Planta (2003) 217: 726–735
DOI 10.1007/s00425-003-1052-1

ORIGINAL ARTICLE

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Induction of parthenocarpy in tomato via specific expression of the *rolB* gene in the ovary

Received: 26 September 2002 / Accepted: 4 May 2003 / Published online: 30 May 2003
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Abstract The molecular signals for the development of the ovary into fruit following ovule fertilization are not clear. However, in many species, including tomato (*Lycopersicon esculentum* Mill.), auxins and auxin transport inhibitors can substitute for fertilization as activators of fruit set, suggesting that this plant hormone plays a key role in this process. In agreement, transgenes for auxin biosynthesis expressed under ovary- or ovule-specific promoters were shown earlier to enable parthenocarpic (i.e. seedless) fruit development. In the present study, we tested an alternative approach for the induction of parthenocarpy that is based on ovary-specific expression of the *Agrobacterium rhizogenes*-derived gene *rolB*. This gene was chosen because *rolB* transgenic plants manifest several syndromes characteristic of auxin treatment. Tomato plants transformed with a chimeric construct containing the *rolB* gene fused to the ovary- and young-fruit-specific promoter *TPRP-F1* developed parthenocarpic fruits. Fruit size and morphology, including jelly fill in the locules of the seedless fruits, were comparable to those of seeded fruits of the parental line. Although it is not known whether ROLB signals for the same cassette of genes involved in fertilization-dependent fruit development, it clearly activates a battery of genes that enable successful completion of seedless fruit development in tomato.

Electronic Supplementary Material Supplementary material is available for this article if you access the article at http://dx.doi.org/10.1007/s00425-003-1052-1. A link in the frame on the left on that page takes you directly to the supplementary material.

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Keywords Fruit set · *Lycopersicon* · Ovary-specific-promoter · Seedless tomato · Transgenic parthenocarpy

Abbreviations IAA: indole-1-acetic acid · TSS: total soluble solids · WT: wild type

Introduction

Fruit set and development normally depend on successful fertilization (Gillaspy et al. 1993). In tomato and many other species, a major limiting factor for fruit set is the extreme sensitivity of microsporogenesis and pollination to moderately high or low temperatures and inadequate humidity (Picken 1984). Any method enabling fruit set to be independent of pollination may circumvent the environmental constraints on tomato fruit production. Currently, auxin, auxin analogs or auxin-transport inhibitors are still used to induce artificial development of parthenocarpic fruits in tomato, and to increase the size of poorly fertilized fruits. However, the auxin has to be applied to each truss separately, since it causes severe morphological malformations when applied to the vegetative organs; it also inhibits further flowering and frequently yields fruits of poor quality (reviewed by Abad and Monteiro 1989).

An alternative solution to the problem of fruit set is genetically controlled facultative parthenocarpy, which enables seeded fruit set if fertilization occurs, and seedless fruit set under pollination-restrictive conditions (George et al. 1984). In tomato, the two best sources for parthenocarpy *pat-2* from the Russian cultivar "Severianin" and *pat-3*/*pat-4* from the German line "75/59" (Philouze and Maisonneuve 1978a, 1978b) are governed by complex recessive systems, for which there are still no associated molecular markers. Pertinent to strong parthenocarpy is the difficulty in maintenance of the

parental lines. Furthermore, there are claims that these genes bear penalty on fruit-quality (reviewed by Lukyanenko 1991, and references therein). Thus, not surprisingly, there are still no elite cultivars containing either of these parthenocarpic systems.

Since the exploitation of existing sources for facultative parthenocarpy is complicated, one possible biotechnological alternative might be expression, under an ovary-specific promoter, of the *Agrobactrium tumefaciens* genes *iaaM* and *iaaH*, which in concert encode for the biosynthesis of the auxin indole-1-acetic acid (IAA) from tryptophan (Inze et al. 1984). Following this rationale, we induced parthenocarpic fruit set in tomato plants expressing the *iaaH* gene specifically in the ovary (Szechtman et al. 1997). This induction occurred subsequent to ovary treatment with the auxin precursor naphthaleneacetamide (NAM), which is hydrolyzed to naphthalene-1-acetic acid (NAA) by the *iaaH*-encoded enzyme indoleacetamide hydrolase (Inze et al. 1984; Klee et al. 1987). Similarly, it was shown that expression of *iaaM* under the ovule-specific promoter *DefH-9* induces parthenocarpy in eggplant, tobacco and tomato (Rotino et al. 1997; Ficcadenti et al. 1999; Donzella et al. 2000).

