THE *DefH9-iaaM*-CONTAINING CONSTRUCT EFFICIENTLY INDUCES PARTHENOCARPY IN CUCUMBER [#]

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Abstract: Parthenocarpy (seedless fruits) is a desirable trait that has been achieved in many plant cultivars. We generated parthenocarpic cucumber fruits by introducing the chimeric *DefH9-iaaM* construct into the cucumber genome using an *Agrobacterium tumefaciens*-mediated protocol. The construct consists of the *DefH9* promoter from *Antirrhinum majus* and the *iaaM* coding sequence from *Pseudomonas syringae*. Transgenic plants were obtained from nine independent transformation events: half of these were tetraploid and did not produce seeds following self-pollination, while the remaining half were capable of displaying parthenocarpy in the subsequent reproductive generation. Of the fruits produced by the transgenic lines, 70-90% were parthenocarpic. The segregation of the marker gene in the transgenic T_1 progeny indicated single gene inheritance. The seed set in the transgenic lines and their F_1 hybrids was lower than in the non-transgenic control plants. Some of the methodological details and the practical significance of the results are discussed.

Key words: Fruit set, Cucumber, Ovary-specific promoter, Transgenic parthenocarpy

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INTRODUCTION

Parthenocarpy, the ability to set fruit in the absence of pollination, is an economically valuable trait in a number of horticultural crops. Consumers often prefer seedless fruits for aesthetic reasons, as many such fruits have a more attractive appearance, and for their ease in preparation and consumption. Manufacturers prefer them because they facilitate processing; for example, the processing of parthenocarpic tomatoes is easier because no seeds have to be removed. Additional advantages for the marketing sector are their simplified growth requirements, particularly when the plants are grown in the greenhouse or in an unfavorable climate. Parthenocarpy in the gynoecious cucumber was proposed as a mechanism for overcoming fruit set inhibition [1]. Although this trait was found to be under genetic control, to date there has been no consensus on the number of genes or mode of gene action involved; proposals have ranged from a single recessive gene through incomplete recessive genes to one dominant gene with three additional major additive genes [2, 3]. Despite this lack of knowledge and the difficulty in genetically managing parthenocarpy due to the influence of the genetic background, commercial greenhouse cucumber varieties carrying the parthenocarpy trait have been successfully developed.

Biotechnology offers new possibilities and easier ways of obtaining parthenocarpic varieties than conventional breeding [4, 5]. Rotino *et al.* [4] reported that transgenic tobacco and eggplants containing the *DefH9-iaaM* transgene produce parthenocarpic fruits in the absence of pollination, and that seeds are generated inside the fruit following pollination. Parthenocarpy has also been achieved in transgenic tomato plants carrying the *DefH9-iaaM* construct [6, 7]. The parthenocarpy produced by the introduction of the *DefH9-iaaM* construct is facultative. Carmi *et al.* [8] also obtained parthenocarpic tomato fruits on plants carrying a transgene consisting of the *rol B* coding region fused to the fruit-specific promoter *TPRP-FL*.

Cucumber (*Cucumis sativus* L.) is a popular market garden vegetable that is grown world-wide. The first transgenic cucumber plants were described two decades ago and were obtained using an *Agrobacterium*-mediated system [9, 10] and by direct gene transfer [11]. At present, different marker and reporter genes and various types of transgenes with agronomic potential have been introduced into the cucumber genome [12]. Enhanced biotic resistance was observed following the introduction of the cucumber mosaic virus coat protein (CMV-cp) gene [13, 14], the zucchini green mottle mosaic virus coat protein (ZGMMV-cp) gene [15], and various chitinase genes [16, 17]. The introduction of the *DHN10* gene, coding dehydrin, caused a slight enhancement of tolerance to abiotic stresses [18] and delivering another construct with SK_3-type dehydrine increased frost tolerance in physiological tests [19]. The fruits of transgenic cucumber plants carrying a construct with the thaumatin II coding sequence had an enhanced sweet taste [20], while the introduction of the *mSOD1* gene gave a higher level of superoxide dismutase (SOD), which might be useful as

a functional cosmetic substance [21]. Transgenic cucumber plants immune to CFMMV infection by mechanical and graft inoculation and to root infection following planting in CFMMV-infested soil were obtained after the introduction of the 54-kDa replicase gene [22]

We report here on the successful production of transgenic cucumber plants carrying the chimeric *DefH9-iaaM* gene. We also present and discuss the subsequent impact of this construct on plant regeneration and on the ability of the transgenic plant to produce parthenocarpic fruit in the first reproductive generation.

