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### Authors

Martinelli, Federico  
Uratsu, Sandra L  
Reagan, Russell L  
et al.

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RESEARCH PAPER

# Gene regulation in parthenocarpic tomato fruit

Federico Martinelli<sup>1</sup>, Sandra L. Uratsu<sup>1</sup>, Russell L. Reagan<sup>1</sup>, Ying Chen<sup>2</sup>, David Tricoli<sup>3</sup>, Oliver Fiehn<sup>4</sup>, David M. Rocke<sup>2</sup>, Charles S. Gasser<sup>5</sup> and Abhaya M. Dandekar<sup>1,\*</sup>

<sup>1</sup> Department of Plant Sciences, Mail Stop 2, University of California, Davis, 1 Shields Ave, Davis, CA 95616, USA

<sup>2</sup> Division of Biostatistics, Med Sci 1C, Room 146, University of California, Davis, 1 Shields Ave, Davis, CA 95616, USA

<sup>3</sup> Plant Transformation Facility, 190 Robbins Hall, University of California, Davis, 1 Shields Ave, Davis, CA 95616, USA

<sup>4</sup> UC Davis Genome Center and Bioinformatics Program, 1315 GBSF, University of California, Davis, 1 Shields Ave, Davis, CA 95616, USA

<sup>5</sup> Department of Molecular and Cellular Biology, University of California, Davis, 1 Shields Ave, Davis, CA 95616, USA

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## Abstract

Parthenocarpy is potentially a desirable trait for many commercially grown fruits if undesirable changes to structure, flavour, or nutrition can be avoided. Parthenocarpic transgenic tomato plants (*cv MicroTom*) were obtained by the regulation of genes for auxin synthesis (*iaaM*) or responsiveness (*rolB*) driven by *DefH9* or the INNER NO OUTER (*INO*) promoter from *Arabidopsis thaliana*. Fruits at a breaker stage were analysed at a transcriptomic and metabolomic level using microarrays, real-time reverse transcription-polymerase chain reaction (RT-PCR) and a Pegasus III TOF (time of flight) mass spectrometer. Although differences were observed in the shape of fully ripe fruits, no clear correlation could be made between the number of seeds, transgene, and fruit size. Expression of auxin synthesis or responsiveness genes by both of these promoters produced seedless parthenocarpic fruits. Eighty-three percent of the genes measured showed no significant differences in expression due to parthenocarpy. The remaining 17% with significant variation ( $P < 0.05$ ) (1748 genes) were studied by assigning a predicted function (when known) based on BLAST to the TAIR database. Among them several genes belong to cell wall, hormone metabolism and response (auxin in particular), and metabolism of sugars and lipids. Up-regulation of lipid transfer proteins and differential expression of several indole-3-acetic acid (IAA)- and ethylene-associated genes were observed in transgenic parthenocarpic fruits. Despite differences in several fatty acids, amino acids, and other metabolites, the fundamental metabolic profile remains unchanged. This work showed that parthenocarpy with ovule-specific alteration of auxin synthesis or response driven by the *INO* promoter could be effectively applied where such changes are commercially desirable.

**Key words:** fruit quality, fruit ripening, *INO*, parthenocarpic, seedless fruit, tomato.

## Introduction

Seeds are an undesirable feature in many fruits because they may have a hard or leathery texture, bitter taste, and in many instances accumulate harmful toxic compounds. Replacing seeds and seed cavities with edible fruit tissue is desirable (Varoquaux *et al.*, 2000). Seedlessness is especially attractive in species with many seeds per fruit such as citrus, one large seed such as mango, or large cavities filled with

numerous seeds such as melon and papaya. In tomato, seeds are in general not considered as a negative trait of fresh market fruits since they contribute in a positive way to the fruit taste. However, seedless fruits would be valuable and improve tomato processing.

Seed formation is an integral component of fruit development: developing seeds promote cell expansion via synthesis

\* To whom correspondence should be addressed. E-mail: [amdandekar@ucdavis.edu](mailto:amdandekar@ucdavis.edu)

Abbreviations: FDR, false discovery rate; GA, gibberellic acid; GSEA, gene set enrichment analysis; INO, INNER NO OUTER; LTP, lipid transfer protein; PCA, principal component analysis; PCR, polymerase chain reaction; PG, polygalacturonase.

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of auxin and other unknown molecules (Gillapsy *et al.*, 1993). Metabolites associated with the developing embryo control the rate of cell division in surrounding fruit tissue, and seed number influences the final size and weight of fruit (Gillapsy *et al.*, 1993). Thus, seedlessness is potentially associated with agronomically undesirable changes in quality.

Parthenocarpy is fruit set in the absence of fertilization. It can be induced with phytohormones, particularly auxins, and is currently used to increase fruit production under adverse conditions for fruit set and growth. Such methods are sometimes used in tomato, where they can cause malformed fruit and vegetative organs, inhibit further flowering, and usually yield poor quality fruit (Abad and Monteiro, 1989).

Genetic strategies offer effective approaches involving specific mutations or introduction of specific genes. In tomato, *pat* mutations that result in parthenocarpy increase gibberellic acid (GA) in ovules during development (Fos *et al.*, 2001). Parthenocarpic fruit has also been generated through ovule-specific expression of the *iaaM* or *iaaH* genes from *Agrobacterium tumefaciens* or the *rolB* gene from *Agrobacterium rhizogenes*, which affect auxin biosynthesis or response, respectively (Rotino *et al.*, 1997; Carmi *et al.*, 2003). Expression of IaaH in ovaries induced parthenocarpic fruit by hydrolysis of the auxin precursor naphthaleneacetamide (NAM) in the ovary. Parthenocarpic eggplant, tobacco, and tomato fruits were also obtained by expressing *iaaM* under the ovule-specific promoter *DefH9* (Rotino *et al.*, 1996; Ficcadenti *et al.*, 1999; Donzella *et al.*, 2000).

Expression of *rolB* under *TRP-F1* (a promoter specific to ovaries and young fruit) induced parthenocarpy in tomato (Carmi *et al.*, 2003). Parthenocarpic fruits have also been obtained with the silencing of SIIAA9 (before IAA4) (Wang *et al.*, 2005) and SIARF7 (de Jong *et al.*, 2009), and mutations in the *ARF8* gene have been shown to induce parthenocarpic development in tomato (Goetz *et al.*, 2007). However, most of the parthenocarpic fruits were heart-shaped and had a rather thick pericarp compared with wild-type fruits (de Jong *et al.*, 2009). The challenge is to develop seedlessness by inducing parthenocarpy without needing supplemental pollination or application of plant growth regulators and without affecting fruit size and morphology. While the effect of the transgenic modifications on gross fruit morphology, productivity, and yield is known (Carmi *et al.*, 2003), the effects on overall gene expression and metabolism in fruit are not known.

The aim of the present study was to address the following questions. Is the INNER NO OUTER (*INO*) promoter from *Arabidopsis thaliana* able to induce parthenocarpy in tomato? Can we avoid undesirable traits often associated with seedlessness such as loss of flavour or nutritional value? What changes result from seedlessness at a transcriptomic and metabolomic level? Which pathways show significant changes in seedless fruit compared with seeded fruit?

The first objective of this study was to verify whether expression of *iaaM* and *rolB* driven by the *INO* promoter

could induce parthenocarpic fruits in tomato (cv Micro-Tom). The second objective was to determine changes in the transcriptome and metabolite profile induced by this genetic modification, comparing them with both wild-type fruits and transgenic parthenocarpic fruits obtained through ovule-specific expression of the same genes driven by the previously described ovule-specific promoter *DefH9*. The third objective was to determine any differences between transgenic and wild-type fruits in soluble solids content and other important morphological parameters.

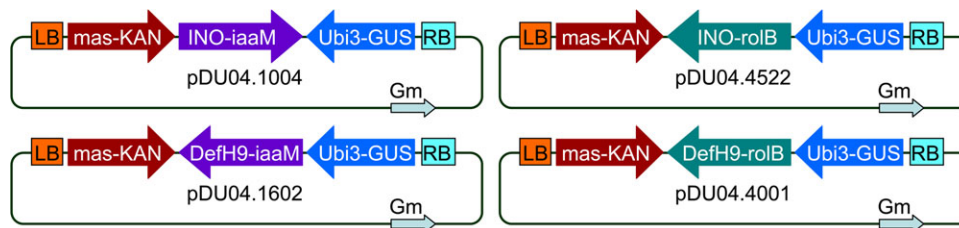
## Materials and methods

### Plant material

Seeds of the tomato cultivar MicroTom were obtained from the Ralph M Parsons Foundation Plant Transformation Facility at UC Davis.

### Plant binary vectors

Four plasmids were constructed, each containing one of two genes, *iaaM* and *rolB*, under the control of one of two ovary-specific promoters, *DefH9* or *INO* (Fig. 1). *iaaM* was obtained by PCR of DNA obtained from the wild-type *Agrobacterium tumefaciens* strain C58 using primers derived from the NCBI sequence (NC003065). The forward primer with a *KpnI* site was GGTACCATGTCAGCTTCAGCTCTCCTTGATAACCAGTGC, and the reverse primer with an *XbaI* site was TCTAGATTAATTTCTATTGCGGTAGTTATATCTCTTCC. *rolB* was obtained by polymerase chain reaction (PCR) of DNA obtained from wild-type *Agrobacterium rhizogenes* strain A4 using primers based on sequences published by Slightom *et al.* (1986). The forward primer with a *HindIII* site was GGAAGCTTATGGATCCCAAATTGCTATTCC. The reverse primer with a *HindIII* site was: GGAAGCTTTTAGGCTTCTTTCTTCAGGTTTACTGC. The *DefH9* promoter was obtained from *Anthriscum majus* DNA using the forward primer AGGCGCGCCAATTTCGGCAGGAGGTCCTTTCTATTTTTGCACAAAGCGTC and the reverse primer GG-TACCGTACCTCAGAAAATAACCTAATCATAATAAAC. These primers were based on the sequences of Spena *et al.* (2002). The above PCR products were TOPO cloned according to Invitrogen (Carlsbad, CA, USA), and then DNA was isolated from selected clones (Qiagen, Valencia, CA, USA) and sequenced (Davis Sequencing, Davis, CA, USA). The *INO* promoter fragment was from pRJM71 (Meister *et al.*, 2004). A cassette containing the *INO* promoter and *nos* terminator was made by deleting the *INO* cDNA from pRJM71. The *iaaM* and *rolB* genes were introduced into this cassette and then ligated into the binary vector pDU99.2215 (Escobar *et al.*, 2001). The constructs were designated pDU04.1004 (INO-*iaaM*) and pDU04.4522 (INO-*rolB*). *DefH9* constructs were made by cloning *iaaM* and *rolB* into cassettes containing the 35S promoter and *ocs* terminator. *iaaM(rolB)-ocs* was then blunt-end ligated into the TOPO-cloned *DefH9* construct. The resulting *DefH9/*