We tested an alternative approach for the induction of parthenocarpy that is based on ovary-specific expression of the *A. rhizogenes*-derived gene *rolB*. This gene was chosen because transgenic plants expressing it manifest several syndromes characteristic of auxin treatment (Schmulling et al. 1988), though parthenocarpic fruit development was not reported in transgenic tomatoes expressing rolB under the control of its native promoter (van Altvorst et al. 1992). Following our preliminary data indicating that, indeed, this transgene induces parthenocarpic fruit development in tomato (Carmi et al. 1997; Barg and Salts 2000), here we report a detailed analysis of the *rolB*-based parthenocarpy.

Materials and methods

Construction of the *TPRP-F1::rolB* binary vector

The *rolB* gene was obtained from Prof. J. Schell in plasmid pUC19-B26 (Slightom et al. 1986). To achieve organ-specific expression of the *rolB* gene, it was harnessed to the promoter of the ovary- and young-fruit-specific gene *TPRP-F1*, which we have previously isolated and analyzed in *TPRP-F1::gus* transgenic tomato plants (Salts et al. 1991, 1992; Carmi 1996; see also Electronic Supplementary Material). It should be noted that the *TPRP-F1::gus* plants did not differ in their phenotype from that of control MP-1 plants either at the vegetative or reproductive stages. The *TPRP-F1* promoter used to drive the expression of both *gus* and *rolB* is 2,543 bp long, including 2,133 bp of the promoter, 344 bp of the 5′ untranslated region and 66 bp of the translated mRNA (GenBank accession X61395). To fuse the *rolB* gene to the promoter, the *Nsi*I restriction site residing 66 bp downstream of the *TPRP-F1* translation initiation site was abolished and changed to a *Kpn*I site, by in vitro mutagenesis via PCR, using the primer TGGTACCGGGCAATGAACAAGTTCCA (the bold letters designate the mutated sequence). The mutated 2,543-bp fragment flanked by this *Kpn*I site and by a *Xba*I site at its 5′ end was fused to *rolB* (a *Kpn*I/*Hin*dIII fragment from the pUC19-B26 plasmid, containing T-DNA spanning bases 11,324 to 9,814 of the sequence published by Slightom et al. 1986). This sequence includes 39 bp of the *rolB* 5′ untranslated region, and 800 bp downstream of the translated region. In this construct, translation initiation of *rolB* is out of frame relative to the *TPRP-F1* translation start site which presumably confers attenuated translation of the authentic ROLB protein from its native initiation codon (the open reading frame starting with the *TPRP-F1* initiation codon terminates after 90 bp). The chimeric gene was inserted into a modified pGA492 binary vector (An 1986), the multiple cloning site of which was replaced by that of pUC19, to generate the binary vector pBSNrolB.

Plant transformation

The binary vector pBSNrolB was transformed into two indeterminate tomato (*Lycopersicon esculentum* Mill.) breeding lines, MP-1 (Barg et al. 1997) and CP-117. The two lines differ substantially in their horticultural make up, including fruit size; MP-1 bears smaller fruits than CP-117 (20–30 and 35–50 g per fruit, respectively). Transformation was done by co-cultivation of cotyledons derived from 10-day-old seedlings with *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993). Essentially, the protocol described by McCormick (1991) was followed, with the modifications described in Barg et al. (1997). Transgenic progeny were selected by germinating sterile seeds on selective medium (1/2 MS medium, 3% sucrose and 100 mg l^{-1} kanamycin), where only transgenic seedlings developed a branched root system.

DNA and RNA analysis

Nucleic acids were analyzed following established procedures (Sambrook et al. 1989). Southern analysis was performed following alkaline transfer of agarose electrophoretograms of restricted DNA to a charged nylon filter (Hybond N+) and hybridization with a radiolabeled fragment of the *rolB* gene. For RNA analyses, fruits were collected from three plants at R_2 (Fig. 1b), and from at least five plants at R_5 (Fig. 1c), fruits of line MPB-4 were from vegetatively propagated R_0 plants. Northern analysis was performed following total RNA fractionation in a formaldehyde–agarose gel. The running buffer and gel contained 10 mM Na-phosphate buffer (pH 6.5), 2 mM EDTA. The steady-state level of *rolB* mRNA was calculated from the ratio between *rolB* and *rRNA* signals quantified for each sample (*rolB*/*rRNA*) by the ImageQuanT program, of phosphorimager images of Northern blots hybridized with ^{32}P-radiolabeled *rolB* and *28S rRNA* (Fig. 1b) or *18S rRNA* probes (Fig. 1c). *rRNA* was probed by a *Bam*HI fragment (1,150 bp) of tomato *28S ribosomal RNA* gene obtained from Prof. E. Lifschitz, or with a 100-bp fragment of the tomato *18S ribosomal RNA* gene (PCR-amplified from reverse-transcribed total tomato RNA). The *Kpn*I/*Hin*dIII fragment (1,510 bp) from pUC19-B26 was used as a probe for *rolB*.

