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Methodology article

Optimisation of transgene action at the post-transcriptional level: high quality parthenocarpic fruits in industrial tomatoes

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Abstract

Background: Genetic engineering of parthenocarpy confers to horticultural plants the ability to produce fruits under environmental conditions that curtail fruit productivity and quality. The *DefH9-iaaM* transgene, whose predicted action is to confer auxin synthesis specifically in the placenta, ovules and derived tissues, has been shown to confer parthenocarpy to several plant species (tobacco, eggplant, tomato) and varieties.

Results: UC82 tomato plants, a typical cultivar used by the processing industry, transgenic for the *DefH9-iaaM* gene produce parthenocarpic fruits that are malformed. UC82 plants transgenic for the *DefH9-RI-iaaM*, a *DefH9-iaaM* derivative gene modified in its 5'ULR by replacing 53 nucleotides immediately upstream of the AUG initiation codon with an 87 nucleotides-long sequence derived from the *rolA* intron sequence, produce parthenocarpic fruits of high quality. In an *in vitro* translation system, the *iaaM* mRNA, modified in its 5'ULR is translated 3–4 times less efficiently than the original transcript. An optimal expressivity of parthenocarpy correlates with a reduced transgene mRNA steady state level in *DefH9-RI-iaaM* flower buds in comparison to *DefH9-iaaM* flower buds. Consistent with the known function of the *iaaM* gene, flower buds transgenic for the *DefH9-RI-iaaM* gene contain ten times more IAA than control untransformed flower buds, but five times less than *DefH9-iaaM* flower buds.

Conclusions:: By using an auxin biosynthesis transgene downregulated at the post-transcriptional level, an optimal expressivity of parthenocarpy has been achieved in a genetic background not suitable for the original transgene. Thus, the method allows the generation of a wider range of expressivity of the desired trait in transgenic plants.

Background

Parthenocarpy, the development of the fruit in the absence of pollination and/or fertilization, is advantageous for horticultural plants grown for the value of their fruits. The trait is advantageous for the consumer, the producer and the food industry (for review, see [1,2]). The main advantage for fruit productivity is that fruit set and growth is not inhibited by environmental conditions adverse for

pollination and/or fertilization. In tomato, for example, pollination does not occur when either the night temperature is lower than 13°C [3] or when the day temperature is higher than 38°C for 5 hours [4,5]. Thus, parthenocarpy ensures yield stability. Moreover, parthenocarpic fruits are seedless. The absence of seeds improves the quality of the fruit in many species, because seeds are usually hard to taste and to digest, and often their presence is associated with bitter substances, e.g. eggplant [6]. Parthenocarpy can also be used to have an earlier harvest (see [2]) or a late ripening of the fruit [7,1].

Parthenocarpic cultivars exist in many species. However, they have not been widely used in horticulture because high yield and good fruit quality have been seldom combined with parthenocarpy [1]. To fully exploit the advantages offered by parthenocarpy, several methods to genetically engineer parthenocarpic plants have been developed (for review, see [1,2]). Using the *DefH9-iaaM* gene [8], parthenocarpy has been, so far, conferred to tobacco, eggplant [9], tomato [10], strawberry, raspberry, melon and chicory (our unpublished results).

The productivity of parthenocarpic eggplant and tomato transgenic for the *DefH9-iaaM* gene, tested in different genetic backgrounds and under different conditions of cultivation, has shown a drastic increase in fruit yield [11,12]. Thus, the *DefH9-iaaM* gene has allowed for the combinantion of parthenocarpy with high yield and high fruit quality in eggplant and in tomato lines grown for the production of fruits for the fresh market.

Tomato is the most important vegetable crop plant. Its worldwide production is approximately 97 million tons [13]. Tomato fruits are consumed either fresh or processed. Processing tomatoes account for most of the tomato production. To meet the different needs for fresh and processed tomato markets, breeders have selected lines and/or cultivars optimised for either type of production. Fresh market tomato flowers, during out of season cultivation, are treated with auxinic phytohormones [14]. Phytohormonal sprays cause parthenocarpic fruit development and production under environmental conditions adverse for fruit set and growth [14]. However, either a higher sensitivity to auxins or an excess of exogenous phytohormones causes malformations of the tomato fruit [3]. Consequently, breeding programs for fresh market tomatoes have usually screened tomato lines for an optimal response of the flowers to phytohormonal sprays.

Industrial tomatoes are cultivated in open field, and consequently their cultivars have not been selected for an adequate response to exogenous auxin treatment, which is a common practice only in greenhouse cultivation. So, flowers from different tomato lines and/or cultivars might differ in their response both to an exogenous phytohormonal treatment and to the action of the *DefH9-iaaM* gene, whose predicted action is to synthesise auxinic phytohormones in the placenta, ovules and tissues derived therefrom [8]. Consistent with these considerations, the *DefH9-iaaM* gene, when introduced in the typical industrial tomato cultivar UC82, caused parthenocarpic development of the fruit, but the tomato fruits were misshapen (see results section). The malformations, i.e. umbonated fruits with an empty cavity, are very similar to those caused either by an excess of exogenous auxin or by a higher sensitivity of the tomato flowers to the hormonal treatment.

Parthenocarpy is a valuable trait for industrial tomatoes also because parthenocarpic tomato fruits can have a higher percentage of soluble solids [15], and parthenocarpy may allow to improve yield and flavour of paste and to reduce the processing costs.

Here, it is described an easy, valid and cost-conscious solution to the problem observed when using the parthenocarpic *DefH9-iaaM* gene in the industrial tomato UC82. The method used represents a genetic tool which allows to optimise, wherever necessary, the parthenocarpic trait and to tailor the expressivity of the trait for the different needs of horticultural production. In general terms, the method allows to modulate transgene expression at the post-transcriptional level.