MATERIALS AND METHODS

Transformation

The *DefH9-iaaM* gene construct [4] was introduced into a highly inbred line of *Cucumis sativus* L. cv. Borszczagowski (line B) using the *Agrobacterium tumefaciens* strain LBA4404/pGA482 [23], as previously described [20]. The plantlets (designated the T_0 generation) that regenerated from the explants successively cultured in kanamycin-containing media (kanamycin-resistant) were transferred to the soil and cultivated under greenhouse conditions from August to November, until seed set. The DNA content was established using flow cytometry according to the procedure previously described [24].

Detection of the transgene in plants

Two pairs of primers specific for the *DefH9* promoter and the 5' end of the *iaaM* coding sequence, 5'-CTTTGGAACTCGTGTTGAGCTCTCA-3' and 5'-GGTGAATTAAAATGGTCATACAT-3', were used for the PCR analysis of the T_0-generation plants and for the PCR-mediated synthesis of the probe used for Southern analysis of T_1-generation plants.

For Southern hybridization, the PCR-amplified DNA fragments were purified using the QIAEX1 kit (Qiagen, Hilden, Germany) and subsequently radio-labeled with alpha-$[^{32}P]$dCTP using the High Prime reagent (Roche Diagnostics, Mannheim, Germany). Labeled probes were purified on Mini QuickSpin DNA columns (Roche).

Cucumber genomic DNA for Southern blot analysis was isolated according to Scott *et al.* [25]. Genomic DNA samples (5 μg each) were digested with *Eco*RI (Roche), separated by electrophoresis in 0.8% agarose gels, transferred onto a Zeta-Probe nylon membrane (Bio-Rad, Hercules, Calif.), and hybridized with the *DefH9-iaaM*-specific probe according to the manufacturer's instructions (Zeta-Probe1; Bio-Rad).

Estimation of parthenocarpy level

Non-transformed plants of the inbred B line comprised the control group, and T_1 plantlets of three transgenic lines were used as the experimental material. The seeds were plated on a solidified growth medium, without antibiotic in the case of the control, and supplemented with 200 mg/l kanamycin in the case of the

transgenics. Thirty seeds were plated for each transgenic line, and the number of kanamycin-resistant and -sensitive seedlings that were regenerated was assessed. Details on the regeneration, selection, and rooting procedures are described in Szwacka *et al.* [20]. Extra light was provided in the greenhouse (a 16/8-h light/dark photoperiod). Four plants in each combination were analyzed from March to mid-April. In order to completely exclude any chance of pollination, we isolated all the female flowers manually by wrapping them in cotton wool. The fruits were left on the plants until they reached a size of 10-12 cm and then removed; the number of dry female flowers was counted at the same time. As line B is monoecious, the percentage of parthenocarpic fruits present on the plants was calculated as the ratio of all of the generated fruits to the total number of female flowers that had been secured against pollination. This calculation is presented as an average for each of the combinations. Between 42 and 80 female flowers were analyzed for each combination, with one exception that contained 28. The data was verified statistically by calculating the standard deviation.

Seed setting after various pollinations
Plants of the three transgenic lines were cultivated in the greenhouse (summer) and either self-pollinated or crossed with a 2gg line, which is a mutant line carrying a recessive femaleness derived from the B line. The latter was used as the non-transgenic control. The number of seeds in five to seven cucumber fruits harvested from two to three plants was compared.

RESULTS

Using the plant transformation procedure of Szwacka *et al.* [20], we obtained nine independent transformation events containing the *DefH9-iaaM* transgene. Of the transgenic plants that developed from these events, 50% were tetraploids (Tab. 1) according to their growth habit, confirmed by flow cytometry analyses (Fig. 1).

Tab. 1. The number of analyzed T_0 cucumber plants grown in the greenhouse, their seed set and growth habit.

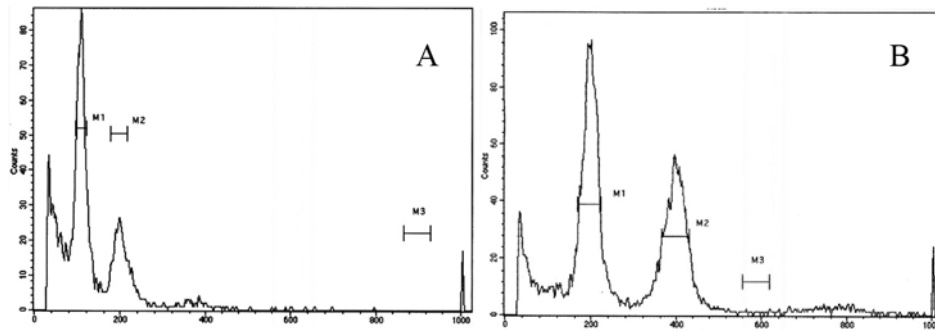| Independent transformants	Number of plants	Mean No. of seeds in fruit	Growth habit
TM1	3	150	diploid
TM2	4	163	diploid
TM3	3	98	diploid
TM4	3	144	diploid
TM5	3	0	tetraploid
TM6	3	0	tetraploid
TM7	2	0	tetraploid
TM8	1	0	tetraploid
TM9	3	0	tetraploid

