf8-4* as determined by real time PCR from anthesis flowers. There was no expression detectable in the Monalbo wild-type flowers. Therefore the expression in line Monalbo + *garf8-4* #81 was set to 1.0 as the reference point.
| T2 generation (emasculated) | Number of fruits | Average fruit size and weight | Percentage parthenocarpic fruit development | No. of *Atarf8-4* genes |
|-----------------------------|------------------|-------------------------------|-----------------------------------------------|------------------------|
|                             | small (<3.2cm; <15g) | medium (<4cm; <30g) | large (>4cm; >30g) |                                     |                       |
| Monalbo Wild-type           | 68% (n=51)       | 21% (n=16)                  | 11% (n=8)                     | 2.8cm ± 0.8 cm 14.15g ± 11.81 g     | 47%                   | 0         |
| Monalbo + *garf8-4* #81-20 | 85% (n=52)       | 15% (n=9)                   | - (n=0)                       | 2.6cm ± 0.5 cm 9.52g ± 4.61 g       | 72%                   | 1         |
| Monalbo + *garf8-4* #81-18 | 83% (n=39)       | 17% (n=8)                   | - (n=0)                       | 2.6cm ± 0.5 cm 10.12g ± 5.38 g      | 79%                   | 1         |
| Monalbo + *garf8-4* #6-9   | 75% (n=36)       | 25% (n=12)                  | - (n=0)                       | 2.8cm ± 0.4 cm 11.88g ± 4.58 g      | 75%                   | 2         |
| Monalbo + *garf8-4* #6-7   | 62% (n=36)       | 14% (n=8)                   | 24% (n=14)                    | 3.2cm ± 1.0 cm 20.91g ± 21.06 g     | 76%                   | 2         |
| Monalbo + *garf8-4* #81-12 | 40% (n=25)       | 27% (n=17)                  | 33% (n=21)                    | 3.3cm ± 0.9 cm 22.09g ± 13.87 g     | 77%                   | 1         |
| Monalbo + *garf8-4* #7-3   | 20.5% (n=9)      | 63.5% (n=28)                | 16% (n=7)                     | 3.5cm ± 0.6 cm 22.65g ± 11.16 g     | 79%                   | 3         |
| Monalbo + *garf8-4* #7-5   | 25% (n=7)        | 46.5% (n=13)                | 28.5% (n=8)                   | 3.5cm ± 0.7 cm 24.17g ± 12.83 g     | 73%                   | 4         |
| Monalbo + *garf8-4* #7-11  | 7% (n=3)         | 52% (n=23)                  | 41% (n=18)                    | 3.8cm ± 0.6 cm 31.63g ± 13.59 g     | 78%                   | 4         |
| Monalbo + garf8-4 #7-8 | 12% (n=6) | 35% (n=17) | 53% (n=26) | 3.9cm ± 0.7 cm | 32.26g ± 18.53 | 79% | 5 |

**Table IV** Analysis of segregating plants from self-pollinated primary transgenics.
|       | AtARF8 | AtARF6 | SIARF8 | OsARF8 | BrARF8 | CsARF8 |
|-------|--------|--------|--------|--------|--------|--------|
| AtARF8 |        | 63%/54%| 76%/66%| 70%/59%| 81%/76%| 64%/55%|
| AtARF6 | 63%/54%|        | 65%/55%| 64%/56%| 61%/53%| 79%/71%|
| SIARF8 | 76%/66%| 65%/55%|        | 71%/60%| 73%/62%| 65%/55%|
| OsARF8 | 70%/59%| 64%/56%| 71%/60%|        | 68%/58%| 64%/55%|
| BrARF8 | 81%/76%| 61%/53%| 73%/62%| 68%/58%|        | 63%/53%|
| CsARF3 | 64%/55%| 79%/71%| 65%/55%| 64%/55%| 63%/53%|        |

**Table V** Protein similarity/identity of ARF proteins from various plants. *At*, *Arabidopsis thaliana*; *Sl*, *Solanum lycopersicon*; *Os*, *Oryza sativa*; *Br*, *Brassica rapa*; *Cs*, *Cucumis sativus*. 