Results

The DefH9-iaaM gene causes the development of misshapen parthenocarpic fruits in the tomato cultivar UC82

In all 47 independent UC82 tomato plants transgenic for the *DefH9-iaaM* gene analysed, the fruits were parthenocarpic and yet malformed (Fig. 1). The malformations consisted in the formation and/or the abnormal accentuation of the umbone in the apical part of the fruit. Such abnormal prominence has been called the "Pickelhauben" phenotype [17] when the tomato fruit ends with a pronounced picked point (Fig. 1a). Malformations consist also in the formation of "box-shaped" fruits and often in a remarkable accentuation of the ribs (not shown). This malformation gives problems of fitness for presentation and it increases the possibility of breaks and fissures, with consequent exposition of the mesocarp to fungal and bacterial infections. When selfed, none of the 47 independent transgenic events produced seeds.

The transgenic state of four independent transgenic plants analysed shows that the copy number of the *DefH9-iaaM* gene ranged from one copy (plant 3; Fig. 2a, Lane 3) to six copies (plant 1; Fig. 2a, Lane 1). RT-PCR analysis performed with mRNA extracted from flower buds (0,5–1 cm long) shows that the transgene is expressed in all four in-

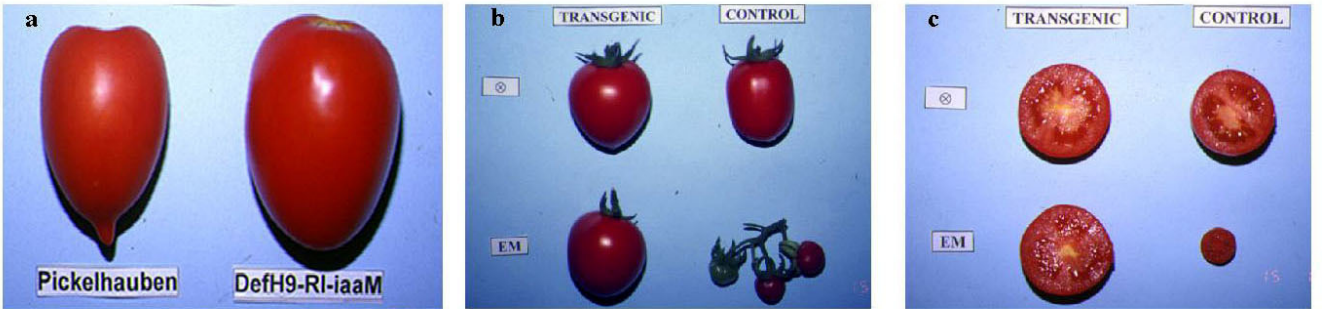

Figure 1
Parthenocarpic fruit development in UC82 tomato fruits. a. Parthenocarpic fruits produced by *DefH9-iaaM* (left) and *DefH9-RI-iaaM* (right) transgenic plants, b. Fruits from pollinated (top) and unpollinated (bottom) flowers from *DefH9-RI-iaaM* transgenic and control untransformed plants, c. Cut fruits from pollinated (top) and unpollinated (bottom) flowers from *DefH9-RI-iaaM* transgenic and control plants. EM = fruits from emasculated and unpollinated ovaries, X = fruits from selfed flowers.

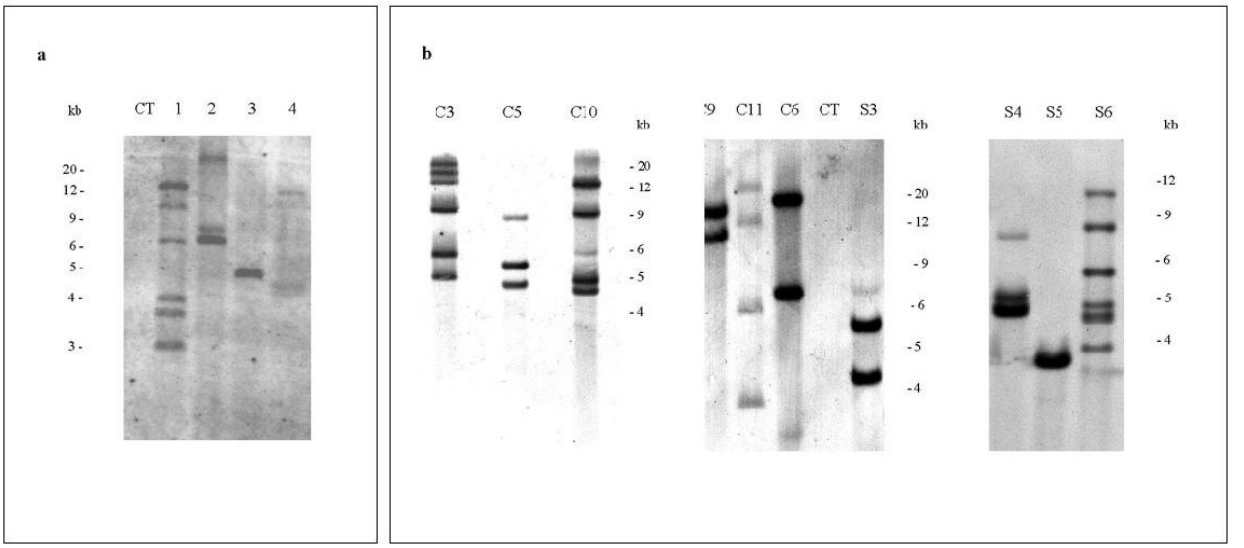

Figure 2
Southern blot analysis of parthenocarpic tomato plants. Genomic DNA digested with *Hina*III from: control UC82 plants (panels a and b, lanes CT), four independent UC82 lines transgenic for *DefH9-iaaM* (panel a, lanes 1,2,3,4) and ten independent lines transgenic for *DefH9-RI-iaaM* (panel b, lanes C3, C5, C6, C9, C10, C11, S3, S4, S5 and S6).

dependent plant clones (Fig. 3b, lanes 1,2,3,4). The steady state level of *DefH9-iaaM* mRNA estimated by competitive (Fig. 3) and real time RT-PCR (not shown) is, on the average, 1×10^{-7} of the total mRNA population, ranging from approximately 3×10^{-7} (transgenic event 1) to 5×10^{-8} (transgenic event 3) in the four independent transgenic events analysed.

