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Research article

The *defH9-iaaM* auxin-synthesizing gene increases plant fecundity and fruit production in strawberry and raspberry

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Abstract

Background: The *DefH9-iaaM* gene fusion which is expressed specifically in placenta/ovules and promotes auxin-synthesis confers parthenocarpic fruit development to eggplant, tomato and tobacco. Transgenic *DefH9-iaaM* eggplants and tomatoes show increased fruit production due mainly to an improved fruit set. However, the weight of the fruits is also frequently increased.

Results: *DefH9-iaaM* strawberry and raspberry plants grown under standard cultivation conditions show a significant increase in fruit number and size and fruit yield. In all three *Rosaceae* species tested, *Fragaria vesca*, *Fragaria x ananassa* and *Rubus idaeus*, *DefH9-iaaM* plants have an increased number of flowers per inflorescence and an increased number of inflorescences per plant. This results in an increased number of fruits per plant. Moreover, the weight and size of transgenic fruits was also increased. The increase in fruit yield was approximately 180% in cultivated strawberry, 140% in wild strawberry, and 100% in raspberry. The *DefH9-iaaM* gene is expressed in the flower buds of all three species. The total IAA (auxin) content of young flower buds of strawberry and raspberry expressing the *DefH9-iaaM* gene is increased in comparison to untransformed flower buds. The *DefH9-iaaM* gene promotes parthenocarpy in emasculated flowers of both strawberry and raspberry.

Conclusions: The *DefH9-iaaM* gene is expressed and biologically active in *Rosaceae*. The *DefH9-iaaM* gene can be used, under cultivation conditions that allow pollination and fertilization, to increase fruit productivity significantly in *Rosaceae* species. The finding that the *DefH9-iaaM* auxin-synthesizing gene increases the number of inflorescences per plant and the number of flowers per inflorescence indicates that auxin plays a role in plant fecundity in these three perennial *Rosaceae* species.

Background

Flowering and fruiting are developmental processes of both heuristic and applied interest. In this regard, modification of flowering and fruiting can improve agricultural production in both a quantitative and qualitative manner. We have developed a biotechnological strategy based on

the *DefH9-iaaM* gene construct, which is composed of the regulatory region of the *DefH9* gene from snapdragon and the *iaaM* coding region from *Pseudomonas syringae* pv *savastanoi*. In horticultural plants grown for the value of their fruits, it has been already shown that: i) the placenta/ovule-specific expression of the *DefH9-iaaM* gene confers parthenocarpic fruit development to eggplant and tomato [1,2]; ii) under the cultivation conditions tested, either protected or open field cultivation, transgenic *DefH9-iaaM* eggplants show a significant increase in fruit production, averaging between 30–35% extra fruit, concomitant with improved fruit quality and a reduction in cultivation costs [3-5]; iii) *DefH9-iaaM* tomato plants, grown under protected cultivation during spring, show a significant increase in fruit productivity ranging between 60% and 250% in the four different lines tested [6]; iv) by using an optimized gene version, namely the *DefH9-RI* (*R*educed by *I*ntron)-*iaaM* gene, high quality fruit development has been achieved also in an industrial tomato cultivar that is hypersensitive to auxin [7] (for a review of patents and methods to induce parthenocarpy, see [6]); v) the increased productivity conferred on eggplant and tomato plants is mainly due to improved fruit set, although the weight of the fruit is also often increased [4,6,7]; vi) consistent with the known function of the *iaaM* gene product, which converts tryptophane to indole-3-acetamide which is then hydrolysed to the auxin indole acetic acid (IAA), *DefH9-iaaM* flower buds have an higher auxin (i.e. total IAA) content than controls [7].

In the present study we have evaluated, under environmental conditions permitting pollination and fertilization, the effects caused by the expression of the *DefH9-iaaM* gene in fruit species belonging to the *Rosaceae*. Both wild and cultivated strawberry and raspberry are fruit-bearing species cultivated for the high quality and value of their fruits. In the present study we have shown that introduction of the *DefH9-iaaM* gene construct causes a significant increase in fruit production which results from an increase in individual fruit weight, an increased number of fruits per inflorescence and an increased number of inflorescences per plant. Fruit production data are based on fruits bearing seeds. Thus, in *Rosaceae*, the increase in fruit production is due neither to parthenocarpic fruit development nor to enhanced fruit set but to increased plant fecundity, and only in minor part to enhanced fruit weight.

Strawberry inflorescences are modified stems emerging from the strawberry crown (i.e. stem) [8]. Each strawberry inflorescence terminates with a primary blossom, typically followed by two secondaries, four tertiaries and, eventually, eight quaternaries [8]. Carpel number ranges from 600 in primary blossoms to 60 in quaternaries. To produce a well-shaped strawberry fruit, at least one-third of the carpels must be fertilized [8]. The growth of the strawberry receptacle, which is what comprises the 'commercial fruit', is controlled primarily by auxin synthesized by the fertilized ovules, the achenes [9]. When the achenes are removed, fruit (i.e. receptacle) growth is inhibited [9]. However, exogenous auxin can replace the achenes in inducing and sustaining growth of the receptacle [10]. Several factors, including poor pollination due to adverse climatic conditions and biotic and/or abiotic injuries to the fertilized ovules, result in the development of malformed strawberry fruits [11]. In this study, we have used both wild strawberry (diploid) and cultivated strawberry (octaploid). The wild strawberry, *Fragaria vesca* cv Alpina W Original, is an ever-bearing plant, i.e. it has an indeterminate flowering habit, whilst the cultivated strawberry (*Fragaria x ananassa*) breeding selection has a determinate flowering habit. Raspberry (*Rubus idaeus*) is another species belonging to *Rosaceae*. This plant has an aggregate fruit composed of multiple drupelets, each one developing from a single ovary. All the drupelets of a raspberry fruit derive from the ovaries of the same flower and adhere to a common receptacle [12].