**Fig. 1.** The *Agrobacterium* binary vectors pDU04.1004 and pDU04.1602 control ovule-specific expression of the *iaaM* gene from *Agrobacterium tumefaciens* while pDU04.4522 and pDU04.4001 regulate ovule-specific expression of the *rolB* gene from *Agrobacterium rhizogenes*. The vectors pDU04.1004 and pDU04.4522 contain the novel *INO* ovule-specific promoter from *Arabidopsis thaliana* and vectors pDU04.1602 and pDU04.4001 contain the reference *DefH9* promoter from *Antirrhinum majus* previously shown to display ovule-specific expression and parthenocarpy with *iaaM* from *Pseudomonas syringae*. Other common components present on all vectors include an *nptII*-selectable marker gene driven by the mannopine synthase 2 promoter (*mas5*) and a *uidA* scorable marker gene driven by the *ubi3* promoter (*ubi3*). Arrows indicate the direction of transcription. LB and RB indicate the left and right T-DNA border sequences.

*iaaM(rolB)-ocs* cassette was then ligated into the binary pDU99.2215. The constructs were called pDU04.1602 (DefH9-*iaaM*) and pDU04.4001 (DefH9-*rolB*). These binary plasmids were then introduced into disabled *Agrobacterium* EHA105 pCH32 as previously described (Wen-jun and Forde, 1989) to create a functional *Agrobacterium* vector for plant transformation.

#### Tomato transformation

Tomato transformation was performed using MicroTom tomatoes following a modified version of the method described by Fillati *et al.* (1987). Primary parthenocarpic transformants ( $T_0$ ) were used for transcriptome and metabolite analysis and all transgenic plants were used for morphological measurements and soluble solids content.

#### Morphological analysis and soluble solid content

Fifteen fully ripe fruits from each transgenic plant and 10 from wild-type plants were picked for analysis of morphology and soluble solids content. Each fruit was weighed and the polar and equatorial diameter, number of locules, and seed number were determined. Brix value, an index of total solids content, was determined from juice squeezed from five separate fruits on each plant with a digital refractometer. Each fruit was also evaluated for shape malformation and colour of pulp. The data were analysed with an analysis of variance (ANOVA) univariate and ‘*post hoc*’ Duncan test ( $P=0.05$ ) using SPSS software.

#### Experimental design and plant material

Gene expression profiles for breaker-stage fruits were generated for wild-type and parthenocarpic lines with the four transgenes. Wild-type fruits with seeds and wild-type fruits from which seeds had been removed were the controls. Three replicates (each one as a pool of eight fruits) were used for each treatment and control, except for DefH9-*rolB*, where only two replicates were available.

Microarrays were used to study gene expression patterns in parthenocarpic fruit. Wild-type fruit with seeds was

compared with transgenic lines INO-*iaaM*, DefH9-*iaaM*, INO-*rolB*, and DefH9-*rolB*. To find genes with seed-specific expression, the control fruit were also compared with wild-type fruit from which seeds had been manually removed. There were three biological replicates for each treatment and control except DefH9-*rolB*, for which only two replicates were available. The metabolites present in parthenocarpic fruit were also studied, using each transgenic line as a separate treatment. Wild-type fruit with seeds were used as the control, and wild-type fruits with seeds manually removed were not considered. There were six replicates of each treatment and control group except DefH9-*rolB*, of which there were only five replicates.

#### RNA extraction

A modified hot borate method (Wan and Wilkins, 1994) was used to extract RNA from the pooled samples. Tissue was ground in liquid nitrogen and PVP-40 (200 mg) and put into a pre-chilled collection tube with 6–8 mg of proteinase K and 10 ml of 80 °C XT buffer (ratio 5 ml  $g^{-1}$ ).

#### Preparation of labelled RNA and hybridization

RNA labelling was performed according to instructions in the GeneChip One Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA, USA). Tomato microarrays were also purchased from Affymetrix. The array has 10 209 *Solanum lycopersicum* (preiously *Lycopersicon esculentum*) probe sets that interrogate >9200 *S. lycopersicum* transcripts. All fragmentation, hybridization, scanning, and image data processing were performed according to Affymetrix protocols.

#### Metabolomic analysis

For transgenes INO-*iaaM*, DefH9-*iaaM*, and INO-*rolB*, two fruits were taken from each of three different plants (representing three independent regeneration events), for a total of six replicates per transgene. For transgene DefH9-*rolB*, only two different plants with very few fruits were available and two fruits were taken from one plant and

three from the other, for a total of five replicates. The transgenic seedless fruits were compared with wild-type seeded controls, two fruits each from six plants. Wild-type fruit without seeds was not analysed.

The replicate fruits were harvested at breaker stage, frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until analysis. For each sample, 20–50 mg of pulp was ground and 1 ml of pre-chilled extraction solvent ( $\text{dH}_2\text{O}:\text{MetOH}:\text{CHCl}_3$  1:2.5:1) was rinsed with argon or gaseous nitrogen for 5 min and then added. After vortexing and centrifugation, the supernatant was analysed with a Pegasus III TOF (time-of-flight) mass spectrometer. The relative concentrations were determined by peak area ( $\text{mm}^2$ ). All peak detections were manually checked for false-positive and false-negative assignments. These mass spectra were then compared with known and commercially available mass spectral libraries. Statistical analysis was performed using pairwise comparison to determine significant differences. Metabolites that showed significant differences were grouped into functional categories.

#### *Real-time quantitative TaqMan PCR systems*

For each target gene, PCR primers and a TaqMan<sup>®</sup> probe were designed using Primer Express (Applied Biosystems, Foster City, CA, USA). The total RNA fraction was incubated with RNase-free DNase I following protocol instructions (Invitrogen, Carlsbad, CA, USA). Absence of genomic DNA contamination was confirmed with a universal 18S TaqMan PCR system. cDNA was synthesized with 50 U of SuperScript III following protocol instructions (Invitrogen). Each PCR contained 20 $\times$  Assay-on-Demand primer, probes for the respective TaqMan system, and TaqMan Universal PCR Mastermix (Applied Biosystems) and was amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Applied Biosystems standard amplification conditions were used: 2 min at  $50^{\circ}\text{C}$ , 10 min at  $95^{\circ}\text{C}$ , 40 cycles of 15 s at  $95^{\circ}\text{C}$ , and 60 s at  $60^{\circ}\text{C}$ . Fluorescent signals were collected during the annealing temperature and CT values extracted with a threshold of 0.04 and baseline values of 3–10. Three common housekeeping genes were examined: plant 18S rRNA (ssrRNA), apple glyceraldehyde phosphate dehydrogenase (GAPDH), and apple ribosomal protein S19. 18S rRNA had the lowest standard deviation across all tissues and its 18S rRNA CT values were therefore used to normalize the target gene CT values.

#### *Statistical analysis of microarray data*

Statistical analyses of microarray data were performed using R statistical software. The RMA method (Irizarry *et al.*, 2003) was used for background subtraction and normalization to pre-process raw probe data and produce the gene expression matrix. To determine which genes were differentially expressed among different groups, one-way ANOVA was used to obtain a *P*-value for each gene. Then all *P*-values were BH adjusted (Benjamini and Hochberg,

2000) for multiple hypotheses. Genes with adjusted *P*-values  $<0.05$  were considered differentially expressed in different groups. The R package LMGene (Rocke, 2004) was used to perform one-way ANOVA and the R package mulltest (Pollard *et al.*, 2004) was used to adjust multiple hypotheses. Annotation of some sequences was supplied by Affymetrix Inc.; additional annotations were found by BLAST comparisons with the NCBI non-redundant protein and TAIR databases. Functional classifications were based on those in the MapMan software, a user-driven tool that displays large data sets onto diagrams of metabolic pathways or other processes. (Thimm *et al.*, 2004). Gene set enrichment analysis (GSEA) was used to elucidate which functional categories of genes were more significantly associated with seeds and induced seedlessness. GSEA is a computational method that determines whether an *a priori* defined set of genes shows statistically significant, concordant differences between two biological states (Subramanian *et al.*, 2005). Affymetrix tomato GeneChip targets matched *Arabidopsis* genes in  $>800$  categories in the MapMan knowledge base. The 51 categories listed contained significant numbers of differentially expressed genes with a false discovery rate (FDR)  $<0.27$ . For each functional category a colour between blue (higher FDR, less significant) and red (lower FDR, more significant) and the number of genes differentially regulated were assigned.

#### *PCA microarray and metabolomic analysis*

Principal component analyses (PCA) was used to reduce the dimensionality of the microarray data (Fiehn *et al.*, 2000). Arrays were considered as observations and genes as variables. PCA was also applied to the metabolomic data. There were 35 samples (one control and 6–8 replicates for each of the four transgenes) and measurements of 234 metabolites. Metabolites that were missing in  $>60\%$  of the samples were ignored.

## Results and Discussion

### *Characterization of transgenic plants*

Parthenocarpy is a desirable trait in many commercially grown fruit crops if undesirable changes to quality, flavour, or nutrition can be avoided. This work is the first to demonstrate the ability of *INO* to induce parthenocarpy and a significant reduction in the number of seeds. The *INO* gene is required for ovule development in *Arabidopsis* and expression is limited to the predictive initiation site and developing outer (abaxial) cell layer of the ovule outer integument (Villanueva *et al.*, 1999; Meister *et al.*, 2004). There are several lines of indirect evidence that suggest *INO* expression is ovule specific in tomato. The tomato *INO* promoter was fused to the *Arabidopsis INO* and green fluorescent protein (GFP) coding regions (P-TOMINO::AtINO-GFP). Expression was observed in only one cell layer in the outer integument. This was fully consistent with *in situ* hybridizations that showed this same expression

pattern for the endogenous tomato *INO* gene (Charles S Gasser, personal communication). Although a thicker pericarp was observed in some parthenocarpic fruits, there were no significant changes to radial pericarp thickness in the four different types of transgenic tomatoes compared with wild-type tissues. Finally, the strongest piece of evidence that *INO* expression in tomato is ovule specific is the comparison with the corresponding expression of *DefH9*. There was a strong overlap in the expression changes triggered by these two promoters, with only a very few specific genes being expressed exclusively in either *INO* or *DefH9* transgenic plants (Fig. 3).