Rationale and construction of the DefH9-RI-iaaM gene: a genetic solution to the "Pickelhauben" problem

Since all 47 independent transgenic events of UC82 tomato transformed with the *DefH9-iaaM* gene had severely malformed fruits, the "Pickelhauben" problem can not be easily resolved by exploiting the "so-called" position effect. Position effect defines both the observation that in-

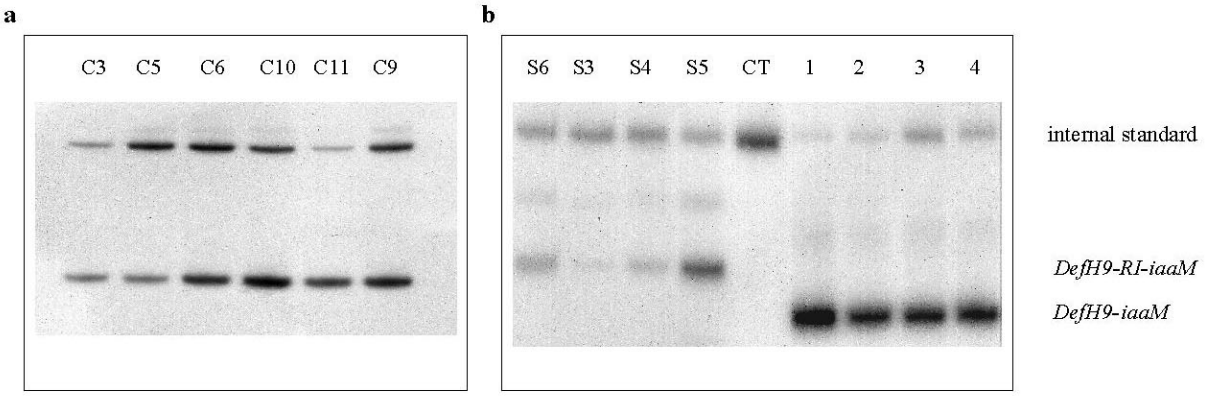

Figure 3
RT-PCR analysis of tomato transgenic floral buds. Analysis was performed with single strand cDNA synthesised from mRNA extracted from young flower buds of UC82 plants transformed with either *DefH9-iaaM* (panel b, lanes 1,2,3,4) or *DefH9-RI-iaaM* (panel a, lanes C3, C5, C6, C9, C10, C11; panel b, S3, S4, S5 and S6) gene. Either 0.05 fg (C3, C5, C6, C9, C10, C11 *DefH9-RI-iaaM* transgenic lines) or 0.2 fg (S3, S4, S5, S6 *DefH9-RI-iaaM and* 1, 2, 3, 4 *DefH9-iaaM* transgenic lines) of a 600 bp *DefH9* cDNA fragment were used as internal standard in the PCR, giving an amplicon of 351 bp. The chimeric fragments are amplicons of 161 and 195 bp respectively, corresponding to the 5' end of the *DefH9-iaaM* and *DefH9-RI-iaaM* mRNAs.

dependent transgenic plants differ for the level of transgene expression, and the interpretation that this variability is due to the random integration of the transgene in different loci of the plant genome. Transgene expression is well known to be affected both by its location in the genome and by neighbouring sequences.

The malformations observed in the tomato fruits of UC82 plants transgenic for the *DefH9-iaaM* gene are similar to those caused by an excess of exogenous auxin [3]. Thus, the working hypothesis to solve the "Pickelhauben" problem has been to develop a genetic tool to reduce the expression and consequently the action of the *DefH9-iaaM* gene. A reduced expression of a transgene could be achieved by either truncating/deleting and/or by mutation(s) in regulatory sequences of the promoter. However, this approach is labour intensive and it might not be suitable for the *DefH9-iaaM* gene considering that its level of expression in transgenic fruits is very low in all species and lines so far investigated [9,10]. Thus, we have chosen to downregulate the level of expression of the *DefH9-iaaM* gene at the post-transcriptional level. For this purpose we have used an 87 bp long DNA sequence derived from the intron of the *rolA* gene of *Agrobacterium rhizogenes* to modify the 5'ULR of the *DefH9-iaaM* gene. The presence of the *rolA* intron in its mRNA reduces *rolA* gene action in *Arabidopsis* and tobacco without affecting *rolA* (pre-)mRNA steady state levels [18,19]. Since such an effect might be restricted to the *rolA* gene context, we have first tested the hypothesis in an *in vitro* translation assay. The 87 bp long DNA sequence corresponding to the *rolA* intron sequence mutated in the splicing sites (GT to GA and AG to AA) has been used to replace the 53 bp immediately before the AUG initiation codon of the *iaaM* gene. The effect of the modified 5'ULR on the translation efficiency of *iaaM* mRNA has been evaluated by using an expression vector with either its original 53 bp or the modified *rolA* intron sequence preceding the AUG start codon of the *DefH9-iaaM* gene (Fig. 4, panel a). In an *in vitro* transcription-translation system, the *DefH9-RI-iaaM* mRNA is translated 3–4 times less efficiently than the *DefH9-iaaM* mRNA (Fig. 4, panel b right, compare lane 4 to lane 5). No difference in RNA levels was detected (Fig. 4, panel b left, compare lane 1 to lane 2). Therefore, the results are not due to a difference in RNA transcription, and one can conclude that the substitution of the original 53 bases immediately upstream the *iaaM* AUG start codon with the 87 bases derived from the *rolA* intron is responsible for the reduced *in vitro* translation efficiency. Thus, the *RI-iaaM* gene was positioned under the control of the *DefH9* promoter, and the *DefH9-RI-iaaM* gene used to transform UC82 tomato plants.