In plants grown for the value of their fruits, an increase in productivity can be achieved by increasing one or more of the following parameters: fruit weight, number of fruits per inflorescence, and/or number of inflorescences per plant. The present study shows that under standard cultivation conditions, i.e. allowing pollination and fertilization, the *DefH9-iaaM* gene increases fruit productivity in three perennial species (i.e. wild strawberry, cultivated strawberry, raspberry) belonging to the *Rosaceae*. The increase in fruit production results from an increase of all three of the aforementioned parameters (fruit weight, fruit number per inflorescence, and number of inflorescences per plant). The increase in fruit production does not affect the total sugar content of fruit, a parameter related to fruit quality. Moreover, our data indicate a new role for auxin in plant fecundity (i.e. number of flowers per inflorescence and number of inflorescences per plant) in these three related perennial crop species. This work also shows that in all three species analyzed, the *DefH9-iaaM* gene causes parthenocarpic fruit development, a finding that confirms and extends the previous results in Solanaceous crops [1,2,6].

Results

Strawberry and raspberry plants transgenic for the DefH9-iaaM *gene*

The transgenic state of plants transformed with the *DefH9-iaaM* gene was analyzed by Southern blot (Fig. 1). The two transgenic *F. vesca* lines used in this study had either one (line 1) or three (line 2) copies of the *DefH9-iaaM* gene (Fig. 1, panel a, lanes 2 and 3, respectively). The transgenic *F. x ananassa* line used in this work had three copies of the

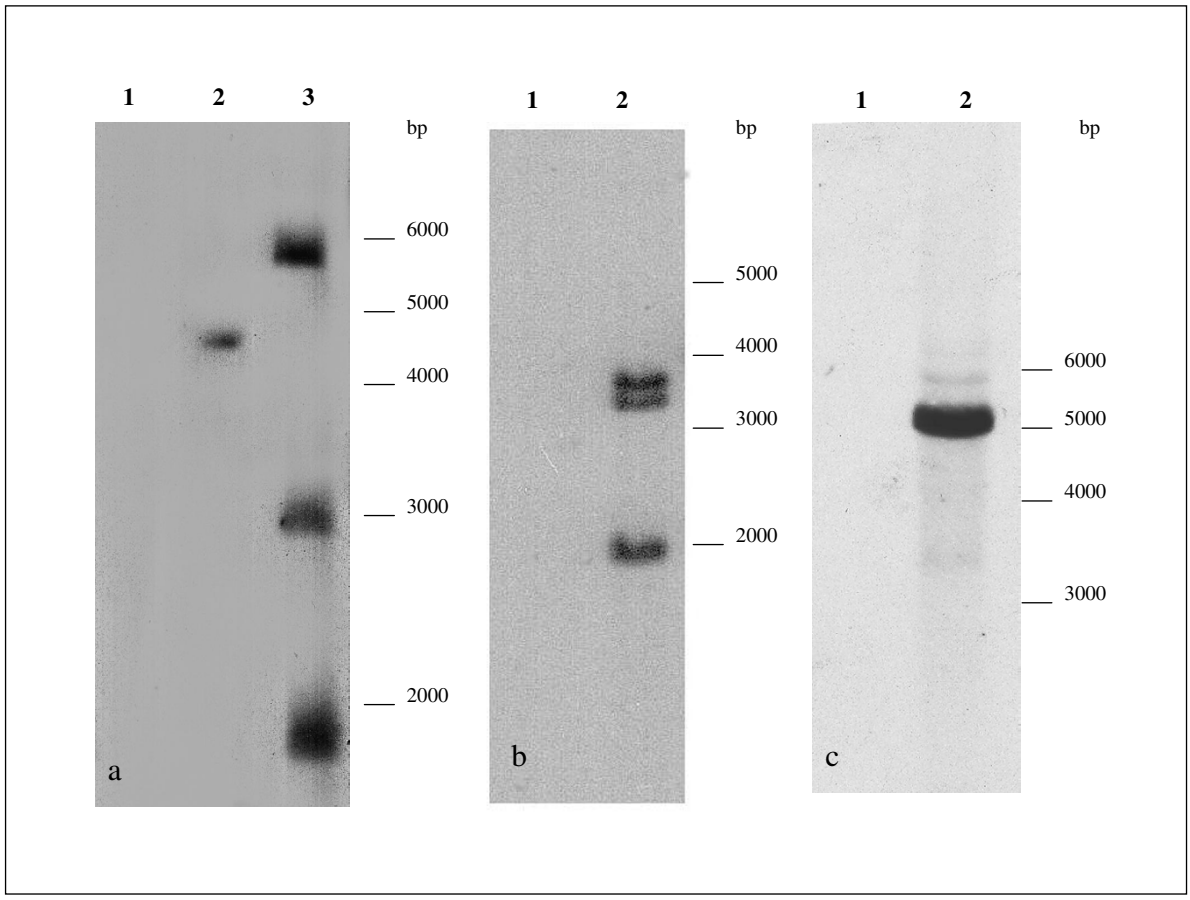

Figure 1
Southern blot analysis of strawberry and raspberry plants transgenic for *DefH9-iaaM*. Genomic DNA digested with *Hind*III from: *F. vesca* control plants (panel **a**, lane1), and transgenic lines 1 and 2 (panel **a**, lanes 2, 3 respectively); *F. x ananassa* control plants (panel **b**, lane1) and transgenic line 1 (panel **b**, lane 2); *R. idaeus* control plants (panel **c**, lane 1) and transgenic line 1 (panel **c**, lane 2). The blots were probed with a 589 bp DNA fragment of the *iaaM* coding region that does not contain the *HindIII* site present in the *iaaM* coding region. The probe was obtained by PCR using the following primers 5'AAACAAGCTTCCCACCACCATCCAG3' and 5'CATGCTCTTTTCACCCGTATTAG3'.