Several transgenic plants were generated for each of the four possible promoter (*INO* or *DefH9*)-gene (*iaaM* or *rolB*) combinations and evaluated for seedlessness. For both promoters, ~25–28% of transgenic tomato lines expressing *iaaM* lacked seeds; another 36–42% had only a few seeds (Table 1; Fig. 2). The remaining 33–36% had greatly diminished seed production. Of the transgenic plants expressing *rolB*, 20–33% lacked seeds, 40–45% had just a few seeds, and the remaining 22–40% had greatly diminished seed production (Table 1). *INO* and *DefH9* have similar effects when controlling expression of *iaaM* or *rolB* and produce similar proportions of seedless and reduced-seed lines.

Since several published works evaluated fruit quality and productivity of transgenic parthenocarpic fruits (Ficcadenti *et al.*, 1999; Acciarri *et al.*, 2002; Pandolfini *et al.*, 2002; Rotino *et al.*, 2005), the focus here was on fruit morphology and soluble solids content in order to measure differences between transgenic and wild-type fruits. MicroTom is a tomato cultivar suitable for analysis of fruit morphology but not fruit productivity. Only plants bearing completely parthenocarpic fruits were used for detailed transcriptome and metabolite analysis. Analysis was restricted to T<sub>0</sub> plants as they produced very few seeds and the germination of these seeds was also low.

### Fruit morphology

No significant morphological differences were observed among *INO-iaaM* and wild-type fruit (Table 2). Partheno-

carpic fruits from plants transformed with *DefH9-iaaM* had reduced weight and equatorial diameter, but seeded and parthenocarpic *INO-rolB* fruits had increased polar and equatorial diameter. In *DefH9-rolB* plants, no clear relationship was observed between the presence of seeds and fruit diameter. No significant differences in soluble solids were found among wild-type and *INO-iaaM*, *DefH9-iaaM*, and *INO-rolB* fruits (Table 2), but *DefH9-rolB* transgenic tomatoes with few seeds/fruit had more soluble solids than control fruit. There were no significant differences among lines in the number of locules in individual fruits. Although differences were observed in diameters and weight between transgenic and wild-type fruits, no clear correlation between number of seeds, transgene, and fruit size was observed. Fruit growth is also controlled by the developing seeds, as parthenocarpic fruits are generally smaller than seeded fruits (Mapelli *et al.*, 1978). The presence of seed-like structures that resemble pseudoembryos was observed. These were also found in auxin-induced fruit (Serrani *et al.*, 2007), and originate from divisions of cells of the inner integument (Kataoka *et al.*, 2003). These seed-like structures in transgenic tomato fruits were hypothesized to substitute for the seeds in stimulating fruit growth (Kataoka *et al.*, 2003).

Carmi *et al.* (2003) found a positive correlation between *rolB* and soluble solids, which was directly affected by seedlessness. Tomatoes transformed with *DefH9-iaaM* had increased soluble solids, probably because seedless fruits re-allocate assimilates from the seeds to the pericarp (Ficcadenti *et al.*, 1999). Seedless fruit are more desirable than seeded: they are less acidic (Lukyanenko, 1991) and have more soluble solids (Falavigna *et al.*, 1978) than seeded cultivars. *DefH9-rolB* lines with few seeds in the present study had increased soluble solids, but no significant changes were observed in the other transgenic lines.

### Gene expression changes in transgenic and control tomato fruit

It is possible that the presence/absence of seeds could have significant effects on the surrounding carpel tissue. The Affymetrix tomato GeneChip was used to compare gene

**Table 1.** Number and type of transgenic plants obtained for each construct, with percentage and mean number of seeds/fruit

Significant differences were calculated using ANOVA univariate ( $P=0.05$ ) among classes for each transgene and control (untransformed). Different letters for each different transgene indicate significant differences in comparison with control plants measured by Duncan multiple range test ( $P=0.05$ ).

	<u>INO-iaaM lines</u>		<u>DefH9-iaaM lines</u>		<u>INO-rolB lines</u>		<u>DefH9-rolB lines</u>	
	No. of lines	Seeds/fruit	No. of lines	Seeds/fruit	No. of lines	Seeds/fruit	No. of lines	Seeds/fruit
All transgenic lines	12 (100%)	5.2	11 (100%)	2.2	9 (100%)	5.4	5 (100%)	5.1
Transgenic lines with fewer seeds	5 (42%)	0.9 a	4 (36%)	0.4 a	4 (45%)	1.3 a	2 (40%)	0.7 a
Transgenic lines without seeds	3 (25%)	0 a	3 (28%)	0 a	3 (33%)	0 a	1 (20%)	0 a
Transgenic lines with seeds	4 (33%)	10.4 b	4 (36%)	6.9 b	2 (22%)	14.6 b	2 (40%)	8.7 b
Control lines	10	16.7 b	10	16.7 c	10	16.7 b	10	16.7 b

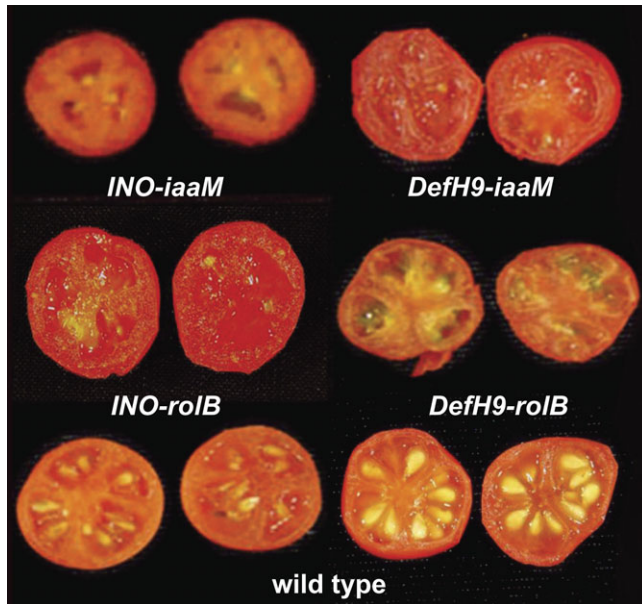
expression profiles of wild-type and transgenic fruits at the breaker stage and identify changes directly induced by transgene expression. At this stage, the formerly green MicroTom fruit had turned yellow, but not red. Although direct changes induced by genetic transformation in both the transcriptome and metabolome are likely to occur at earlier stages of development, it was expected to see long-term effects at the breaker stage, where the fruit has a clear and distinct phenotype that allows physiologically similar

fruits to be compared. At this very active physiological stage, many gene expression changes occur, emphasizing differences among transgenic and wild-type untransformed fruits.

Using one-way ANOVA and multiple hypothesis testing, 1748 of 10 209 genes (17%) with significant variation ( $P < 0.05$ ) were identified among wild-type and transgenic fruits. Thus, 83% of the genes analysed showed no significant differences in expression due to parthenocarpy.

Pairwise comparison (one-way ANOVA,  $P < 0.001$ ) of the most differentially expressed genes showed 98 and 101 down-regulated genes (0.96% and 0.98% of all genes represented on the microarray), respectively, for *rolB*- and *iaaM*-transformed plants. There were also 13 and 21 up-regulated genes (0.13% and 0.21% of represented genes), respectively, for *iaaM*- and *rolB*-transformed plants (Fig. 3). Thus, only a small proportion of genes were regulated differently in all transgenic fruits than in wild-type fruits with seeds removed, and fewer genes were up-regulated than were down-regulated. Among promoters controlling the same transgene, there was much overlap between down-regulated and up-regulated genes in *DefH9*- and *INO*-transformed plants, suggesting very similar effects of the two promoters on gene expression. This evidence strongly supports the hypothesis that the *A. thaliana INO* promoter drives ovule-specific expression in tomato.

PCA was applied to 1748 genes with significant differences in expression (Fig. 4). Principal component 1, accounting for 53% of the variance, was probably the result of genes affected by the transgene's ovule-specific expression or by the absence of seeds. Principal component 2, accounting for 12.6% of the variance, represents gene expression modifications induced in different ways by *iaaM* and *rolB* ovule-specific expression, which act by different molecular mechanisms. *iaaM* encodes a tryptophan



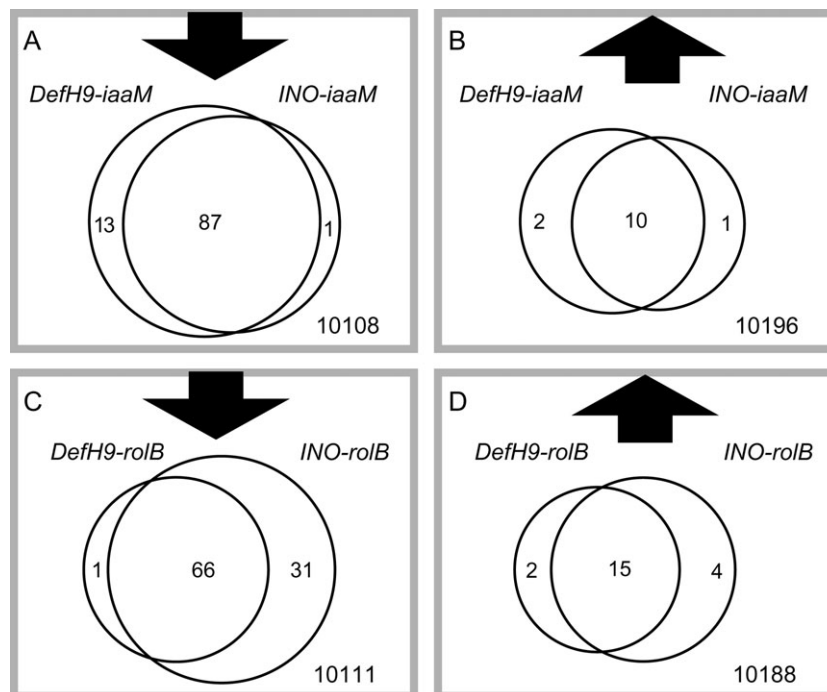
**Fig. 2.** Wild-type and parthenocarpic transgenic tomato fruit generated with four different constructs (*INO-iaaM*, *DefH9-iaaM*, *INO-rolB*, and *DefH9-rolB*). No seeds are visible in the parthenocarpic lines.