UC82 tomato plants transgenic for the DefH9-RI-iaaM gene produce high quality parthenocarpic fruits

A total of 44 tomato UC82 plants transgenic for the *DefH9-RI-iaaM* gene were raised by using two versions of the gene (*DefH9-RI-iaaM* C and *DefH9-RI-iaaM* S) identi-

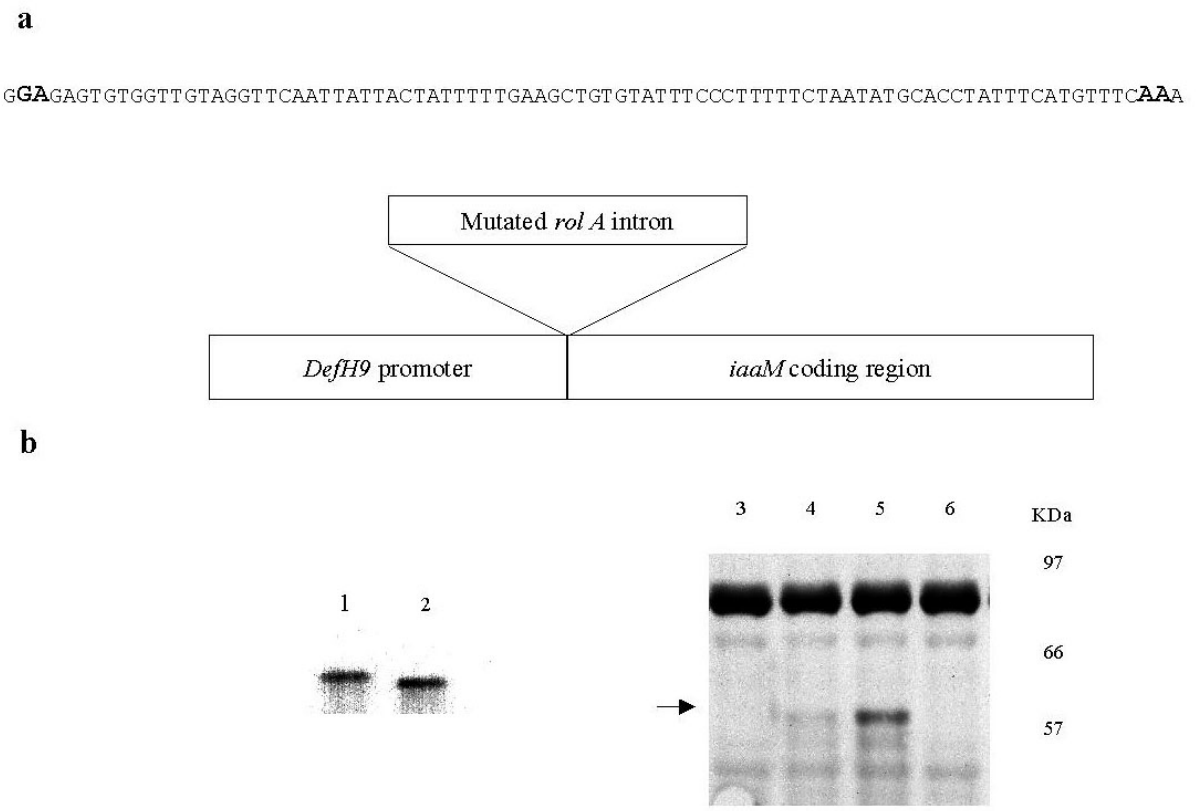

Figure 4
a. Nucleotide sequence of the mutated *rolA* intron and schematic drawing of the *DefH9-RI-iaaM chimeric* gene, derived from the *DefH9-iaaM* gene. The mutated splicing sites (GT and GA changed to GA and AA, respectively) are indicated in bold. In vitro transcription (left panel) and in vitro translation analysis (right panel) of the DNA fragments corresponding to the transcribed regions of *DefH9-RI-iaaM* (lanes 1 and 4) and *DefH9-iaaM* (lanes 2 and 5) genes, subcloned in bluescript vector. Lanes 3 and 6: the *in vitro* translation analysis was performed either without any added DNA or with the vector alone, respectively. The predicted molecular mass of iaaM is 61.8 kDa.

cal except for the termination sequences (i.e. 32 plants with termination sequences of the *rolC* gene and 12 plants with the termination sequence of the *nos* gene). All 44 independent plants showed parthenocarpic fruit development, irrespective of the termination sequence used. In 39 out 44 independent transgenic events, the parthenocarpic fruits were morphologically perfect (Fig. 1a and 1b). Moreover, the malformations observed in the fruits of five *DefH9-RI-iaaM* plants were much less severe than in *DefH9-iaaM* UC82 tomato plants (not shown). When the 39 independent transgenic plants with morphologically perfect fruits were selfed, 8 of them did produce seeds. Although pollinated, the remaining 31 transgenic plants produced seedless fruits (Fig. 1c) Thus, by using the *DefH9-RI-iaaM* gene both facultative and obligatory parthenocarpic plants were obtained.

The transgenic state of ten independent plants (four with the *DefH9-RI-iaaM* gene containing the termination sequence of the *nos* gene, and six with the termination sequence of the *rolC* gene) was analysed by Southern blot analysis (Fig. 2b). The copy number of the transgene ranged from one (Fig. 2b, plant S5; Lane S5) to six copies (Fig 2b, plants C3 and S6; lanes C3 and S6). All ten transgenic plants analysed displayed an optimal parthenocarpic development of the fruit irrespective of transgene copy number.

The fruit set percentage of unpollinated ovaries was on the average 85%, ranging from 73% to 100% in the 18 independent *DefH9-RI-iaaM* transgenic plants analysed (Table 1). The fruit set percentage was lower than the value (97%) obtained from *DefH9-iaaM* transgenic plants, but 7 fold higher than the fruit set percentage (12%) of control untransformed plants. The average weight of fruits ob-

Table 1: Number and percentage of fruits set per number of emasculated flowers, average weight and diameters of fruits obtained from selfed and emasculated flowers of *defh9-iaam, defh9-ri-iaam c* and *defh9-ri-iaam s* transgenic plants and untranformed control. *def h9-ri-iaam c* and *def9-ri-iaam s* transgenic plants were raised by using two versions of the gene identical but for the termination sequences (with termination sequences of the rolc gene and the termination sequence of the nos gene, respectively).