DefH9-iaaM gene (Fig. 1, panel b, lane 2), while the transgenic raspberry line had a single copy of the *DefH9-iaaM* gene (Fig. 1, panel c, lane 2). *DefH9-iaaM* transgenic strawberry plants and their corresponding controls were micropropagated and after acclimatization grown under greenhouse conditions. *DefH9-iaaM* raspberry plants after acclimatization were transferred to the field and grown under open field conditions.

Expression of the DefH9-iaaM *gene in transgenic plants*

Expression of the *DefH9-iaaM* gene was analyzed by RT-PCR (Fig. 2). All transgenic plants showed an amplicon of the expected size (i.e. 149 bp; Fig. 2, panels a and b), and sequence (not shown). The steady state level of *DefH9-iaaM* mRNA was estimated by real time RT-PCR by using mRNA extracted from young flower buds. *F. vesca* line 1 had a *DefH9-iaaM* mRNA steady state level of 0.5×10^{-9} of the total mRNA population present in young flower buds, while *F. vesca* line 2 had a steady state level of 4×10^{-9}. In *DefH9-iaaM F. x ananassa* flower buds, the mRNA steady state level was estimated to be 0.5×10^{-9}. In the transgenic raspberry, the steady state level of *DefH9-iaaM* mRNA, as estimated by RT-PCR, was 2×10^{-9} of the total mRNA population of young flower buds. Thus, the *DefH9-iaaM* gene is expressed in strawberry and raspberry flower buds at a steady state level comparable to that found in tomato [2,7] and eggplant [1].

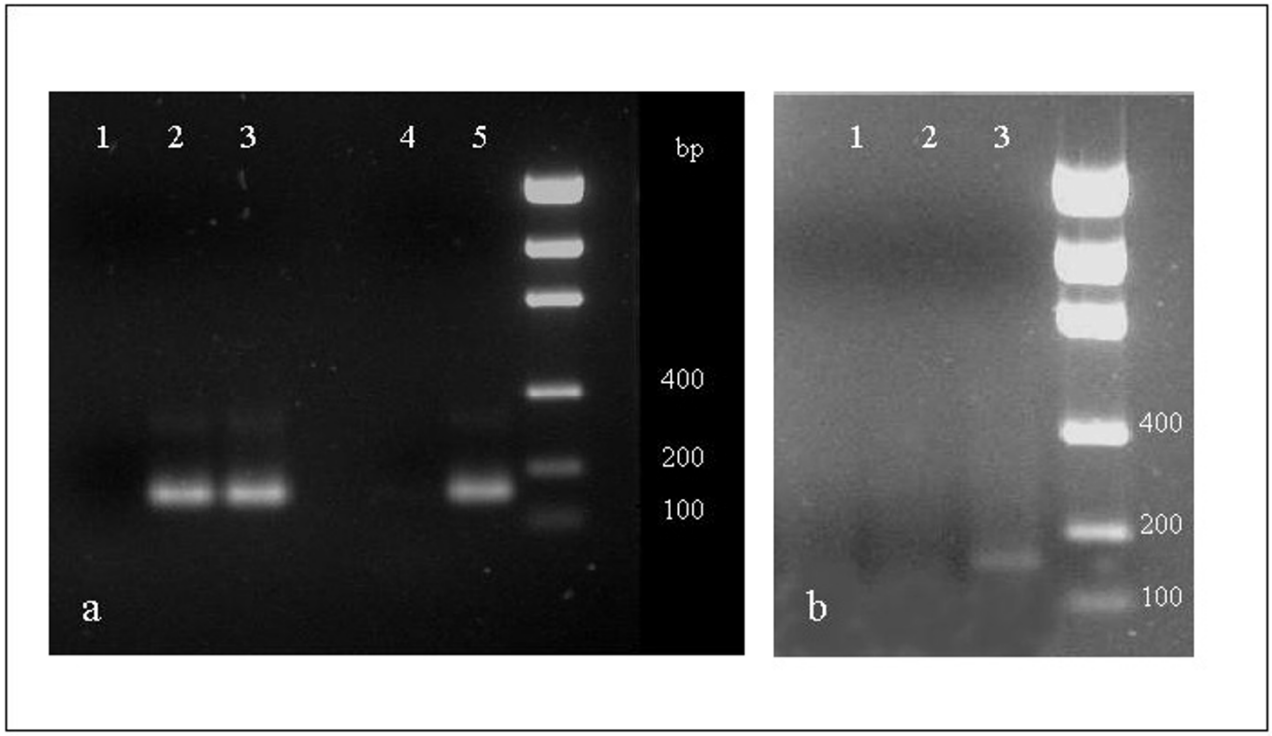

Figure 2
RT-PCR analysis of flower buds from strawberry and raspberry plants transgenic for the *DefH9-iaaM* gene. Analysis was performed with single strand cDNA synthesized from mRNA extracted from young flower buds of: control and transgenic *F. vesca* plants (panel a, lane 1: untrasformed plants; lanes 2 and 3: transgenic lines 1 and 2, respectively); control and transgenic *F. x ananassa* plants (panel b, lane 1: mock reaction without template; lane 2: untransformed plant: lane 3; transgenic line 1); control and transgenic *R. idaeus* (panel a, lane 4: untransformed plants, lane 5: transgenic line 1). The resulting 149 bp amplicon corresponds to the 5'end portion of the *DefH9-iaaM* mRNA.