Fig. 1. Flow cytometry analyses of the regenerated transgenic plants: A - control diploid plant and B - regenerated tetraploid plant.
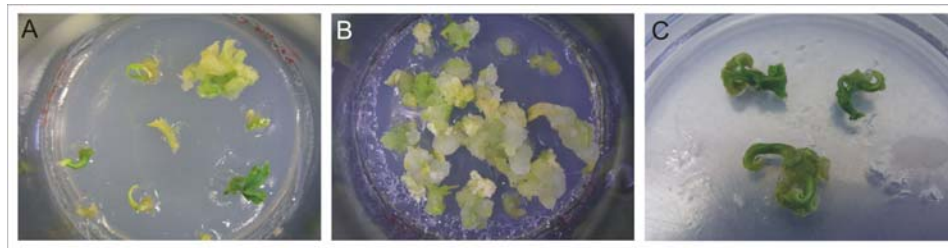

Fig. 2. Three types of abnormal regeneration shown by cucumber explants transformed with the *DefH9-iaaM* construct: A - no shoots or only the development of leaflet structures; B - overgrowth with various types of callus; C - green structures resembling shoots without an apical meristem.
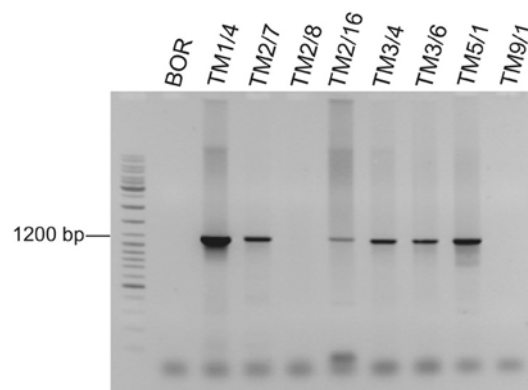

Fig. 3. PCR analysis of the T_0 progeny of cucumber transformants. The DNA size marker standard (Gene Ruler Mix Fermentas, Vilnius, Lithuania) (in base pairs) is given on the left. *BOR* is the control plant – wild-type cucumber cv. Borszczagowski.
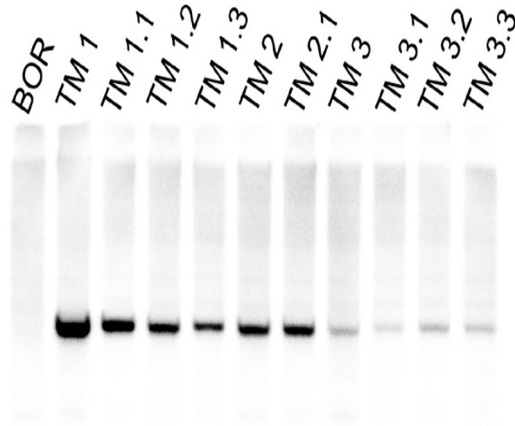
Fig. 4. Southern blot analysis of the control and transgenic cucumber lines evaluated for parthenocarpic fruit development. A specific signal indicating the presence of the transgene was detected in all the tested plants, except for the control (*BOR*).
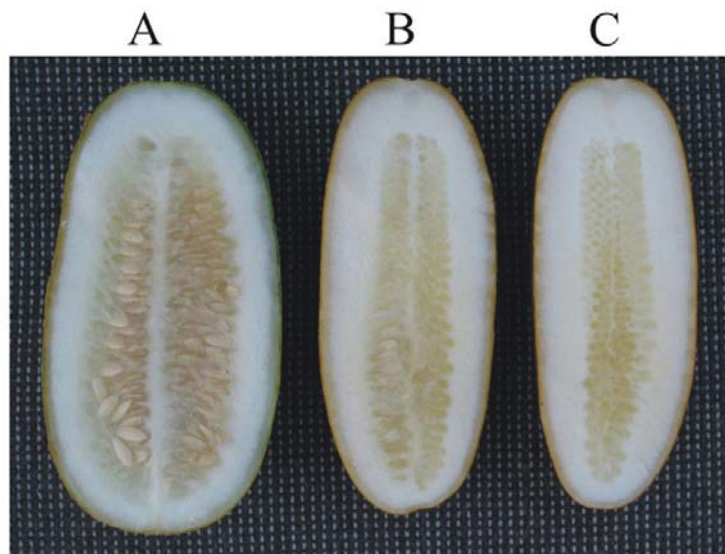

Fig. 5. A comparison of the fruits of the pollinated control (A) and those of seedless transgenic lines (B, C). Some enlarged, empty embryo sacs are visible in line TM1 (B); no enlarged, empty embryo sacs are found in line TM2 (C).