**Table 2.** Phenotypes of wild-type and transgenic fruit produced on  $T_0$  plants

Weight, polar and equatorial diameter, number of locules, and soluble solids were analysed by one-way ANOVA ( $P=0.05$ ). Different letters in the same column indicate different groups by the Duncan multiple range test. No letters in the same column means no significative differences between classes.

Classes		Weight (g)	Polar diameter (mm)	Equatorial diameter (mm)	No. of locules	Brix value
INO-iaaM	With few seeds	2.1	15.5	13.0	2.7	9.0
	Without seeds	2.1	13.1	13.9	3.0	9.8
	With seeds	2.0	13.0	13.9	2.6	7.7
Control	All lines	1.8	12.2	13.2	2.4	8.2
DefH9-iaaM	With few seeds	2.2 b	13.7 b	15.0 c	2.7	9.3
	Without seeds	1.1 a	11.0 a	10.6 a	2.7	8.3
	With seeds	2.0 b	12.9 b	12.5 b	2.4	10.0
Control	All lines	1.8 b	12.2 a,b	13.2 b	2.4	8.2
INO-rolB	With few seeds	1.6	14.7 b	14.0 a,b	2.5	8.9
	Without seeds	2.1	15.5 b	15.8 c	2.2	10.1
	With seeds	1.8	16.4 c	15.0 b,c	2.2	8.6
Control	All lines	1.8	12.2 a	13.2 a	2.4	8.2
DefH9-rolB	With few seeds	1.8 a	14.5 a,b	14.7 a,b	2.6	11.5 c
	Without seeds	1.8 a	16.5 b	15.2 a,b	2.5	8.0 b
	With seeds	2.5 a	17.0 b	16.7 c	2.6	6.9 a
Control	All lines	1.8 a	12.2 a	13.2 a	2.4	8.2 a,b

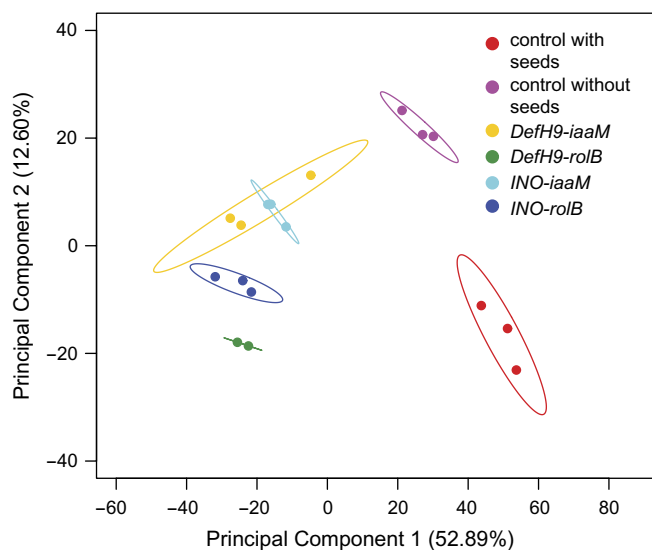




**Fig. 3.** Pairwise comparison of each transgenic sample with control fruits without seeds. (A) and (B) the number of down-regulated and up-regulated genes (adjusted  $P$ -value  $<10^{-4}$ ) of samples from DefH9-iaaM and INO-iaaM lines. (C) and (D) Number of down-regulated and up-regulated genes (adjusted  $P$ -value  $<10^{-4}$ ) of samples from DefH9-rolB and INO-rolB lines. Much overlap exists between down-regulated and up-regulated genes in DefH9-iaaM and INO-iaaM samples. This is also the case in DefH9-rolB and INO-rolB samples.

monoxidase producing indolacetamide, which is converted either chemically or enzymatically to indole-3-acetic acid (IAA) (Inze *et al.*, 1984). RolB is a putative tyrosine phosphatase operating in auxin signalling (Carmi *et al.*, 2003). Despite extensive research, the actual function of the product of the *rolB* gene is still not clearly understood. Cluster analysis of individual genes confirmed the induction of six different gene expression patterns in transgenic lines (Fig. 5A). Genes in group 6 were up-regulated in at least one transgenic line compared with seeded or seedless wild-type fruits. These genes do not have seed-specific expression, but are involved in pathways affected by the ovule-specific expression of *iaaM* and *rolB*. Thus, they may play important roles in fruit quality and warrant further investigation. Functions of other genes up-regulated specifically in *iaaM*-transformed (group 2) or *rolB*-transformed fruits (group 5) may be of interest to better characterize gene expression differences induced by ovule-specific expression of these genes. The roles of genes down-regulated in parthenocarpic transgenic fruits (group 3) and those with seed-specific expression (group 4) may also be of interest. Functional analysis of the significantly differently regulated genes was performed using MapMan software (Fig. 6).

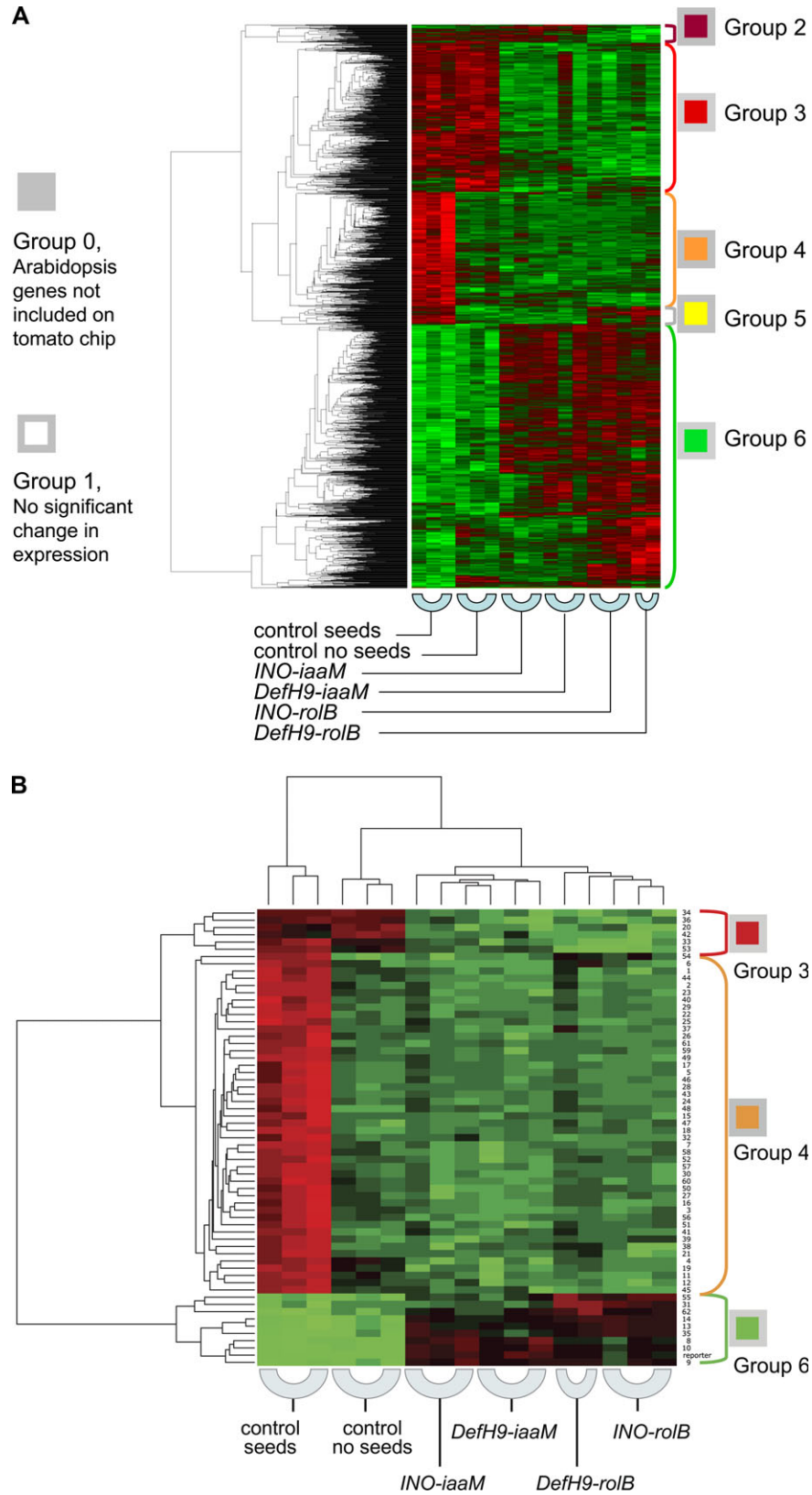
GSEA was very useful for showing the most important changes taking place in seedless transgenic fruits (Fig. 7). Among the 1748 differentially regulated genes, those that followed a similar pattern in GSEA analysis and had similar functions were strongly supposed to be affected by the absence of seeds. Interestingly, one effect of the transgene modification was the up-regulation of lipid transfer proteins