The DefH9-iaaM *gene is biochemically and biologically active in flower buds of strawberry and raspberry*

The *DefH9-iaaM* gene product is known to cause parthenocarpy in *Solanaceae* by converting tryptophan to indole-3-acetamide which is then hydrolysed to IAA. Thus, to establish whether the *DefH9-iaaM* gene has a similar biological function in *Rosaceae* we have evaluated fruit development in emasculated flowers of the transgenic lines. To confirm that it is biochemically active we have measured the total IAA content of fruit. This value includes both free IAA and IAA produced by the hydrolysis of IAM and conjugated IAA.

In emasculated flowers, the presence of the *DefH9-iaaM* gene in the genome of both *F. vesca* and *F. x ananassa* plants caused parthenocarpic development of the achenes and some swelling of the receptacle (Fig. 3, panels a-d). All emasculated flowers of *DefH9-iaaM F. vesca* plants were able to sustain achene development (Fig. 3, panel a), whilst 80% (24 out of 30) of emasculated flowers in control plants did not develop achenes (Fig. 3, panel b). A similar result was found in *F. x ananassa* (Fig. 3, panels c and d), where 28 out of 30 *DefH9-iaaM* emasculated flowers developed achenes, whereas only 4 out of 30 emasculated control flowers developed achenes. Raspberry plants transgenic for the *DefH9-iaaM* gene showed parthenocarpic fruit development from emasculated flowers (Fig. 3, panels e and f). Twenty-seven out of 30 emasculated flowers from *DefH9-iaaM* raspberry plants developed fruits, whereas only 3 out of 30 emasculated flowers of control plants showed fruit development. Thus, in three species belonging to *Rosaceae*, the *DefH9-iaaM* gene conferred parthenocarpic fruit development to emasculated flowers. However, the commercial fruits obtained from emasculated flowers of both strawberry (i.e. receptacle)

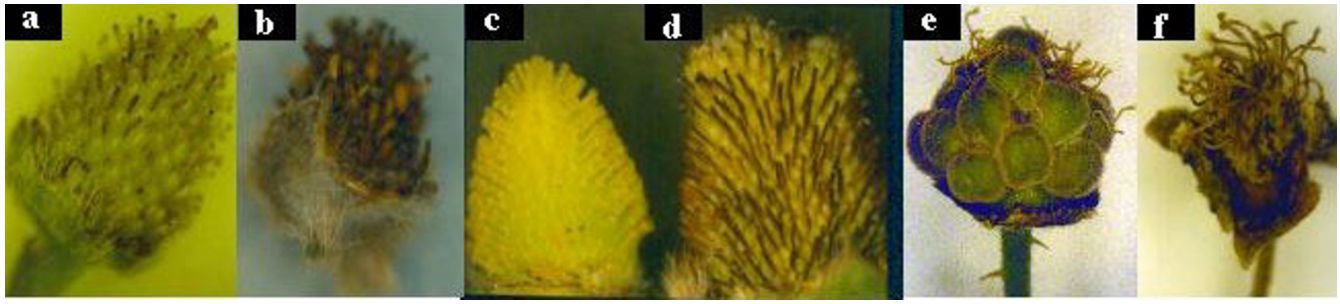

Figure 3
Parthenocarpy in strawberry and raspberry transgenic for the *DefH9-iaaM* gene. Parthenocarpic development of the achene in emasculated flowers of *F. vesca* and *F. x ananassa* transgenic for the *DefH9-iaaM* gene (panel a and c) in comparison with emasculated flowers from control plants (panels b and d). Parthenocarpic development of raspberry drupelets in emasculated flowers of *DefH9-iaaM* raspberry plants (panel e), in comparison with emasculated flowers from control plants (panel f). Pictures were taken, for *F. vesca* 21 days after emasculation, and for *F. x ananassa* and raspberry 30 days after emasculation.

and raspberry did not develop fully. In all three species, the percentage fruit-set of self-pollinated flowers, both transgenic and control, was 100% (data not shown).

The total auxin (IAA) content of young flower buds of strawberry (wild and cultivated) and raspberry plants was analysed by GC-MS. Flower buds transgenic for the *DefH9-iaaM* gene had an IAA content higher than controls. In *F. vesca*, IAA content after hydrolysis was 40.1 and 24.4 picomoles/gram fresh weight in *DefH9-iaaM* (line 2) and control flower buds, respectively. In *F. x ananassa*, *DefH9-iaaM* young flower buds contained 446 picomoles of IAA per gram fresh weight, while flower buds from control untransformed plants contained 301 picomoles/gram of fresh weight. In *R. idaeus*, transgenic flower buds contained 89.4 picomoles/gram fresh weight while untransformed flower buds contained 12.8 picomoles/gram fresh weight. Thus, in *DefH9-iaaM* transgenic flower buds of all three species, the total IAA content was increased in comparison to untransformed controls. From the aforementioned results, we conclude that the *DefH9-iaaM* gene is biologically and biochemically active in the flower buds of plants belonging to the *Rosaceae*.

DefH9-iaaM *strawberry and raspberry fruits have increased weight and size*

Under standard cultivation conditions, i.e. allowing pollination and fertilization, the *DefH9-iaaM* gene increases both weight and size of the receptacle, which is equivalent to the "commercial" strawberry fruit (Fig. 4, panel a; Table 1). The weight of strawberry fruits from *DefH9-iaaM F. vesca* plants was significantly higher and was increased by an average of 24% (18% and 30% increased in line 1 and line 2, respectively) in comparison to control fruits (Table 1). The increase in weight of *DefH9-iaaM F. vesca* fruits correlated with an increase in fruit size (fruit height and diameter; Fig. 4, panel a; Table 1). For cultivated strawberry (*F. x ananassa*), the average fruit weight was increased by 62% in *DefH9-iaaM* plants (Fig. 4, panel b; Table 1). The presence of the *DefH9-iaaM* gene did not modify the total sugar content of transgenic strawberry fruits compared to control fruits (Table 1). *DefH9-iaaM* raspberry fruits were 14% heavier than control fruits (Table 1) and the increase in fruit weight correlated with an increase in fruit size (Fig. 4, panel c; Table 1). *DefH9-iaaM* raspberry fruits had a total sugar content that was not significantly different from that of fruits from control plants (Table 1).