Lines	Fruit set/emasculated flowers		Average weight(g)		Average diameter(cm)			
	(N°)	(%)			Polar		Equatorial	
			Emasc.	Selfed	Emasc.	Selfed	Emasc.	Selfed
DefH9-iaaM								
#1	10/10	100	105.6a	80.5b-d	6.3a	4.9c-m	6.0ab	5.9ab
#2	10/10	100	81.8bc	65.35e-g	5.6b-d	5.7b	5.1cd	4.9c-g
#3	7/8	87	32.24o-r	38.5l-r	3.8pq	4.31-p	3.8k-p	4.1i-o
#4	10/10	100	91.4ab	85.7b	5.5b-e	5.4b-g	6.1a	5.4bc
DefH9-RI-iaaM C.								
#1	10/10	100	34.72n-r	43.4j-p	4.4k-p	5.0b-m	3.4o-q	3.7m-q
#2	13/14	93	45.3j-o	50.7h-m	4.8e-o	5.4b-g	4.2h-n	4.3e-m
#3	8/11	73	25.4r	52.9f-k	3.4qr	4.31-p	3.8l-p	4.7d-j
#4	19/24	79	27.3qr	48.7h-n	3.5q	4.5i-p	3.1q	3.7l-q
#5	14/16	87	27.9qr	55.8e-j	3.4q	4.9d-n	3.5n-q	4.4e-l
#6	13/15	87	65.2e-g	70.17c-e	5.1b-j	5.5b-e	4.7d-i	4.7d-i
#7	8/11	73	45.7i-o	55.5f-j	4.7g-o	5.2b-j	4.2g-n	4.4e-l
#8	9/11	82	37.1m-r	62.5e-h	4.7f-o	5.2b-i	4.5d-k	4.6d-j
#9	11/15	73	53.6f-k	67.0d-f	4.7f-o	5.2b-h	4.3e-l	4.4e-l
# 10	18/20	90	44.5j-q	56.11e-j	4.1qp	4.7f-o	4.3f-m	4.6d-j
#11	8/8	100	52.32g-l	59.8e-i	3.9pq	4.1n-p	4.7d-i	4.8c-h
DefH9-RI-iaaM S								
#1	7/9	78	60.0e-i	91.4ab	5.6bc	6.3a	4.6d-j	5.0c-e
#2	12/15	80	29.8p-r	42.6j-p	4.31-p	4.4j-p	3.4o-q	4.1i-o
#3	10/11	91	88.9b	89.0b	5.5b-e	5.1b-k	5.4bc	4.9c-f
#4	8/8	100	53.5f-k	55.9e-j	4.5h-p	5.4b-c	4.3e-m	4.1i-o
#5	14/16	87	48.1i-n	52.7f-l	4.6h-o	4.9b-l	4.4e-l	4.3f-m
#6	9/10	90	40.7k-q	45.0j-o	3.3qr	4.2m-p	3.4p-q	4.3f-m
#7	8/10	80	44.9j-o	49.6h-m	4.9d-m	5.1b-k	4.0j-p	4.2h-n
Control	6/51	12	10.2s	48.2h-n	2.8r	4.8e-o	2.4r	4.1i-n

For each trait, means followed at least by one common letter are not significantly different according to Duncan's Multiple Range Test ($\alpha = 0.05$)

tained from unpollinated ovaries of *DefH9-RI-iaaM* plants was 46 g, ranging from 25 to 88 g in the independent transgenic events analysed (Table 1), whilst selfed fruits had an average weight of 58 g, ranging from 42 to 91 g in independent transgenic plants. All transgenic fruits obtained from unpollinated ovaries were significantly heavier than those ones from emasculated untransformed control (average fruit weight 10 g). Moreover, in 13 out of 18 independent transgenic plants the weight of the fruits derived from unpollinated ovaries was not statistically not different from the selfed fruits produced by the same plant. The weight of *DefH9-RI-iaaM* fruits was usually lower than the weight of *DefH9-iaaM* fruits, which was on the average 77 g (unpollinated) and 67 g (selfed).

Dry matter content of transgenic *DefH9-RI-iaaM* fruits ranged from 5 to 7 % of fresh weight in comparison to 6% in control fruits (Table 2). The sugar content (Brix°) (Table 2) was, in 15 out of 18 plants analysed, lower than that of fruits from control untransformed plants. However, in three *DefH9-RI-iaaM* transgenic plants, the sugar content, either in selfed or emasculated fruits, was not significantly different from that of selfed control fruits. Fruit acidity (Table 2) was not significantly changed in *DefH9-RI-iaaM* fruits in comparison to control fruits. Different independent transgenic events show also a variability in fruit characteristics (see also fruit diameters, Table 1), which could be used, following field validation, to breed parthenocarpic lines dedicated to specific requirements of production.

The expression of the *DefH9-RI-iaaM* gene was analysed by RT-PCR. All ten transgenic plants analysed showed an amplicon of 195 bp (Fig. 3, panels a and b). The steady

Table 2: soluble solids concentration (brix°) pH and dry matter of fruits obtained from selfed and emasculated flowers of *defH9-iaam, defH9-ri-iaam c* and *defH9-ri-iaam- s* transgenic plants and untransformed control. *defH9-ri-iaam c* and *defH9-ri-iaam s* transgenic plants were raised by using two versions of the gene identical but the termination sequences (with termination sequences of the rolc gene and the termination sequence of the nos gene, respectively).