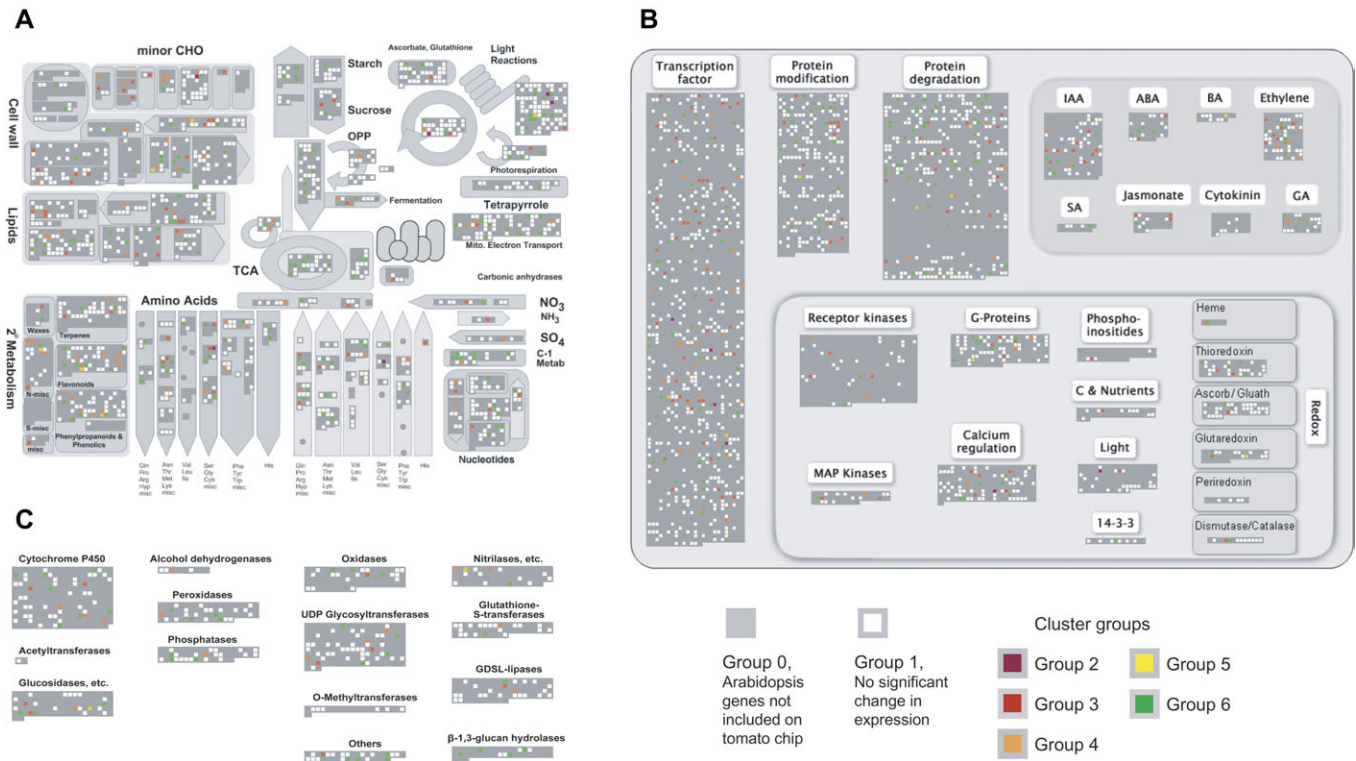
**Fig. 4.** Principal component analysis of 1748 genes with significant differences at  $P < 0.05$  in expression among transgenic and control fruits. The dots represent each of the 17 individual microarrays used in this study, and the ellipses represent the 95% confidence limit for the replicates in each group.

(LTPs) in all four different transgenic fruit types compared with wild-type fruits with seeds removed. LTPs and puroindolines can inhibit the growth of fungal pathogens *in vitro* and they are capable of synergistically enhancing the antimicrobial properties of other antimicrobial peptides such as defensins and thionins (Marion *et al.*, 2004). LTPs





**Fig. 5.** Cluster analysis of gene expression in control fruit with or without seeds and transgenic fruit with *INO-iaaM*, *DefH9-iaaM*, *INO-rolB*, or *DefH9-rolB*. Genes were clustered based on differential expression using the *made4* package of R statistical software. Three biological replicates were used for each genotype, except for *DefH9-iaaM*, from which only two biological replicates were available. (A) Expression data for 1748 (18%) target genes with  $P < 0.05$  could be divided into five groups based upon expression patterns. (B) Hierarchical clustering and heat map for 62 (0.6%) target genes with  $P < 10^{-4}$ . Three of the five groups appeared in the latter.



**Fig. 6.** Functional categorization of the six expression pattern groupings obtained from pairwise comparison of each transgenic sample with control fruits without seeds. MapMan display of pathway assignments for gene groups created in previous cluster analysis: (A) Metabolism overview, (B) Regulation overview, (C) Large enzyme families. Colours in small squares were assigned based on positions of target genes in the cluster analysis, indicated on the right margin of heat map figures. White squares indicate target genes whose expression did not pass the  $P < 0.05$  cut-off.

are also involved in the signalling of the defence mechanism of plants against their pathogens, recognizing membrane receptors involved in the transduction pathways of local defence responses (Blein *et al.*, 2002). Although the accumulation of these proteins has to be confirmed, the up-regulation of the transcript is intriguing for the possibility to increase the resistance of plants to biotic stresses. In this regard, overexpression of these antimicrobial proteins induces significantly increased resistance of plants toward microbial pathogens (Marion *et al.*, 2004). In addition, these antimicrobial proteins can help preserve fruit products.

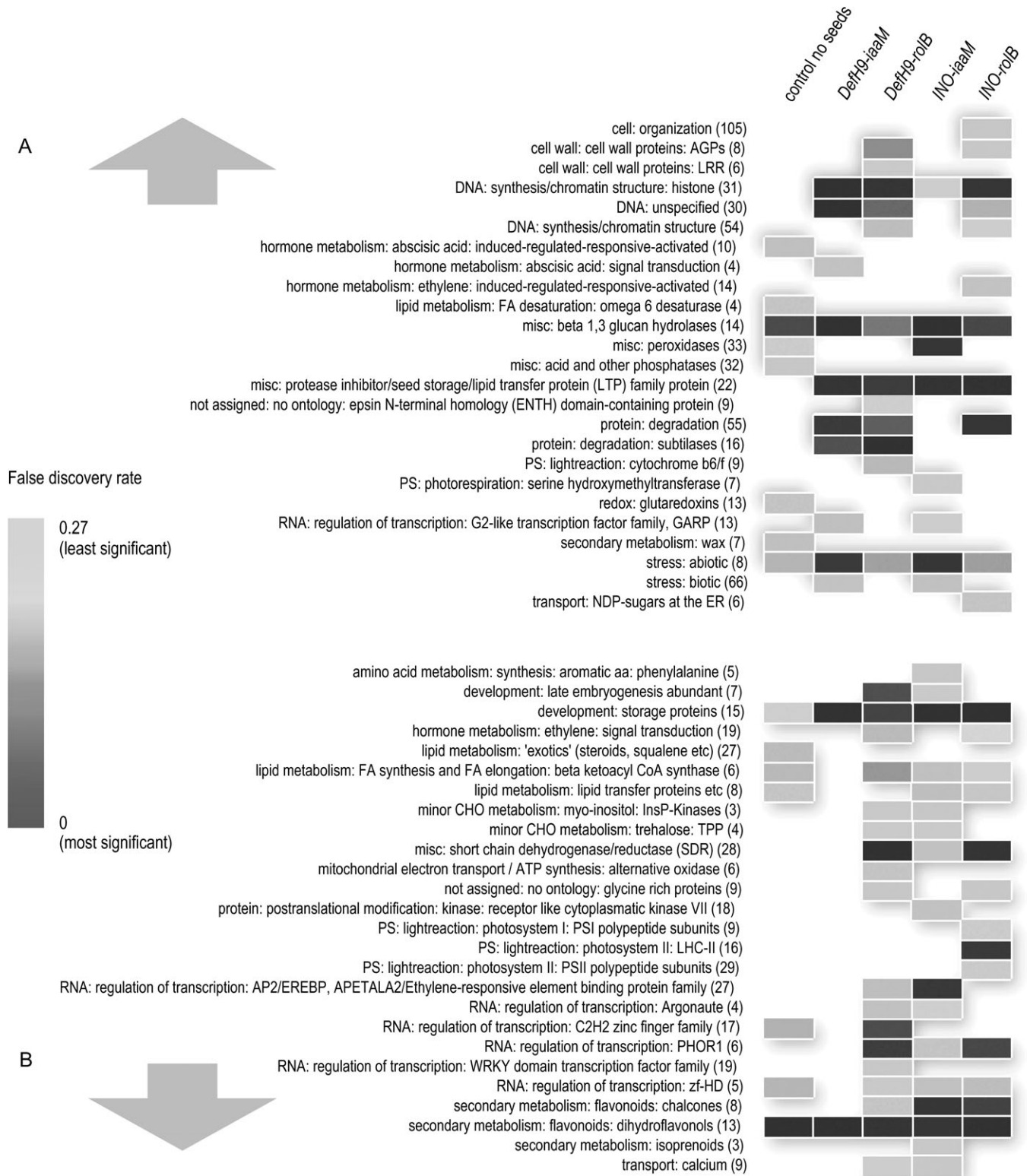
The two analyses (GSEA and MapMan) were complementary and showed important similarities, providing a clearer picture of which gene categories were more affected by transgenic induction of parthenocarpy. The important conclusions of both analyses are outlined (Table 3). Interestingly, *iaaM*-transformed fruits up-regulated genes involved in abiotic stress and three of the four types of transgenic fruits up-regulated genes involved in protein degradation. These effects are also important topics of further investigations. Among the down-regulated genes, short chain dehydrogenase genes and PHOR1 transcription factors were associated with *rolB*. Transgenic potato plants expressing an antisense PHOR1 construct had a semi-dwarf phenotype, displayed reduced response to GA application, and had more endogenous GAs than control plants

(Amador *et al.*, 2001), supporting the hypothesis that PHOR1 is a positive regulator of GA signalling (Thomas and Sun, 2004).

Another specific analysis of individual genes was performed by determining NCBI accession annotations of the 62 most differentially regulated genes with adjusted  $P$ -values  $< 10^{-4}$  (ANOVA model) and clustering them into 19 functional categories (Table 4). Among down-regulated genes, the functional categories most affected by gene expression changes were those involved in light reactions, transcription factors, and redox reactions (*rolB* fruits), and flavonoid metabolism and storage proteins (all seedless fruits).

Excluding genes with unknown function, these genes clustered primarily into the minor CHO metabolism, DNA synthesis, and transcription factor categories. In addition, several differentially regulated genes were involved in secondary metabolism and hormone metabolism. Another interesting category, transport/transporters, was represented by four differentially regulated genes: a cation exchanger, two sugar transporters, and nitrate transporter NRT1-3.