DefH9-iaaM *strawberry and raspberry plants have an increased number of fruits per inflorescence and an increased number of inflorescences per plant*

DefH9-iaaM strawberry plants developed more fruits per inflorescence (Table 2). Inflorescences of *DefH9-iaaM F. vesca* plants developed 45% more fruits per inflorescence, while *DefH9-iaaM F. x ananassa* plants developed 18% more fruits per inflorescence than control plants. *DefH9-iaaM* raspberry inflorescences developed 47% more fruits per inflorescence (fruiting lateral) than controls (Table 2)

DefH9-iaaM strawberry plants also developed a larger number of inflorescences per plant compared to untransformed control plants (Table 2). *DefH9-iaaM F. vesca* plant line 2 had an average of 42% more inflorescences per plant (Table 2). *DefH9-iaaM F. vesca* plant line 1 showed a 27% increase in the number of inflorescences, although the increase observed in line 1 compared to controls was not statistically significant (Table 2). *DefH9-iaaM F. x ananassa* plants developed 49% more

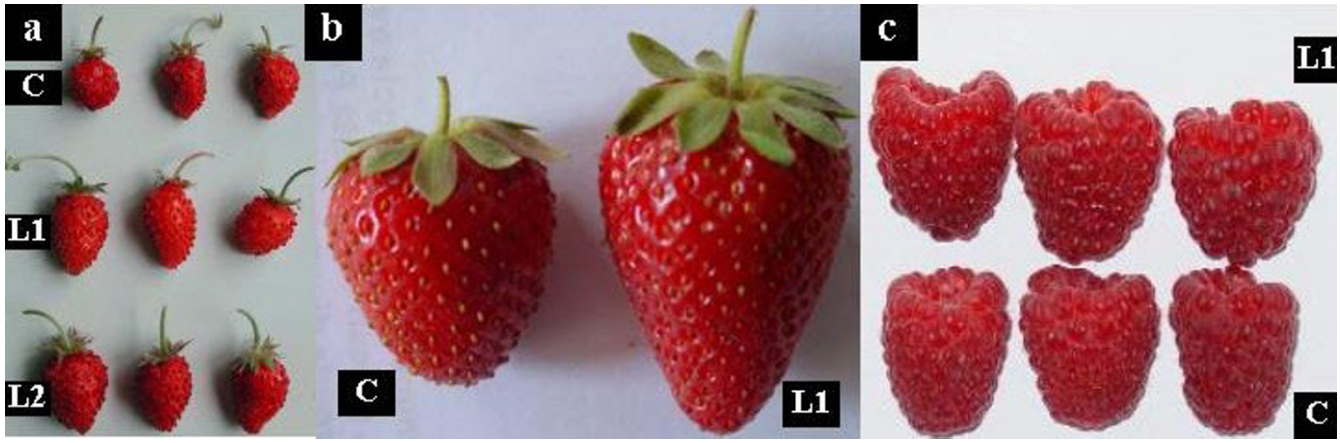

Figure 4
Strawberry and raspberry fruits from control and *DefH9-iaaM* plants. Wild strawberry *F. vesca* fruits (panel a): control plant, fruits in the top row; *DefH9-iaaM* line 1, fruits in the middle row; *DefH9-iaaM* line 2, fruits in the bottom row. Cultivated strawberry *F. x ananassa* (panel b): control plant (left fruit) and *DefH9-iaaM* (right fruit). Raspberry *R. idaeus* fruits (panel c): control plant (the three fruits in the bottom row) and *DefH9-iaaM* (the three fruits in the top row). Annotation: C = untransformed control; L = transgenic line.

Table 1: Fruit parameters: size (average height and average diameter), weight, total sugar content from control and *DefH9-iaaM* transgenic lines of strawberry (*F. vesca* and *F. x ananassa*) cultivated in greenhouse and raspberry (*R. idaeus*) cultivated in open field.

	Fruit size		Fruit weight	Total Sugar
Lines	Height (mm)	Diameter (mm)	g	°Brix
		F. vesca – greenhouse		
Control	11.90 ± 0.09 c	11.69 ± 0.44 c	0.76 ± 0.01 c	11.46 ± 0.54 a
DefH9-iaaM 1	13.14 ± 0.10 b	13.05 ± 0.47 b	0.90 ± 0.01 b	11.72 ± 0.41 a
DefH9-iaaM 2	13.99 ± 0.10 a	13.81 ± 0.47 a	0.99 ± 0.02 a	11.34 ± 0.49 a
		F. x ananassa – greenhouse		
Control	30.77 ± 0.91 b	23.77 ± 0.77 b	7.76 ± 0.69 b	7,87 ± 0.12 a
DefH9-iaaM 1	37.55 ± 1.02 a	29.11 ± 0.69 a	12.55 ± 0.78 a	7,91 ± 0.15 a
		R. idaeus – open field		
Control	19.82 ± 1.12 b	21.80 ± 1.10 b	2.19 ± 0.05 b	11.73 ± 0.18 a
DefH9-iaaM 1	21.89 ± 1.30 a	24.30 ± 1.11 a	2.50 ± 0.07 a	13.50 ± 0.13 a

Values are means ± SE calculated on at least 30 fruits for each production cycle (see methods for details). For each trait, means followed by at least one common letter are not significantly different according to Duncan's Multiple Range Test ($p < 0.01$). SE = Standard Error.

inflorescences than control plants (Table 2). *DefH9-iaaM* raspberry plantshad an average of 22% more inflorescence (fruiting laterals) per cane (Table 2).