Lines	Brix°		pH		Dry matter	
	Emasc.	Selfed	Emasc.	Selfed	Emasc.	Selfed
DefH9-iaaM						
#1	4.19e-i	5.50a	4.25b-f	4.05b-i	5.83d-i	6.75bc
#2	3.70i-m	5.52a	4.16b-i	4.22b-h	4.81j-k	6.45b-d
#3	4.08f-j	3.13n	4.05b-i	3.93f-i	4.84jk	4.66k
#4	5.10a-c	5.47a	5.7a	5.8a	6.10c-g	6.21b-f
DefH9-RI-iaaM C						
#1	4.03g-k	4.07g-j	4.33b-e	4.17b-i	nt	nt
#2	2.52o	3.59j-n	4.13b-i	4.11b-i	5.09h-k	6.17b-f
#3	3.57j-n	5.23ab	4.37b-d	4.07b-i	6.44b-d	5.97c-h
#4	5.47a	4.60c-f	4.03b-i	4.17b-i	7.76a	6.99b
#5	3.87h-m	3.93g-l	4.19b-i	4.24b-g	7.91a	5.87c-i
#6	4.05g-k	3.90g-m	4.10b-i	4.07b-i	5.03i-k	6.43b-d
#7	4.13f-i	3.97g-l	4.08b-i	4.06b-i	nt	nt
#8	4.07g-j	4.80b-d	3.83i	3.86hi	nt	nt
#9	5.00a-c	5.47a	4.13b-i	4.03c-i	5.77d-i	6.22b-f
#10	4.70c-e	3.69i-m	3.95f-i	4.07b-i	6.29b-e	5.80d-i
#11	3.40m-n	4.00g-l	4.18b-i	4.41b	5.20g-k	5.07h-k
DefH9-RI-iaaM S						
#1	4.27e-h	4.4d-g	3.97e-i	3.87g-i	5.41e-k	4.74k
#2	3.50l-n	3.40mn	4.04b-i	4.07b-i	4.99i-k	5.07h-k
#3	3.73i-m	3.53k-n	4.24b-g	4.37b-c	5.39e-k	5.57d-k
#4	4.8b-d	3.70i-m	4.05b-i	3.99d-i	5.68d-j	4.96i-k
#5	4.03g-k	3.97g-l	4.15b-i	4.04b-i	5.77d-i	5.80d-i
#6	4.30d-h	3.97g-l	4.07b-i	4.15b-i	5.50e-k	5.67d-j
#7	4.30d-h	4.27e-h	3.90f-i	3.90f-i	f-k	5.33f-k
Control	-	5.49a	-	4.17b-i	-	6.01c-g

For each trait, means followed at least by one common letter are not significantly different according to Duncan's Multiple Range Test ($\alpha = 0.05$). nt, not tested.

state level of the *DefH9-RI-iaaM* mRNA has been estimated by both semicompetitive (Fig. 3) and real time RT PCR analysis (data not shown) to be on the average 1×10^{-8} of the total mRNA population present in young flower buds. In independent transgenic plants it ranged from approximately 4×10^{-8} to 2×10^{-9} (Fig. 3, plants S6 and C5, respectively; panel b, lanes S6, and panel a, C5).

In conclusion, the *DefH9-RI-iaaM* gene causes parthenocarpic development without any fruit malformations in 39 out of 44 independent transgenic UC82 tomato plants. Thus, the Pickelhauben problem has been solved by modifying the 5' ULR of the *DefH9-iaaM* gene.

IAA content of flower buds from parthenocarpic tomato plants

The predicted action of the product of the *iaaM* gene is to cause the synthesis of indoleacetamide (IAM) and consequently to increase the IAA content by a hydrolytic release of IAA from IAM. To test whether parthenocarpy correlates with a higher IAA content, the IAA content of control and flower buds transgenic for the parthenocarpic genes was evaluated. The IAA content was measured after hydrolysis, and consequently the values include both free IAA and IAA generated by hydrolysis from IAM and IAA conjugated forms. The average values for control untransformed plants was about 0.47 nmol/g fresh weight, while in flower buds from parthenocarpic plants transgenic for the *DefH9-RI-iaaM* gene the average value was ten times higher (5.3 nmol/g fresh weight) (Table 3). The IAA content of flower buds from *DefH9-iaaM* parthenocarpic plant evaluated in plant 1, which shows a strong "Pickelhauben" phenotype, was approximately 60 times higher (30 nmol/g fresh weight) than controls and 5–6 times higher than that one found in *DefH9-RI-iaaM* flower buds. Such finding is consistent with the hypothesis that fruit malformations are caused by a too high auxin content.

Table 3: IAA cotent in flower buds from untransformed control and *defH9-ri-iaam* transgenic plants. the IAA amounts has been-measured using D_5-IAA as internal standard. *defH9-ri-iaam c* and *defH9-ri-iaam s* transgenic plants were raised by using two versions of the gene identical but for the termination sequences (with termination sequences of the rolc gene and the termination sequence of the nos gene, respectively).

Lines	IAA nmols/g FW
Untransformed control	
	0.5
	0.6
	0.5
	0.3
DefH9-RI-iaaM C	
#6	3
#10	4
#11	11
DefH9-RI-iaaM S	
#4	4
#6	4

Discussion

Gene expression and action can be regulated at several levels. To properly regulate expression and action of parthenocarpic genes acting by conferring auxin synthesis to the ovary, it has been proposed to modify the regulatory sequences present in the promoter to build derivative promoters with different specificity and/or strength of expression [20]. Alternatively, it has been also suggested to use translational enhancers to improve translation efficiency of the parthenocarpic gene of interest [20]. Moreover, to resolve the problem of an adequate strength of gene expression, plant genetic engineering can also exploit the so-called position effect, i.e. different level of transgene expression in independent transgenic events. In this regard, however, when the *DefH9-iaaM* parhenocarpic gene was introduced in the genetic background of UC82 tomato, all 47 independent transgenic plants analysed produced parthenocarpic fruits, but the fruits were misshapen with the hallmarks of the "Pickelhauben" phenotype [17]. Such malformations are similar to those caused by an excess of exogenous auxin [3], and consequently they have been interpreted as due either to hormonal excess caused by the *DefH9-iaaM* gene or to a higher sensitivity of the fruits to auxins. Hormonal dependent malformations might be faced not only in other tomato cultivars, but also in other plant species and/or varieties (e.g. melon) transgenic for parthenocarpic genes based on auxin synthesising genes.