The regeneration of plants from callus was difficult, since in many cases there was a strong overgrowth of the callus, and deformed embryo- and sprout-like structures appeared that were incapable of further development (Fig. 2).

All of the regenerated plants were transferred to the greenhouse, and those that possessed the transgene (Fig. 3) and generated seeds passed the transgene on to the next (T_1) generation (Fig. 4).

The segregation pattern observed in plants of the transgenic TM1 and TM2 lines was compatible with a single-gene inheritance segregation (22:8 and 24:6, resistant:sensitive). The plants of the transgenic lines were not visibly different from those of the non-transgenic control, but we did not carry out any in-depth analysis.

The parthenocarpic fruits of the transgenic lines were seedless and did not markedly differ in size from the seeded fruits that originated from the control plants (Fig. 5 A, B). The embryo sacs generally remained undeveloped, but a few continued to grow despite the absence of embryos. The tendency to form these larger embryo sacs differed among the individual lines. About 10% of the fruits produced by the wild-type control plants were parthenocarpic, while the transgenic lines produced between 70% and 90% seedless fruits, i.e. a seven- to nine-fold increase (Fig. 6).

The seed set ability following pollination of the transgenic lines decreased relative to that of the non-transgenic control (Tab. 1). The F_1 progeny of crosses between the non-transgenic control and 2gg female line and each of the three transgenic lines also showed a reduced ability to set seed (Fig. 7).
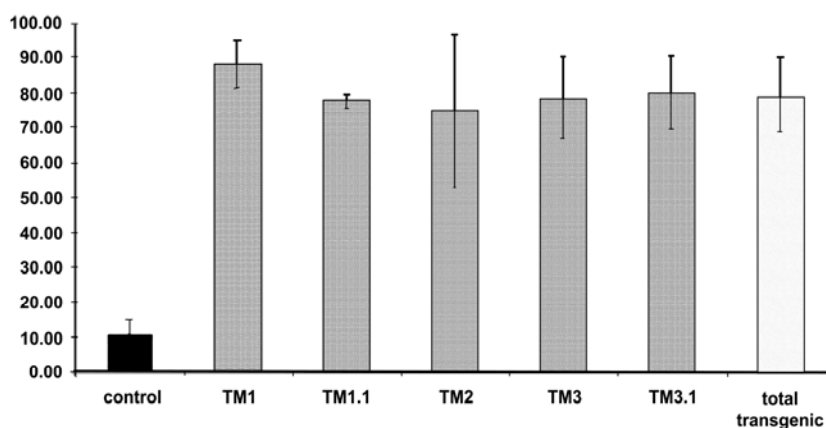

Fig. 6. The percentage of parthenocarpic fruits in the control and various transgenic cucumber lines bearing the *DefH9-iaaM* transgene. The data was obtained from greenhouse-grown plants.
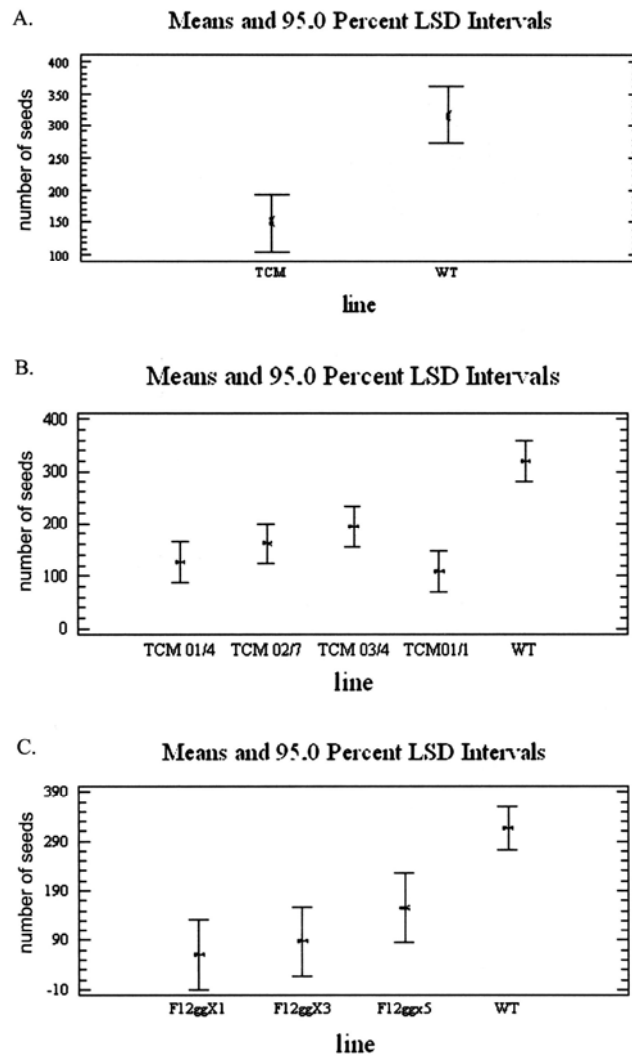
A.