The data for *F. vesca* relating to fruit weight and size, number of fruits per inflorescence, and number of inflorescences per plant result from trials over three harvesting seasons (i.e. 2000, 2001 and 2002) under greenhouse cultivation conditions. The *F. x ananassa* data were collected during one harvesting season (2003) under greenhouse cultivation conditions. The raspberry data on fruit weight, fruit size and plant fecundity derive from trials over two

Table 2: Plant fecundity and fruit yield: number of inflorescences per plant/strawberry – cane/raspberry, number of fruits per inflorescence and total plant (strawberry) – cane (raspberry) fruit production from control and *DefH9-iaaM* transgenic lines of strawberry (*F. vesca* and *F. x ananassa*) cultivated in greenhouse and raspberry (*R. idaeus*) cultivated in open field.

Lines	Inflorescence per plant/cane	Fruit per inflorescence	Fruit Production g/plant – cane
		F. vesca – greenhouse	
Control	8.43 ± 0.79 b	2,41 ± 0.07 b	15.55 ± 1.28 c
DefH9-iaaM 1	10.71 ± 0.68 ab	3.44 ± 0.09 a	32.94 ± 1.62 b
DefH9-iaaM 2	11.94 ± 0.65 a	3.53 ± 0.09 a	41.32 ± 2.64 a
		F. x ananassa – greenhouse	
Control	4.12 ± 0.66 b	4.27 ± 0.33 b	135.13 ± 12.48 b
DefH9-iaaM 1	6.15 ± 0.47 a	5.03 ± 0.35 a	383.36 ± 36.56 a
		R. idaeus – open field	
Control	9.43 ± 0.37 b	9.50 ± 0.52 b	195.76 ± 17.29 b
DefH9-iaaM 1	11.50 ± 0.45 a	13.96 ± 0.56 a	408.53 ± 34.94 a

Values are means ± SE collected from 15 plants of *F. vesca*, 15 plants of *F. x ananassa*. For raspberry, four plants from each of the four plots were analysed, and five canes per plant were evaluated (see methods for details). For each trait, means followed by at least one common letter are not significantly different according to Duncan's Multiple Range Test ($p < 0.01$). SE = Standard Error.

harvesting seasons (2002 and 2003). Raspberry plants were cultivated under open field conditions.

DefH9-iaaM *strawberry and raspberry plants show increased fruit productivity*

Fruit production of *F. vesca*, an ever-bearing strawberry, was evaluated under greenhouse cultivation conditions for three consecutive years (i.e. 2000, 2001 and 2002; see methods). Each harvesting period was five weeks long (Table 2). The increase in fruit production of *DefH9-iaaM* wild strawberry averaged 139% (112% in line 1 and 166% in line 2). Fruit production of *F. x ananassa* was evaluated during one year of cultivation under greenhouse conditions. The increase in fruit production of *DefH9-iaaM* cultivated strawberry was 184% (Table 2).

DefH9-iaaM raspberry plants were evaluated under open field cultivation conditions and the data were obtained from two five-week harvesting seasons (i.e. 2002 and 2003). *DefH9-iaaM* raspberry plants showed, on the average, a 108% increase in fruit production (Table 2).

In all three species, the highly significant increase in fruit productivity resulted from an increase in all three parameters relevant to fruit productivity, namely; number of inflorescences per plant, number of fruits per inflorescence, fruit weight (Tables 1 and 2). We wish to stress that environmental and cultivation conditions allowed pollination and fertilization. Fruits bore seeds. Thus, the productivity data are based on fruits that are not parthenocarpic.

Discussion

In the present work, the effects caused on fruit production by the *DefH9-iaaM* gene in three plant species belonging to the *Rosaceae* and bearing fruits of different types have been analyzed. In the two strawberry species tested (*F. vesca* and *F. x ananassa*), the *DefH9-iaaM* gene that is expressed specifically in placenta and ovules [1] promotes parthenocarpic development of the achenes, in emasculated flowers. Similarly, *DefH9-iaaM* raspberry plants show, in emasculated flowers, parthenocarpic development of their fruit. Thus, consistent with results obtained in other species, e.g. eggplant, tomato, tobacco [6], the *DefH9-iaaM* gene also promotes parthenocarpy in *Rosaceae*. However, pathenocarpic fruits (i.e. from emasculated flowers) did not develop fully. As previously observed in tomato [7] and consistent with the biochemical function of the *DefH9-iaaM* gene [13], strawberry and raspberry flower buds transgenic for *DefH9-iaaM* showed increased total auxin (IAA) contents.

In strawberry, the commercial fruit is the receptacle. Thus, despite its biological interest, achene parthenocarpy is not of biotechnological significance in strawberry. Increased production of strawberry fruit (i.e. the receptacles) does have an applied interest. Auxin synthesized by the fertilized ovules is known to sustain the growth of strawberry fruit (receptacles) [9]. Thus, the increased auxin synthesis

of *DefH9-iaaM* flower buds might promote the growth of strawberry fruit. We have shown that, under standard cultivation conditions, i.e. allowing pollination/fertilization, the *DefH9-iaaM* gene improves fruit growth and production in both species of strawberry tested. The weight of *DefH9-iaaM* wild strawberry (*F. vesca*) fruits was increased by the average of 24%, while the weight of *DefH9-iaaM* cultivated strawberry (*F. x ananassa*) fruits increased by 62%. Thus, in strawberry plants cultivated under standard conditions, and consequently under conditions allowing pollination/fertilization, the *DefH9-iaaM* gene improved fruit growth in comparison to control untransformed plants. The increase in weight and size of *DefH9-iaaM* strawberry fruits is consistent with a role of auxin in sustaining fruit growth [10,14]. In strawberry, exogenous auxin is known to replace fertilized ovules in stimulating the growth of the receptacle, but inhibits fruit ripening [15]. We did not observe any effects of the *DefH9-iaaM* auxin-synthesizing gene on strawberry fruit ripening, however.