Analysis of fruit quality parameters

Flowers were tagged on the day of anthesis, and fruits were picked when fully ripe. Each fruit was weighed and cut transversely to evaluate seed content. Total seed number per fruit was calculated from total seed weight divided by average seed weight (determined by weighing a sample of a counted number of seeds). Brix value, which is an index of total soluble solids (TSS) in the fruit, was measured in juice squeezed from each fruit separately, using a digital refractometer (model PR-100; Atago, Japan).

Results

Characterization of the *TPRP-F1::rolB* regenerated plants

Plants transformed for the *rolB* gene under the control of the ovary-specific promoter *TPRP-F1*, were

Table 1 Seed-bearing phenotype of the various *TPRP-1::rolB* transgenic MP-1 tomato (*Lycopersicon esculentum*) plants (R_0) and their progenies (R_1)

Transgenic plant	Seed-bearing phenotype in R_0	Ratio of Kan^r seedlings in R_1[a]	Seed-bearing phenotype in R_1[b]
MPB-4	All fruits seedless (>80 fruits)[c]	–	– (no sexual progeny)
MPB-5	All fruits seeded (>30 fruits)	≈3/4	All plants yielded fruits full of seeds
MPB-11	All fruits seeded (>20 fruits)	≈3/4	In five of the plants all the fruits were highly seeded (>20 seeds/fruit). In one plant most fruits were seeded (>15 seeds), two fruits bore few seeds (<10 seeds). In one plant most fruits were seeded, three were seedless
MPB-12	Most fruits seedless, a few seeded (>10 fruits)	≈3/4	In all of the plants, most of the fruits were seedless and few (2–4 fruits) were seeded (2–40 seeds/fruit)
MPB-13	Most fruits seedless, a few seeded (>10 fruits)	16/17	In two plants all fruits were seedless, in two plants most of the fruits were seedless, several bore few seeds (<10 seeds), and a few fruits bore 10–20 seeds. In two plants about half of the fruits were seedless, and half were seeded (5–20 seeds/fruit). In one plant all the fruits were seeded (5–20 seeds/fruit)
MPB-19	All fruits seeded (>15)	≈3/4	Not tested

[a]The ratio of kanamycin-resistant (Kan^r) progenies in R_1 was determined by scoring ca. 30 germinating seedlings on selective medium (detailed in Materials and methods)

[b]Seven kanamycin-resistant plants were tested in R_1

[c]Complete seedlessness in all fruits of eight plants propagated from cuttings of the MPB-4 regenerated plant

Table 2 Fruit characteristics of CP-117-rolB (R_o) transgenic tomato plants

Plant No.	Fruit weight (g)	Seed-bearing phenotype
CP-117[a]	38.0 (25–56, $n=19$)[b]	Varying seed number, reduced seed number associated with decreased fruit weight
CP-117B-6	12.0 (3–18, $n=17$)	Most seedless (“nuts”)
CP-117B-9	54.2 (35–67, $n=6$)	All seedless
CP-117B-5	33.7 (15–62.5, $n=3$)	Mostly seedless (strong parthenocarpy)
CP-117B-4	44.3 (16–72, $n=14$)	All seedless (obligate parthenocarpy)
CP-117B-4A	28.7 (15–55, $n=6$)	Most seedless, 2 contained <10 seeds (strong parthenocarpy)
CP-117B-7	34.0 (22–44, $n=11$)	6-seedless, 5 contained <8 seeds (strong parthenocarpy)

[a]Fruits of CP-117 were collected from two plants

[b]The numbers in parenthesis specify the range of fruit weight and n = number of weighed fruits

obtained from both MP-1 and CP-117 tomato lines. Among the six transgenic plants regenerated from breeding line MP-1, three (MPB-4, MPB-12 and MPB-13) manifested a clear parthenocarpic phenotype in the R_0 generation (Table 1). Among the eight transgenic CP-117 plants, six were completely parthenocarpic, bearing predominantly seedless fruits (Table 2), most of which did not differ in size from seeded fruits of the parental line (e.g. Fig. 2i). In the parthenocarpic plants, CP-117B-4A and CP-117B-7, the few generated seeds germinated poorly and these lines could not be further characterized. Possible approaches for sexual propagation of obligate parthenocarpic lines are discussed below. Two of the transgenic plants set only seeded fruits not differing from the non-transformed line (data not shown).