Hormone metabolism genes such as those encoding Dwf1 (Dwarf1/Diminuito), ERF/AP2 transcription factor, and allene oxide synthase (AOS) were differentially regulated between transgenic and wild-type fruits. Interestingly, transgenic plants that overexpress *dwarf4* in the brassinosteroid



**Fig. 7.** Gene set enrichment analysis (GSEA) of microarray expression data. (A) Functional categories up-regulated in at least one of the transgenic constructs, compared to controls with seeds. (B) Functional categories down-regulated in at least one of the transgenic constructs, compared to control with seeds. Affymetrix tomato GeneChip targets matched *Arabidopsis* genes in >800 categories in the MapMan knowledge base. The 51 categories listed contained significant numbers of differentially expressed genes with a false discovery rate (FDR) of <0.27. Numbers in parentheses indicate the number of genes in each set.

**Table 3.** Main gene expression changes between transgenic and seedless and seeded wild-type fruits, subdivided into functional categories

A comparison between two different methods was performed: gene set enrichment analysis and MapMan functional categorization analysis. These gene expression changes are strongly supposed to be linked to parthenocarpy induced by mechanical or genetically engineered removal.

Gene set enrichment analysis	MapMan analysis
<b>Up-regulated</b>	
<b>DNA synthesis</b>	
More up-regulation in DefH9 and INO-rolB fruits	Several genes involved in nucleotide synthesis were shown to be up-regulated (green colour, Fig. 5A)
<b>Protein degradation</b>	
Up-regulated in DefH9-transformed fruits	Many genes were shown to be up-regulated in transgenic fruits (green colours, Fig. 5B)
<b>Lipid metabolism</b>	
More up-regulation in <i>iaaM</i> fruits but also in <i>rolB</i> fruits and wild-type seedless	Several genes were shown to be up-regulated in transgenic seedless fruits (green colours, Fig. 5A)
<b>Protein modification</b>	
Acid and other phosphates up-regulated in all transgenic fruits	More genes were shown to be up-regulated than down-regulated (three genes were up-regulated in transgenic fruits, green colour, Fig. 5C)
<b>Cell wall</b>	
Weak up-regulation <i>rolB</i> fruits (more up-regulation DefH9- <i>rolB</i> fruits)	Two genes up-regulated in <i>rolB</i> fruits (yellow colour, Fig. 5A)
<b>Secondary metabolism: waxes</b>	
More up-regulation in <i>iaaM</i> fruits but also up-regulation in <i>rolB</i> fruits	Four genes analysed: one up-regulated and one down-regulated in transgenic seedless fruits.
<b>Down-regulated</b>	
<b>Light reaction</b>	
Genes were down-regulated in <i>rolB</i> fruits	Two genes were down-regulated in <i>rolB</i> fruits (cytochrome P450, orange colour, Fig. 5C)
<b>Transcription factors</b>	
Down-regulation of several RNA regulation factors in <i>rolB</i> fruits	Four genes down-regulated in <i>rolB</i> fruits, many others down-regulated in all transgenic seedless fruits
<b>Secondary metabolism (flavanoid)</b>	
Down-regulated in all transgenic and wild-type seedless fruits (seed-specific expression)	Many genes down-regulated in all seedless fruits (orange colour, Fig. 5A)
<b>Ox/redox reaction</b>	
Down-regulation in <i>rolB</i> fruits	A gene involved in oxidase down-regulated in <i>rolB</i> fruits
<b>Storage proteins</b>	
Down-regulation in all seedless fruits: above all the transgenic ones	Several genes involved in lipid and protein metabolism were down-regulated in seedless fruits

biosynthesis pathway showed increased vegetative growth and seed yield, consistent with the result found here that a putative *dwarf4* gene was highly down-regulated in transgenic seedless fruits. Although some transgenic tomatoes showed lower internodes, no clear correlation was observed between seedlessness and reduction of vegetative growth. MicroTom plants are naturally bushy and short, however, so any possible effect of *rolB* and *iaaM* ovule-specific expression on brassinosteroid biosynthetic genes and vegetative growth must be investigated in other tomato cultivars.

Interestingly, the functional characterization showed that several ethylene- and IAA- associated genes were also down-regulated in transgenic parthenocarpic fruits (Fig. 6, group 3), while others were up-regulated (group 6). Possible interactions between auxin and ethylene metabolism and perception are also of interest. EREBPs are both transcriptional activators and repressors in plants (Fujimoto *et al.*, 2000), and constitute a large gene family in tomato with important consequences for fruit softening and shelf life. Since some EREBPs induce ripening and others are repressed, Fei *et al.* (2004) proposed a model in which EREBPs dynamically regulate fruit ripening using antagonistic mechanisms.

Among the IAA-responsive genes, down-regulation of an auxin-regulated protein (BT013913.1) in transgenic parthenocarpic fruits was confirmed using real-time RT-PCR. This evidence agrees with previously published data that showed down-regulation of IAA-responsive genes associated with parthenocarpy such as the silencing of an auxin-responsive factor (SIARF7) (de Jong *et al.*, 2009). Mutations in *Arabidopsis ARF8*, also referred to as Fruit Without Fertilization (FWF), cause fruit set in the absence of pollination and fertilization (Goetz *et al.*, 2007). Parthenocarpic fruits have been obtained through down-regulation of IAA9, a tomato Aux/IAA family member (Wang *et al.*, 2005). Recently it has been shown that auxins induce fruit set and growth in tomato, partially enhancing GA biosynthesis and decreasing GA inactivation, leading to more GA<sub>1</sub> as observed in parthenocarpic fruits induced by 2,4-D. These conclusions were made after observation of more transcript for genes encoding copalylidiphosphate synthase (SICPS), S1GA20ox1, S1GA20ox2, S1GA20ox3, and S1GA3ox1 in unpollinated ovaries treated with 2,4-D than in unpollinated untreated ovaries (Serrani *et al.*, 2008).

Seedless fruit often has a longer shelf life than seeded fruit because seeds produce hormones such as ethylene that trigger senescence (Fei *et al.*, 2004). Interestingly, the present data showed that IAA-responsive genes were down-regulated in all transgenic fruits compared with seedless or seeded wild-type fruits, implying that the ovule- and ovary- driven expression of IAA and *rolB* induces down-regulation of other auxin-associated genes irrespective of seeds. It is possible that these negative regulators induce parthenocarpy as in the down-regulation of SIARF7 ovary transcript after pollination in tomato (de Jong *et al.*, 2009).

**Table 4.** Predicted functions of 62 highly differentially regulated genes with adjusted  $P < 10^{-4}$  in ANOVA model, belonging to clusters (Fig. 5) indicated in the third column

Predicted functions are based on tblastx to TAIR and NCBI nr databases (NSH, no significant hit, at  $10^{-5}$  expectation value threshold). Functional categories assigned by MapMan knowledge base. Numbers in the first column correspond to those near the right-hand margin of Fig. 5B.

	MapMan categorization, from tblastx versus TAIR	Cluster	NCBI accession	Annotation
1	Fermentation.aldehyde dehydrogenase	4	AW032379	Aldehyde dehydrogenase (ALDH1a)
2	Gluconeogenesis/glyoxylate cycle.malate synthase	4	AW649829	Strong similarity to glyoxysomal malate synthase from <i>Brassica napus</i>
3	Mitochondrial electron transport	4	AI898816	Alternative oxidase 2, mitochondrial (AOX2)
4	Metal handling	4	BT013123	Selenium-binding family protein
5		4	BI203983	Similar to ferric-chelate reductase (FRO1) ( <i>Pisum sativum</i> )
6	Redox.haem	4	AY026344	Non-symbiotic haemoglobin
7	DNA synthesis/chromatin structure	4	BE462343	High-mobility-group protein/HMG-I/Y protein
8		6	BT013634	Minichromosome maintenance family protein
9		6	BG626714	Prolifera protein (PRL)/DNA replication licensing factor Mcm7 (MCM7)
10		6	BT014477	ATRPA2;ROR1;replicon protein A;suppressor of ROS1
11		4	BG123861	AT-rich element -binding factor 3 [ <i>Pisum sativum</i> ]
12		4	BT013761	MAR-binding protein [ <i>Nicotiana tabacum</i> ]
13	Protein degradation	6	BI931445	Peptidase M20/M25/M40 family protein, similar to acetylornithine deacetylase
14		6	BI935106	Acetylornithine deacetylase, putative [ <i>Brassica oleracea</i> ]
15		4	AI898251	Ubiquitin-protein ligase/zinc ion binding [ <i>Arabidopsis thaliana</i> ]
16	Signalling	4	BT012984	Contains eukaryotic protein kinase domain
17		4	AA824763	Contains IQ calmodulin-binding motif, Pfam:PF00612
18	Transport/transporter	4	BG126449	Cation exchanger, putative (CAX3), similar to high affinity calcium antiporter CAX1
19		4	AI780345	Integral membrane protein, putative/sugar transporter family protein
20		3	BE458971	Sugar transporter, putative, similar to ERD6 protein, <i>Arabidopsis thaliana</i>
21		4	BT012913	Putative nitrate transporter NRT1-3 [ <i>Glycine max</i> ]
22	Miscellaneous	4	AW934450	SSXT protein-related/glycine-rich protein
23		4	AF143742	CBS domain-containing protein
24		4	CK714819	Hydrolase, alpha/beta fold family protein
25		4	BI921484	Transducin family protein/WD-40 repeat family protein
26		4	AI773541	Contains integral membrane protein domain, Pfam:PF01988
27		4	AI781043	Low similarity to SP:P30043 flavin reductase ( <i>Homo sapiens</i> )
28		4	BG131258	Cytochrome P450 71B23, putative (CYP71B23)
29		4	BI928574	GDSL-motif lipase/hydrolase family protein, similar to family II lipase EXL3
30		4	BG734983	Putative zinc-binding domain (DUF701)
31		6	CN385216	Metalloprotease inhibitor [ <i>Solanum tuberosum</i> ]
32	Cell wall	4	BT014503	GDP-mannose pyrophosphorylase (GMP1)
33		3	AF154420	Beta-galactosidase, putative/lactase
34	Lipid metabolism	3	BT014559	Long-chain acyl-CoA ligase/synthetase family protein
35		6	CK715596	Phospholipase/carboxylesterase family protein
36	Amino acid metabolism	3	BT013418	Proline oxidase, putative/osmotic stress-responsive proline dehydrogenase
37	Secondary metabolism	4	AI486965	Tropinone reductase/dehydrogenase, putative
38		4	BM535633	Chalcone-flavanone isomerase family protein
39		4	BG129167	Undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase
40		4	BG631118	Undecaprenyl pyrophosphate synthase [ <i>Leptospirillum</i> sp. Group II UBA]
41	Hormone metabolism	4	BG791226	Cell elongation protein/DWARF1/DIMINUTO (DIM)
42		3	AY192367	ERF (ethylene response factor) subfamily B-3 ERF/AP2 transcription factor
43		4	AF454634	Allene oxide synthase/hydroperoxide dehydrase/cytochrome P450 74A
44	RNA.regulation of transcription	4	BG125438	Basic helix-loop-helix (bHLH) family protein
45		4	AI780243	Zinc finger (C2H2 type) family protein
46		4	AW031142	MYB60;myb family transcription factor
47		4	AW934591	Zinc finger homeobox family protein
48		4	BM412250	Rcd1-like cell differentiation protein, putative
49	Minor CHO metabolism	4	AW650462	Trehalose-6-phosphate phosphatase, putative
50		4	CN384702	Inositol polyphosphate 6-/3-/5-kinase 2a (IPK2a)
51		4	AI897093	Inositol polyphosphate 6-/3-/5-kinase 2b (IPK2b)
52		4	BG627650	Inositol-3-phosphate synthase isozyme 2