B.

C.

Fig. 7. The seed set in F1 between line B, its female 2gg mutant and the transgenic lines. The transgenics were used as a male parent.

DISCUSSION

The transformation procedure that we routinely use to obtain transgenic cucumber plants harboring various constructs [12] was also efficient in producing transgenic cucumber plants carrying the *DefH9-iaaM* construct. However, unlike with the other constructs used with the same cucumber line, the introduction of *DefH9-iaaM* disturbed plant regeneration and resulted in the production of several malformed shoots. In addition, 50% of the transgenic plants displayed a typical tetraploid appearance and were not able to set seed. As

a result, the transformation efficiency was decreased by approximately 50%. The time required to obtain greenhouse-ready plants was approximately 30% longer than that required for cucumber plants transformed with other constructs; this is probably an effect of the partial *DefH9-iaaM* expression and auxin production during the early steps of plant regeneration.

The non-transgenic control that we used for the transformations described here is monoecious and has a tendency towards parthenocarpy. Introduction of the *DefH9-iaaM* construct, which ensures expression of the bacterial indoleacetic acid-monooxygenase in the ovules and the placenta of the fruit increased the number of parthenocarpic fruits by seven- to nine-fold. This demonstrates that the ovule-specific *DefH9* promoter from *Antirrhinum majus*, which has been shown to confer specific expression of the transgene in species of tobacco, eggplant, and tomato, also confers this expression in cucumber, and perhaps in other *Cucurbitaceae*, like sugar melon and watermelon. The possibility of also inducing parthenocarpy in species with a different fruit type than tobacco and *Solanum melongena* (eggplant) broadens the spectrum for the agricultural application of the *DefH9-iaaM* construct, as suggested by Rotino *et al.* [4].

The occurrence of a low level of natural parthenocarpy in the wild-type control cucumber line B was most likely enhanced by the *in vitro* culture of the seeds. The reason for applying this treatment to the control seeds was to ensure that both the control and transgenic seeds were subjected to identical conditions. The literature provides several examples of the impact of various environmental factors on the level of parthenocarpy in cucumber [26].

A significant proportion of the original transformants was tetraploid. We did not obtain this high a proportion in earlier transformation experiments in which we developed nearly 100 transgenic T_1 lines and subsequent generations that differentially carried nine different transgenes [12]. It is possible that the formation of tetraploids was a consequence of the partial expression of the construct during the early steps of the regeneration procedure, which would result in an increased IAA level and polyploidization in some of the tissues. This suggestion is also supported by the developmental disturbances observed during the regeneration process.

The cucumber plants bearing the *DefH9-iaaM* construct appeared to have a decreased fertility. This is suggested by the number of seeds within the control and by the lack of seeds in the tetraploid plants. On the diploid level, the number of seeds within the control plant was about 40% greater than within the obtained transgenic line. The tetraploids usually also generate several seeds within the fruit, depending on the genotype [27]. The same was observed for F_1 hybrids between the control and transgenic lines. This suggests that the construct has an additional effect on the seed/embryo formation. However, this phenomenon of decreased seed setting requires further detailed analysis.

The data obtained from the investigation reported here indicates that the *DefH9-iaaM* construct causes strong parthenocarpy in cucumber and, consequently, should provide a very valuable means for obtaining parthenocarpic varieties in

the *Cucurbitaceae*. An additional positive side benefit of parthenocarpy is the overcoming of fruit-set inhibition; however, this needs to be elucidated via separate studies using the homozygous transgenic 2gg females. This and the production of fruits in the absence of fertilization in a gynoecious cucumber have still to be shown, although the experimental material we produced in this investigation will be very valuable in such studies.

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