This limitation of the method based on organ and/or tissue specific synthesis of auxin to confer parthenocarpy has been resolved by developing the *DefH9-RI-iaaM* gene, obtained by replacing the 53 nt present in the 5'ULR of the *DefH9-iaaM* gene just before the AUG start codon with an 87 nt long sequence derived from the *rolA* intron. In an *in vitro* translation assay, the modification of the 5'ULR causes a 3–4 times reduction in the translation efficiency of *iaaM* mRNA.

The reduced gene action of the *DefH9-RI-iaaM* gene correlates with an optimal expressivity of the parthenocarpic trait in 39 out of 44 independent transgenic UC82 tomatoes. The steady state level of *DefH9-RI-iaaM* mRNAs was on the average 1×10^{-8} of the total mRNA population of young flower buds, a value ten times lower than that one observed in flower buds from *DefH9-iaaM* UC82 tomatoes.

The optimal expressivity of the parthenocarpic trait in tomato UC82 was associated with a ten fold increase of IAA content in *DefH9-RI-iaaM* transgenic flower buds in comparison to untransformed controls. This finding was expected and it is in agreement with the function of the product of the *iaaM* gene, a tryptophan mono-oxygenase which converts tryptophan to indoleacetamide. *In planta*, indoleacetamide is then hydrolysed to IAA either by chemical hydrolysis and/or by endogenous hydrolyses. *DefH9-iaaM* flower buds, doomed to produce "Pickelhauben" fruits, had a 60 times higher IAA content than controls.

The working hypothesis that an optimal expressivity of the parthenocarpic trait in the UC82 genetic background can be easily achieved by modifying the 5'ULR of the *DefH9-iaaM* gene has been validated by the results shown. Barring an effect on transcription rate *in planta*, the reduced *in vitro* translation efficiency of *DefH9-RI-iaaM* mRNA in comparison to *DefH9-iaaM* mRNA and the lower steady state level in transgenic flower buds of *DefH9-RI-iaaM* mRNA in comparison to *DefH9-iaaM* mRNA are consistent with a downregulation of gene action at the post-transcriptional level. In eukaryotes, a reduction in translation efficiency, either by modification of the AUG context and/or by introducing step-loop secondary structures in the 5'ULR of mRNAs usually decreases mRNA stability [21].

Previous findings on the inhibitory effect of *rolA* intron on *rolA* gene action in plants [18,19] are confirmed also for the *DefH9-iaaM* gene. However, in the context of the *rolA* gene, the presence of the *rolA* intron did not affect the steady state level of *rolA* pre-mRNA, but reduced only its *in vitro* translation efficiency. Inhibition of translation caused by alterations in the 5'ULR of poorly translated mRNA, such as MFA2 of yeast, has little effect on the decay rate of its mRNA [22]. In this regard, *rolA* mRNA appears

to be poorly translated in a wheat germ translation assay [18,19].

Conclusions

The described modification of the 5' ULR of *DefH9-iaaM* mRNA has been used to solve the so-called "Pickelhauben" problem [17] in an engineered parthenocarpic cultivar (UC82) typically cultivated for the production of processing tomatoes. Thus, the method has allowed to produce high quality parthenocarpic fruits also in industrial tomatoes. Selfed UC82 tomato fruits transgenic for the *DefH9-RI-iaaM* gene are bigger than control fruits and they contain either a reduced number of seeds or no seed at all. Such features bear relevance for the production in open field cultivation of high quality tomato fruits in cultivars/lines used by the processing industry. Other possible modifications of the transgene (e.g. promoter deletions and/or rearrangements), even if successful, are bound to be much more expensive in terms of cost and time.

Interest is usually granted to increase transgene expression in plants. The described method reduces transgene expression and it might solve problems of a too high expressivity of a trait(s) that might arise when using transgenes affecting phytohormone synthesis. Modifications of the 5'ULR, such as that achieved by the introduction of the 87 nt long sequence derived from the *rolA* intron upstream the AUG start codon, of any transgene can be conveniently used to modulate the range of its expression and consequently the expressivity of the desired trait. The use of either a translational enhancer or a translational silencer sequence in the 5'ULR of a transgene is a rather easy and cost conscious strategy which can assist the breeding of cultivated crops better tailored for the different quality requirements needed to meet the requests of consumers, farmers and processing industry.

Materials and methods

Bacterial strains, recombinant plasmid vectors, plant transformation, analysis of parthenocarpic fruit development

The *DefH9-RI-iaaM* gene was derived from *DefH9-iaaM* gene by replacing 53 bp of the *iaaM* 5'ULR with an 87 bp-long DNA sequence containing the 85 bp long *rolA* intron mutated in the two splicing sites (AG and GT changed in AA and GA, respectively). The two chimeric genes were subcloned in the binary vector pPCV002 [16], and introduced into *Agrobacterium tumefaciens* strain C58 GV3101 [16] using standard techniques. Plant transformation was performed according to Ficcadenti et al. [10].

Phenotypic expression of the parthenocarpic trait was evaluated by monitoring fruit setting in emasculated flowers and by measuring the fruit fresh weight and the polar and equatorial diameters of fruits obtained from emasculated and self-pollinated flowers. Dry matter content was measured on fine-chopped fruits kept in an oven at 85°C until (36–48 h later) their weight did not change in two consecutive surveys. pH values were measured for each fruit by dipping the electrode directly into the squeezed flesh and soluble solids (Brix°) by putting a drop of juice in the refractometer. For pH and Brix° each fruit was analysed three times. Flower buds of transgenic and untransformed tomatoes were emasculated before dehiscence of anthers (closed flowers).

Data collected from emasculated and selfed fruits were subjected to a two-way analysis of variance according to a randomised experimental block design with three replications and Duncan's Multiple Range test ($P > 0.05$) was used for mean separations when the ANOVA-F ($P = 0.05$) value was significant for the treatment.