Table 4. Continued

MapMan categorization, from tblastx versus TAIR	Cluster	NCBI accession	Annotation
53	3	AW933452	PfkB-type carbohydrate kinase family protein
54 Drought/salt stress response	4	AF500011	Dehydration responsive element-binding protein, <i>Lycopersicon esculentum</i>
55 Unknown function	6	BT012940	Unknown protein [ <i>Arabidopsis thaliana</i> ]
56	4	BT013091	Hypothetical protein Osl_007083, <i>Oryza sativa</i>
57	4	AW442644	Contains similarity to cotton fibre expressed protein 1
58	4	BG629826	NSH
59	4	AW092459	Os07g0631100, <i>Oryza sativa</i>
60	4	BG628576	Allantoin transporter [ <i>Arabidopsis thaliana</i> ]
61	4	AW650005	Harpin-induced 1 [ <i>Medicago truncatula</i> ]
62	6	BG630221	NSH

A gene encoding an AOS in the jasmonate biosynthesis pathway was highly down-regulated in seedless transgenic fruits. Since published data associate jasmonates with early stages in climacteric fruit ripening and triggering ethylene production (Janoudi and Flore, 2003), it is of interest to determine whether seedless transgenic fruits differ in the rate of fruit ripening or in shelf life.

Among genes with differential expression in transgenic fruit, some highly down-regulated genes may have important functions in fruit development. Fifteen down-regulated genes were found in parthenocarpic transgenic fruits that were involved in cell wall metabolism (Fig. 6). Two of these, GDP-mannose pyrophosphorylase (GMP1) and  $\beta$ -galactosidase, were highly down-regulated, and a  $\beta$ -1,3 glucan hydrolase was significantly up-regulated in seedless fruits. The effect of these expression changes merits further investigation, since in tomato many genes may cause fruit softening (Giovannoni *et al.*, 1989). Indeed, additional cell wall hydrolases and expansins have been associated with tomato fruit softening (Smith and Gross, 2000).

Another important functional category among differentially expressed genes was minor CHO metabolism involved in fruit sugar partitioning. The metabolomic analysis found no differences in sugars, but this was not verified in ripe fruit. Many proteins in these pathways are allosterically regulated, so their activity in the fruit may be less affected by changes in transcript level.

To validate these data with TaqMan real-time PCR analysis, 17 genes were analysed for correspondence between microarrays and real-time PCR (Table 5A, B). Twelve of the 17 genes showed a microarray versus real-time RT-PCR correlation of  $>0.75$  and the remaining five genes showed a lower correlation. These five genes were the high affinity calcium antiporter CAX1 (BG126449), sugar transporter (BE458971), L-lactate dehydrogenase (BT013913.1), short-chain dehydrogenase reductase (BT014398.1), and putative vicilin (BT013421.1) (Table 5C). However, genes involved in auxin and ethylene biosynthesis and signalling were confirmed to be differentially regulated between transgenic and wild-type fruits.

#### Metabolomic analysis of transgenic and control fruit

The next step was to address how the changes in gene expression altered the overall metabolomic profile of parthenocarpic transgenic fruit. The concentrations of  $>400$  metabolites in parthenocarpic fruits transformed with the four different constructs were compared (in total six replicates for each construct except DefH9-rolB, from which only two different plants with five fruits were available) with those from 12 wild-type fruits containing seeds.

The acquired data sets were compared using PCA (Fiehn *et al.*, 2000) to determine differences and similarities among transgenic seedless fruits and seeded wild-type fruits at the breaker stage. Linear combination of metabolic data generated new vectors or groups to best explain overall variance in the data set without prior assumptions about whether and how clusters might form. It was immediately clear that the overall metabolomic data did not show clear differences among the different fruit genotypes (Fig. 8). PCA could not separate the four transgenic lines and the controls: the 95% confidence intervals of the four treatments and two controls overlapped. Principal component 1, which accounted for  $\sim 38\%$  of the variance, partly distinguished the control from some parthenocarpic lines such that all negative values were from transgenic lines, but the separation was not complete. Principal component 2 did not clearly separate any treatments from the controls.

The relative concentrations of  $>400$  metabolites were determined by peak area in transgenic and control fruits. However, many of them do not have a completely determined structure and could not be identified as a known molecule. Metabolites with known structure were divided into important functional categories (amino acids, sugars, fatty acids, other acids, and other compounds) and they were compared with transgenic seedless and wild-type seeded fruits using ANOVA univariate analysis ( $P=0.05$ ). It was expected that most differences at a metabolomic level induced by the transgene expression might occur at the beginning of fruit set and before fruits reached their final size. However, some changes are also expected when fruits



**Table 5.** Comparison of normalized intensity values from microarray experiments (A) and corresponding expression values from real time-PCR (B) for 17 genes showing significant expression changes between control and seedless types

Correlation values comparing the overall expression pattern between the two experiments are given in the last column in (B). Functional descriptions for the 17 genes are given in (C). Genes that showed a microarray versus real-time RT-PCR correlation <0.75 are indicated in bold

<b>(A)</b>								
Affymetrix Probe Set ID	Microarray data							
	NCBI accession	Control no seeds	Control with seeds	INO-iaaM	DefH9-iaaM	INO-roIB	DefH9-roIB	ANOVA P-value
Les.4140.1.S1_at	AY192367.1	-0.1	3.2	-4	-3	-7.3	-4.4	0.000166
<b>LesAffx.23546.1.S1_at</b>	BG126449	0.3	-17.8	-12.1	-2.5	-11.9	-3.4	0.011324
Les.5021.1.S1_at	BT013123.1	-0.3	0.6	-6	-7.9	-4.8	-3.9	0.018553
Les.3642.1.S1_at	U17972.1	0.3	1.8	-5.5	-19.2	-5.9	-3.2	0.005197
<b>LesAffx.56785.1.S1_at</b>	BE458971	0.3	2.2	-15.7	-10.4	-35.4	-58.8	0.0207
Les.2767.1.S1_at	U18678.1	-0.3	-6.5	-45.9	-59.8	-57.5	-18.6	0.002179
Les.3492.1.S1_at	AY013256.1	-0.3	1.9	-7	-6.5	-10	-9.3	0.000119
Les.3122.2.A1_at	S66607.1	-0.3	-0.2	2252.2	3528	5033	3839.4	1.09E-06
Les.2832.1.S1_at	CN384480	0	3.3	64.6	56.6	151.7	89.1	0.000174
Les.3766.1.S1_at	U77719.1	0	2	-16.9	-12.6	-2.3	-7.6	0.006146
LesAffx.70635.1.S1_at	BI421189	-0.3	1.9	-7	-10.2	-6	-2.9	9.85E-06
<b>Les.97.1.S1_at</b>	BT013913.1	-0.3	3.1	-4	-15.5	0.6	2.3	0.049221
Les.3486.1.S1_at	AF416289.1	-0.2	1.5	-5.9	-5.9	-2	-1.5	2.89E-05
LesAffx.58308.1.S1_at	BG129227	-0.3	0.7	-38	-23.9	-12.2	-8.3	0.014537
Les.3330.2.S1_at	BE458823	0	14.2	47.4	1193.1	2032	3304	0.000761
<b>Les.5694.1.S1_at</b>	BT014398.1	-0.3	-5.6	-27.5	-109.8	-82	-18.7	0.003884
<b>Les.5168.1.S1_at</b>	BT013421.1	0.2	-17.1	-136.9	-201.3	-337	-332.4	0.013441
Les.5024.1.S1_at	BT013126.1	0	13.6	100.9	336.6	1377.4	786.9	0.002116

<b>(B)</b>								
Affymetrix Probe Set ID	Real time RT-PCR data							Microarray versus real-time RT-PCR correlation
	Control no seeds	Control with seeds	INO-iaaM	DefH9-iaaM	INO-roIB	DefH9-roIB	ANOVA P-value	
Les.4140.1.S1_at	6.5	7.5	5.1	5.2	4.6	4.8	3.78E-05	0.97
<b>LesAffx.23546.1.S1_at</b>	6.4	3.1	3.4	3.3	3.6	3.6	1.24E-07	0.63
Les.5021.1.S1_at	9.8	8.8	7.7	7.6	7.8	8.2	1.69E-05	0.87
Les.3642.1.S1_at	7.5	8	4	4.9	4.5	4.4	0.000117	0.56
<b>LesAffx.56785.1.S1_at</b>	6.1	6.6	4.7	4.6	4.6	4.7	5.18E-05	0.67
Les.2767.1.S1_at	12.3	9.9	7.6	7.7	6.9	7.8	0.000176	0.83
Les.3492.1.S1_at	9.2	9.6	7.6	7.7	8	8	0.000414	0.89
Les.3122.2.A1_at	6.1	6.6	7.3	8	8.6	8.2	0.000569	0.98
Les.2832.1.S1_at	6.9	7.4	7.9	7.8	9.1	8.7	0.001232	0.96
Les.3766.1.S1_at	11.5	12.2	8.1	9.1	10.5	9.7	0.0017	0.98
LesAffx.70635.1.S1_at	9.2	9.3	6.1	7	6.6	7.2	0.003298	0.85
<b>Les.97.1.S1_at</b>	6.9	6.3	5.6	5.3	5.7	5.8	0.003809	0.54
Les.3486.1.S1_at	10.8	10.7	8.3	9	8.8	9.1	0.003948	0.84
LesAffx.58308.1.S1_at	12	11.8	7.8	9.2	8.2	9	0.003929	0.8
Les.3330.2.S1_at	5.3	5.7	6	6.3	6.5	7.6	0.00633	0.94
<b>Les.5694.1.S1_at</b>	12.7	9	6	5.9	6.8	7.8	0.010221	0.68
<b>Les.5168.1.S1_at</b>	11.9	7.5	6.8	5.7	6.5	7.6	0.012949	0.58
Les.5024.1.S1_at	5.6	6.3	6.9	7	7.5	7.7	0.035691	0.76