DefH9-iaaM strawberry plants also developed more inflorescences per plant (34% and 49% more in wild and cultivated strawberry, respectively) and more flowers/fruits per inflorescence (45% and 18% more in wild and cultivated strawberry, respectively). The increased fruit weight, the increased number of fruits per inflorescence, and the increased number of inflorescences per plant resulted in a significant net increase (i.e. more than 100% increase) in strawberry fruit production.

In raspberry, the weight of *DefH9-iaaM* fruits was increased by 14% in comparison to control, non-transgenic fruits. In raspberry the presence of the *DefH9-iaaM* gene also caused an increase in the number of inflorescences per plant (22%) and in the number of flowers/fruits per inflorescence (47%). The overall effect of the *DefH9-iaaM* gene doubled (+108%) the yield of raspberry fruit. The sugar content of the fruit was not altered by the presence of the *DefH9-iaaM* gene in any of the three species tested in the present study. As already observed in other plant species analyzed (e.g. eggplant [1], tomato [2], tobacco [1], grape [16]), the *DefH9-iaaM* gene did not affect the vegetative growth of strawberry and raspberry plants compared to controls. The data obtained with these three perennial plants show that the *DefH9-iaaM* gene can be used, under standard cultivation conditions allowing pollination and fertilization, to improve fruit yield in plants belonging to the *Rosaceae* (e.g. strawberry and raspberry). Moreover, the novel finding that the presence of the *DefH9-iaaM* gene construct causes an increase in plant fecundity (i.e. number of flowers/fruits per inflorescence and number of inflorescences per plant) indicates that auxin has a role in plant fecundity in at least three *Rosaceae*. Since this seems to be a novel role of auxin, it needs to be further analysed in these and other plant species.

Conclusions

The data from strawberry (wild and cultivated) and raspberry plants transgenic for the *DefH9-iaaM* gene show that the *DefH9-iaaM* gene can be used, under standard cultivation conditions, to greatly improve fruit yield in perennial plants belonging to the *Rosaceae*. The fruits produced are not parthenocarpic, they do bear seeds. Consequently, the increased fruit yield is caused by an increased plant fecundity and an enhanced fruit growth. The novel finding that the presence of the *DefH9-iaaM* auxin-synthesizing gene causes an increase in plant fecundity (i.e. increased number of flowers/fruits per inflorescence and increased number of inflorescences per plant) indicates that, at least in these three perennial plants, auxin plays a role in plant fecundity. We wish to stress that an increase in plant fecundity has never been observed in any of the varieties of tomato and eggplant tested to date [6,7]. Thus, experiments using plants with novel genetic backgrounds are in progress to evaluate further the fecundity of *DefH9-iaaM* plants belonging to *Solanaceae* and *Vitaceae* [16].

Methods

Plant material and genetic transformation

In vitro proliferating shoots of the diploid strawberry (*Fragaria vesca*) cultivar Alpina W. Original, of the octaploid strawberry (*Fragaria x ananassa*) breeding selection AN93.231.53, and of the raspberry (*Rubus idaeus*) cultivar 'Ruby' were used for the experiments of genetic transformation. Regeneration protocols have been previously described [17]. The *DefH9-iaaM* gene has been previously described [1]. The *nptII* gene under the control of the nopaline synthase promoter, which is linked in the T-DNA to the *DefH9-iaaM* gene and confers resistance to the antibiotic kanamycin, was used as selectable marker. The *Agrobacterium*-mediated transformation protocol described by James et. al. [18] was used for all three species. After selection *in vitro*, regenerants were isolated and transferred to rooting medium (hormone-free MS medium for strawberry and MS supplemented with 0.5 mg/l IBA for raspberry) supplemented with 50 mg/l of kanamycin. Putative transgenic clones were acclimatized and characterized by Southern blot analysis.

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 each plant were digested with *Hind*III. The DNA was separated by 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 hybridized with 100 ng of fluorescein-labeled probe pre-

pared using the Amersham "Random prime labeling module" kit. 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.

RT-PCR analysis

Flower buds (0.5 cm long) were frozen in liquid nitrogen. Total RNA was extracted by using NucleonPhytopure (Amershan) system, modified by adding Polyclar AT (95 mg/g of fresh tissue) and $Na_2S_2O_5$ (0.4 %) during homogenization, and recovered after LiCl precipitation. PolyA+RNA was isolated from total RNA using oligo d(T) Dynabeads (Dynal) following the manufacturer's protocol. The amount of mRNA extracted was determined spectrophotometrically.

RT-PCR analysis was carried out using as template 30 ng of first strand cDNA primed with an oligonucleotide starting 305 b downstream the AUG initiation codon of the *iaaM* gene, on mRNA extracted from flower buds. The cDNA was first amplified using the 5' primer (5'-TTTC-CGAACAAGACAGGTTATTTTT-3') and the 3' primer (5'-ACTATCGCTACCCGAGGGGTGGGC-3'). The resulting amplicon spans parts of the untranslated leader and coding region of the *DefH9-iaaM* gene. An aliquot of the first PCR was diluted and re-amplified with the following nested primers: 5' primer 5'-CCAAAGAATCGTAATCCG-GGTAGCACG-3' and 3'primer 5'-AATAGCTGCCTAT-GCCTCCCGTCAT-3'. The 149 bp amplicon resulting from the nested PCR reaction was checked by DNA sequencing (data not shown). Real-time PCR was performed by using Gene Amp 5700 system (PE Applied Biosystem). A 600 bp long *DefH9* cDNA fragment was used as standard in the Real-time PCR experiments. The expression level was estimated as ratio between transgene mRNA and total mRNA used as template in the RT-PCR reaction.