In vitro translation

In vitro translation analysis was performed using a coupled transcription/translation wheat germ system (Promega). DNA fragments corresponding to the transcribed regions of the two chimeric genes, *DefH9-iaaM* and *DefH9-RI-iaaM*, were subcloned in bluescript vector. Linearized plasmids were used as template for coupled transcription/translation. The addition of ε-labelled biotinylated lysine-tRNA complex to the reaction mixture allowed the biotinylation of the translated proteins. After SDS-PAGE and electroblotting, the biotinylated proteins were visualised by binding Streptavidin-Horseradish Peroxidase, followed by chemilumiscent detection. The *in vitro* transcription assay of the two chimeric genes was performed with radioactive ^{32}P-α-CTP. The transcripts were separated on a 1% agarose-formaldehyde gel and transferred to a nylon membrane (Hybond N, Amersham). Signals were detected using Kodak X-AR5 films.

RT-PCR analysis

Flower buds (0.5 cm long) were frozen in liquid nitrogen and poly(A$^+$)RNA was isolated using oligo d(T) Dynabeads (Dynal) following the manufacturer's protocol. The amount was determined spectrophotometrically.

Semiquantitative (competitive) PCR analysis was carried out using as template 10 ng of first strand cDNA primed with an oligonucleotide starting 97 bp downstream the ATG initiation codon of the *iaaM* gene, on mRNA extracted from flower buds. The 5' primer was 5'-CTTTGGAACTCGTGTTGAGCTCTCA-3', and the 3' primer was 5'-GGGTGAATTAAAATGGTCATACAT-3'. The reactions were performed for 38 cycles in the presence of radioactive [^{32}P]-α-dCTP. The amplicons of 161 and 195 bp, corresponding to fragments of the spliced *DefH9-iaaM* and *DefH9-RI-iaaM* mRNAs, respectively, were subcloned and

verified by DNA sequencing (data not shown). Either 0.05 or 0.2 fg of a 600 bp long *DefH9* cDNA fragment was used as template in the PCR to produce, as internal standard, an amplicon of 351 bp. The *DefH9-iaaM* and *DefH9-RI-iaaM* mRNAs were quantified comparing the intensity of their electrophoretic band with that of the internal standard by means of an Instant Imager (Packard). Real-time PCR quantification was performed using Gene Amp5700 system (PE Applied Biosystem) and SYBR Green as DNA binding dye. The expression level was estimated as ratio between each transgene mRNAs and total mRNA used as template in the RT-PCR reaction.

Southern blot analysis

Genomic DNA was extracted from 1 g of frozen leaves using Nucleon PhytoPure system (Amersham Pharmacia) according to the manufacturer's instructions. 10 μg of DNA from transgenic plants were digested with 70 Units of *Hind*III. The DNA was subjected to electrophoresis through a 0.7% agarose gel at 4.5 V cm^{-1} and transferred to a nylon membrane (Hybond N, Amersham). The membrane was hybridised with 100 ng of fluorescein-labelled probe prepared using the Amersham kit "Random prime labelling module". Detection was performed with anti-fluorescein AP conjugate (Amersham) and the chemiluminescent alkaline phosphatase CDP-Star substrate (Amersham) according to the manufacturer's instructions. The membranes were exposed for 1 h using Kodak XAR-5 films.

IAA analysis from flower buds

Flower buds (0.5 cm long) were frozen in liquid nitrogen and ground to a fine powder. Samples were extracted with 80% methanol/water (v/v) containing 1 μM butylate hydroxytoluene (BHT) overnight at 4°C. The extracts, after centrifugation at 1350 xg for 10 min at 4°C, were reduced to the aqueous phase under N_2 flow. Then, 60 nmoles of deuterated IAA (D5-IAA) were added to each extract. To hydrolyse amidic and esteric conjugated of IAA, the extracts were left in 3 M NaOH at 37°C for three hours. Samples were adjusted to pH 9 by adding 2 M HCl, then 2 ml of ethylacetate were added and the extraction was performed for 30 min with stirring. The aqueous phases were extracted and were partitioned against ethylacetate and then were spiked with 2 M HCl to give pH 2,5. 2 ml of diethylether were added in each acidified extract and the samples were left to extract for 30 min with stirring. This operation was done twice and the ether phases were combined and evaporated to dryness with a N_2 flow.

The dry samples were dissolved into 100 μl of acetonitrile (CH_3CN) and then transferred in vials to be derivatised. 100 μl of N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) were added to each sample and the reaction of derivatisation was performed for 30 min 50°C. At the end of the reaction the mixtures were dried with a N_2 flow.

The dry samples were dissolved in 20 μl of hexane and 1 μl was injected in a capillary chromatografic column (J e W Scientific DB-5, 30 m long, 0.25 mm ID, 0.25 μm film thickness). Chromatography was performed using helium as carrier gas with flow rate of 1 ml/min. The analytical conditions were: injector temperature 250°C, temperature rate begins with 60°C for 3 min, continues arriving at 130°C with a slope of 30°C/min, then goes to 235°C with a slope of 7°C/min and finally reaches 280°C with a slope of 30°C/min.

Electron impact spectra were obtained from a quadrupolar mass spectrometer TRIO 2000 (Micromass, Manchester, UK). The spectra were acquired in a mass range included between 40 and 500 uma, with a scan time of 0.6 sec and an interscan time of 0.08 sec using Mass Lynx software for data acquisition and elaboration. To have a relative quantitative analysis, peaks with spectra containing ions at m/e 202, 319 and 207, 324 were identified. The first two values were monitored for IAA (respectively they are the base peak and the molecular ion) and the last two for D5-IAA: by integrating their peak areas and calculating the ratio (202+319)/(207+324) we obtained the ratio IAA/D5-IAA and so the relative concentration of IAA in the samples. The ratio obtained from the wild type untransformed plant has been compared with that obtained from the transgenic plants.

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