<b>(C)</b>		
Affymetrix Probe Set ID	NCBI accession	Possible function, based on Blast analysis
Les.4140.1.S1_at	AY192367.1	Ethylene-responsive transcription factor 1
<b>LesAffx.23546.1.S1_at</b>	BG126449	High affinity calcium antiporter CAX1
Les.5021.1.S1_at	BT013123.1	Selenium-binding family protein

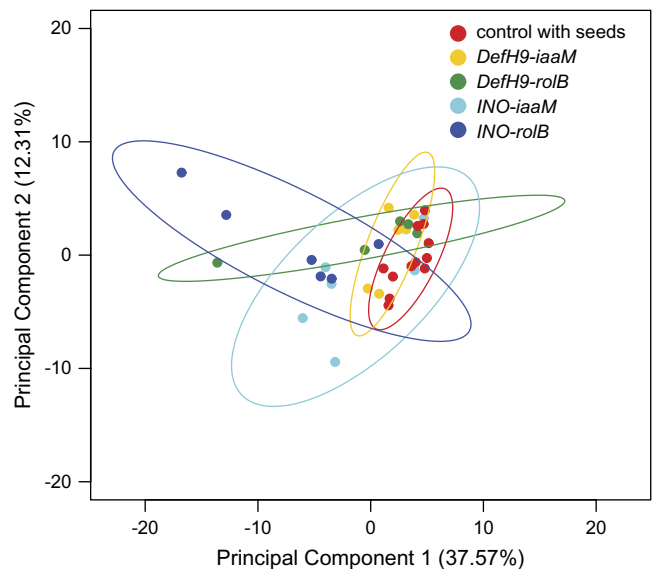
Table 5. Continued

(C) Affymetrix Probe Set ID	NCBI accession	Possible function, based on Blast analysis
Les.3642.1.S1_at	U17972.1	ACC synthase
<b>LesAffx.56785.1.S1_at</b>	BE458971	Sugar transporter
Les.2767.1.S1_at	U18678.1	Isocitrate lyase
Les.3492.1.S1_at	AY013256.1	Phospholipase PLDb2
Les.3122.2.A1_at	S66607.1	Pectinesterase-1 precursor
Les.2832.1.S1_at	CN384480	Peroxidase precursor
Les.3766.1.S1_at	U77719.1	Ethylene-responsive late embryogenesis-like protein
LesAffx.70635.1.S1_at	BI421189	Wound-responsive AP2 like factor 1
<b>Les.97.1.S1_at</b>	BT013913.1	L-Lactate dehydrogenase
Les.3486.1.S1_at	AF416289.1	Auxin-regulated protein
LesAffx.58308.1.S1_at	BG129227	C-repeat-binding protein 4
Les.3330.2.S1_at	BE458823	Fasciclin-like arabinogalactan protein FLA2
<b>Les.5694.1.S1_at</b>	BT014398.1	Short-chain dehydrogenase reductase
<b>Les.5168.1.S1_at</b>	BT013421.1	Putative vicilin
Les.5024.1.S1_at	BT013126.1	MADS-box transcription factor FBP29

reach the breaker stage. This stage is physiologically very active, crucial for the ripening process, and important for the development of fruit quality phenotypes. Among 400 compounds analysed, only 16 showed significant differences between transgenic and wild-type fruits (Table 6). Three of 19 amino acids showed significant differences (serine,  $\beta$ -alanine, and asparagine). INO-rolB-transformed fruits had higher concentrations of these three amino acids than other seedless and seeded fruits. Six of the 18 acids determined revealed significant differences among different fruits. INO-rolB fruits had significantly more glutamate, malate, fumarate, and ascorbate than the other transgenic and seeded wild-type fruits. Among fatty acids, DefH9-iaaM and rolB-transformed fruits had significantly more stearic acid and palmitic acid than seeded wild-type fruits. Linoleic acid was also significantly higher in all transgenic fruits than in seeded wild-type fruits.

Among other metabolites, rolB fruits had more oxoproline and ethanolamine and INO-rolB fruits had more putrescine than seeded wild-type fruits. There were no significant differences in sugars (sucrose, glucose, fructose, or sorbitol) among transgenic and wild-type fruits. Despite the differences in gene expression among transgenic and control fruit, PCA analysis of 400 metabolites showed that the overall metabolomic analysis did not distinguish transgenic fruit from untransformed controls (Fig. 8). Analysis was performed in fruits at a breaker stage and it would be interesting to determine what occurs also in the ripe fruits.

Since only 16 metabolites showed significant differences between transgenic and wild-type fruits, the fundamental metabolism of the fruit seemed to be mostly unchanged. However, some important metabolites were higher in parthenocarpic than in wild-type fruits, especially in INO-rolB fruits, which had the most variability among biological replicates. Although all fruits were harvested at the breaker stage, such biological variability



**Fig. 8.** Principal component analysis of the relative abundance of significantly regulated metabolites obtained from a profile of 400 metabolites sampled for in control and transgenic tomato fruit. The dots represent the biological replicates of the different lines and ellipses define the 95% confidence limits of the metabolite data.

was expected due to unavoidable small differences in fruit developmental stage. However, it is also possible that these metabolite differences were due to ovule-driven expression of *rolB* regulating *rolB*-specific fruit metabolic pathways.

Fatty acids were significantly higher in DefH9-iaaM and rolB-transformed fruits than in seeded wild-type fruits. Linoleic acid was also significantly higher in all transgenic fruits than in seeded wild-type ones. These data are coincident with differences observed in transcripts related

**Table 6.** Relative amounts of metabolites with significant differences among seedless fruits transformed with four different constructs and seeded wild-type fruits

Metabolites were divided in functional categories. Mean values are reported as peak area determined by a Pegasus III TOF mass spectrometer. Differences in letters for the same row for each metabolite indicated significant differences between treatments using ANOVA univariate ( $P=0.05$ ).

Functional category	Seeded wild type	INO-iaaM	DefH9-iaaM	INO-rolB	DefH9-rolB
<b>Amino acids</b>					
Serine	3930.6 a	20 349.5 a,b	37623.0 a	54 467.5 c	8959.2 a,b
$\beta$ -Alanine	488.0 a	5006.8 a,b	1555.7 a	18 304.2 c	6522.0 a,b
Asparagine	9306.4 a	33 263.3 a	17 305.5 a	107 133.7 b	47 176.0 a,b
Glutamate	9549.2a	25 995.0 a	13 940.0 a	68 592.8 b	18 695.6 a
<b>Fatty acids</b>					
Linoleic acid	903.4 a	1767.2 b	2109.5 b	2204.0 b	2151.0 b
Palmitic acid	6286.6 a	12 087.7 a,b	17 665.8 b	17 531.5 b	15 484.8 b
Stearic acid	1959.9 a	3123.2 a,b	4200.3 b	3872.5 b	3672.6 b
<b>Other acids</b>					
Maleic acid	11798.6 a	195 041.2 a	45 742.6 a	402 413.6 b	40 503.8 a
Fumaric acid	684.7 a	8142.4 a	2517.7 a	28 979.0 b	2072.7 a
Aconitic acid	472.7 a	3031.4 a,b	1245.0 a,b	5884.3 b	366.0 a
Succinic acid	2164.8 a	8746.5 b	4019.8 a,b	77 24.0 a,b	3070.0 a,b
Ascorbic acid	654.7 a	976.2 a	761.7 a	3485.4 b	–
<b>Other compounds</b>					
Oxoproline	11 833.7 a	140 969.0 a,b	64 641.5 a,b	348 373.40 c	195 140.0 b,c
Ethanolamine	7212.0 a	9499.4 a,b	11 658.0 b	15 232.67 c	14 706.2 c
Putrescine	9353.7 a	57 317.5 a,b	24 391.3 a,b	70 000.17 c	13 695.0 a
GABA	125 835.2 a	308 213.6 a,b	105439.0 a	453 237.00 b	223 787.2 a

to lipid metabolism. In *Arabidopsis*, auxins and cytokinins induce *FAD3*, a desaturase gene that alters fatty acid composition (Matsuda *et al.*, 2001). Several genes involved in auxin metabolism were differentially regulated in our transgenic seedless fruits than in wild-type fruits: some were down-regulated and some up-regulated. Although Yamamoto (1994) reported that fatty acid desaturases are auxin regulated in mung bean, auxin regulation of fatty acid biosynthesis is not fully understood.

### Conclusions

This study showed that the *INO* promoter from *Arabidopsis* fused with the *iaaM* or *rolB* gene effectively induced parthenocarpy in tomato. At both transcriptomic and metabolomic levels, changes were detected between transgenic parthenocarpic and wild-type fruits. Significant differences were observed in gene expression profiles and in several differentially regulated genes, such as those involved in the cell wall, hormone metabolism and response (auxin in particular), and metabolism of sugars and lipids. Interesting results such as the up-regulation of LTPs in transgenic seedless fruits open up the possibility of investigating their roles in response to biotic stresses. The down-regulation of several ethylene- and IAA- associated genes in transgenic parthenocarpic fruits is also intriguing for its possible effects on fruit shelf life and softening. Only 16 of 400 metabolites analysed at the breaker stage showed significant differences between transgenic and wild-type fruits. The overall metabolomic analysis performed at a breaker stage did not distinguish transgenic fruit from untransformed

controls, implying that fruit metabolism remained essentially unchanged.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE14358 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14358>).

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