IAA analysis

Samples (flower buds) were ground to a fine powder and extracted with 80% methanol/water (v/v) containing 1 μM buthylate hydroxytoluene (Sigma) overnight at 4°C. The extracts were centrifuged for 15 min at 4°C and dried under N_2. 100 nmol of indole propionic acid were added to the acqueous solutions, as internal standard. The hydrolysis of amidic and esteric IAA conjugated were carried out in 3 M NaOH at 37°C for 3 h.

The pH of samples was adjusted to 9 by adding 2 M HCl, and extraction was performed with 2 ml of ethylacetate for 30 min. The aqueous phases were extracted and partitioned against ethylacetate and the pH adjusted to 2.5. The samples were then extracted twice with diethylether for 30 min and dried under N_2. The samples were then dissolved in 100 μl of acetonitrile and 200 μl of N, O-bis.(trimethylsilyl) trifluoroacetamide (BSTFA, Pierce) were added to each sample for 30 min at 50°C. The samples were then dried under N_2, dissolved in 20 μl of hexane. Aliquots (1 μl) were analysed by GC-MS.

The TMS GC-MS analysis was performed on Hewlett Packard 5890 instrument by using a HP-5 (Agilent technologies) fused silica capillary column (30 m, 0.25 mm ID, Helium as carrier gas), with the temperature programme: 70°C for 1 min, 70°C→150°C at 20°C/min, 150°C→200°C at 10°C/min, 200°C→280°C at 30°C/min, 280°C for 15 min. The injection temperature was 280°C. Electron Ionisation (EI) mass spectra were recorded by continuous quadrupole scanning at 70 eV ionisation energy.

Phenotypic analysis

In all three species, *DefH9-iaaM* and control plants were propagated by standard propagation techniques and transferred either to the greenhouse or to open field for the analysis of vegetative and reproductive plant growth. Plants of *F. vesca* were cultivated under greenhouse conditions in single pots (20 cm diameter/18 cm height) and analyzed for three consecutive years (i.e. 2000, 2001 and 2002). The first two production cycles were performed with *F. vesca* plants just acclimatized. The *F. vesca* plants analyzed in the third cycle (i.e. 2002) had been acclimatized the previous year, and consequently the third cycle of production refers to strawberry plants in the second year of growth. To monitor the biological effects caused by the *DefH9iaaM* gene in cultivated strawberry (*F. x ananassa*), one cultivation/production cycle (i.e. 2003) was performed, under greenhouse conditions, using plants vegetatively propagated *in vivo* by runners and grown in single pots (24 cm diameter/22 cm height). In all experiments, 15 strawberry plants were used for each line.

Raspberry plants were analyzed under open field conditions for two consecutive years (i.e. 2002 and 2003). The plants were propagated *in vitro*, acclimatized in the greenhouse in single pots (24 cm diameter/22 cm height) and then transferred in the open field in 2001. Forty plants transgenic for the *DefH9-iaaM* gene and forty plants of control line were arranged in four plots, each one consisting of ten plants. Fruit productivity in two consecutive years (i.e. 2002 and 2003) was evaluated by harvesting the fruits produced during five weeks from canes originating from the same plant pruned in late winter (February) and thinned before blossoming (on average 10 canes per linear meter). Greenhouse and open field experiments were carried out at the Experimental Farm of the Marche Polytechnic University.

To evaluate plant fecundity and fruit yield of *DefH9-iaaM F. vesca* plants, the number of fruits per inflorescence, number of inflorescences per plant and fruit production were recorded during the harvest period corresponding to five weeks of each production cycle (i.e. each year for three years). For cultivated strawberry (*F. x ananassa*), these parameters were recorded during the entire production cycle (one year). In raspberry, for each year of cultivation (i.e. two years), the number of inflorescences per cane, the number of fruits per inflorescence, and fruit production were measured on a total of 160 randomly-chosen canes (5 canes from 4 plants from each plot; i.e. 20 canes per plot every year) for *DefH9-iaaM* and control line. Fruits were harvested for five weeks in order to evaluate fruit production.

In *F. vesca*, the average fruit weight, fruit size parameters (average fruit basal diameter and fruit height) were measured on 30 fruits for each year and line. Total sugar content (°Brix) was measured on three extracts, each one obtained from 10 fruits. The fruits were sampled randomly from the fruits harvested during the second and third week of harvest of every year of cultivation. For *Fragaria x ananassa*, the fruit weight and size data were obtained from 50 fruits collected during the second week of harvest (the whole harvest lasted three weeks). °Brix was evaluated on two extracts, each one obtained from 25 fruits. From each extract, two measurements were made. For raspberry, the fruit weight and size data were obtained from 40 fruits sampled randomly from the fruits harvested during the second and third week of harvest of every year of cultivation. °Brix was evaluated, each year of cultivation, on two extracts each one obtained from 20 fruits. Two measurements were made of each extract.

Data were subjected to one-way ANOVA for means comparison, and significant differences were calculated according to Duncan's Multiple Range Test, $P < 0.01$.

Parthenocarpy was monitored by evaluating fruit set in emasculated flowers from transgenic and control untransformed plants. Flower buds (30 flower buds/line) from transgenic and control plants of both wild and cultivated strawberry were emasculated before dehiscence of anthers (closed flowers) and covered with a small paper bag until achene formation and receptacle development. The same technique was used to evaluate the formation of aggregated drupelets in emasculated raspberry flowers.

Authors' contributions

BM performed and supervised plant transformation, plant propagation and plant evaluation. LL performed plant transformation, plant propagation and plant evaluation. TP performed the molecular analysis of transgenic plants. AS coordinated the project and drafted the manuscript. All authors have read and approved the final manuscript.

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