Analysis of segregation for kanamycin resistance among R_1 progenies of transgenic MP-1 plants (Table 1) indicated that lines MPB-5, MPB-11, MPB-12 and MPB-19 contained a single insertion locus at R_0, whereas MPB-13 contained two independently integrated loci.

Based on the preliminary evaluation of the regenerated plants, lines MPB-4, MPB-12 and MPB-13 were further analyzed. Lines MPB-5 and MPB-19 showed no parthenocarpic phenotype, probably due to position effect, and were not further analyzed.

Southern analysis (Fig. 1a) was performed on the MPB-4 R_0 plant and on R_2 plants of both MPB-12 and MPB-13 each derived from a single facultative parthenocarpic R_1 plant. This analysis indicated that the MPB-4 R_0 plant contained four or five inserts of the chimeric gene. MPB-12 contained a single insertion locus, and the tested MPB-13 offspring maintained only one of the two insertion loci. In both lines, the insertion consisted of two copies of the transgene in a tandem head-to-tail orientation, as determined by additional Southern analyses performed on genomic DNA digested with several restriction enzymes. This was confirmed in successive generations where the two copies were found to co-segregate as a single locus (data not shown).

Obligate parthenocarpy of plant MPB-4

This plant manifested absolute (i.e. obligate) parthenocarpy. All its fruits (>80 tested from plants propagated

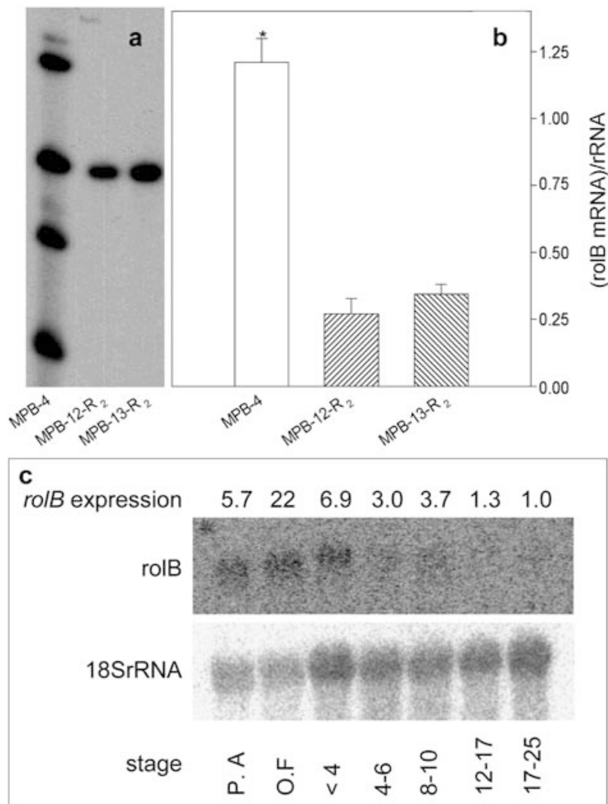


Fig. 1a–c Molecular analysis of *rolB* in transgenic plants. **a** Southern analysis of three tomato (*Lycopersicon esculentum*) lines, MPB-4-R_0, MPB-12-R_2 and MPB-13-R_2 transgenic for *TPRP-F1::rolB*. The DNA samples (ca. 10 μg) were digested with *Kpn*I and hybridized with ^{32}P-labeled *rolB*. **b** Northern analysis of *rolB* mRNA in the ovaries at 7 days post-anthesis. The steady-state level of *rolB* mRNA is expressed as the ratio between the quantified phosphor images of membranes hybridized with ^{32}P-labeled *rolB* and *28S rRNA* probes (*rolB* mRNA/rRNA). The value for MPB-4 (*) differs significantly from those for MPB-12-R_2 and MPB-13-R_2, which do not differ from each other (analysis included: two samples of MPB-4-R_0, two samples of MPB-12-R_2 and three samples of MPB-13-R_2). **c** Northern analysis of *rolB* mRNA in developing fruits of line MPB-12 (tested at R_5). Ovaries were sampled at the stages of: 1 day before anthesis (*P.A*), open flowers (*O.F*) and fruits of: < 4, 4–6, 8–10, 12–17 and 17–25 mm in diameter. Phosphor image was quantified as in **b**, except for using ^{32}P-labeled *18S rRNA* fragment as a reference. *rolB* expression: the level of expression at each stage is expressed relatively to the expression at the 17- to 25-mm stage

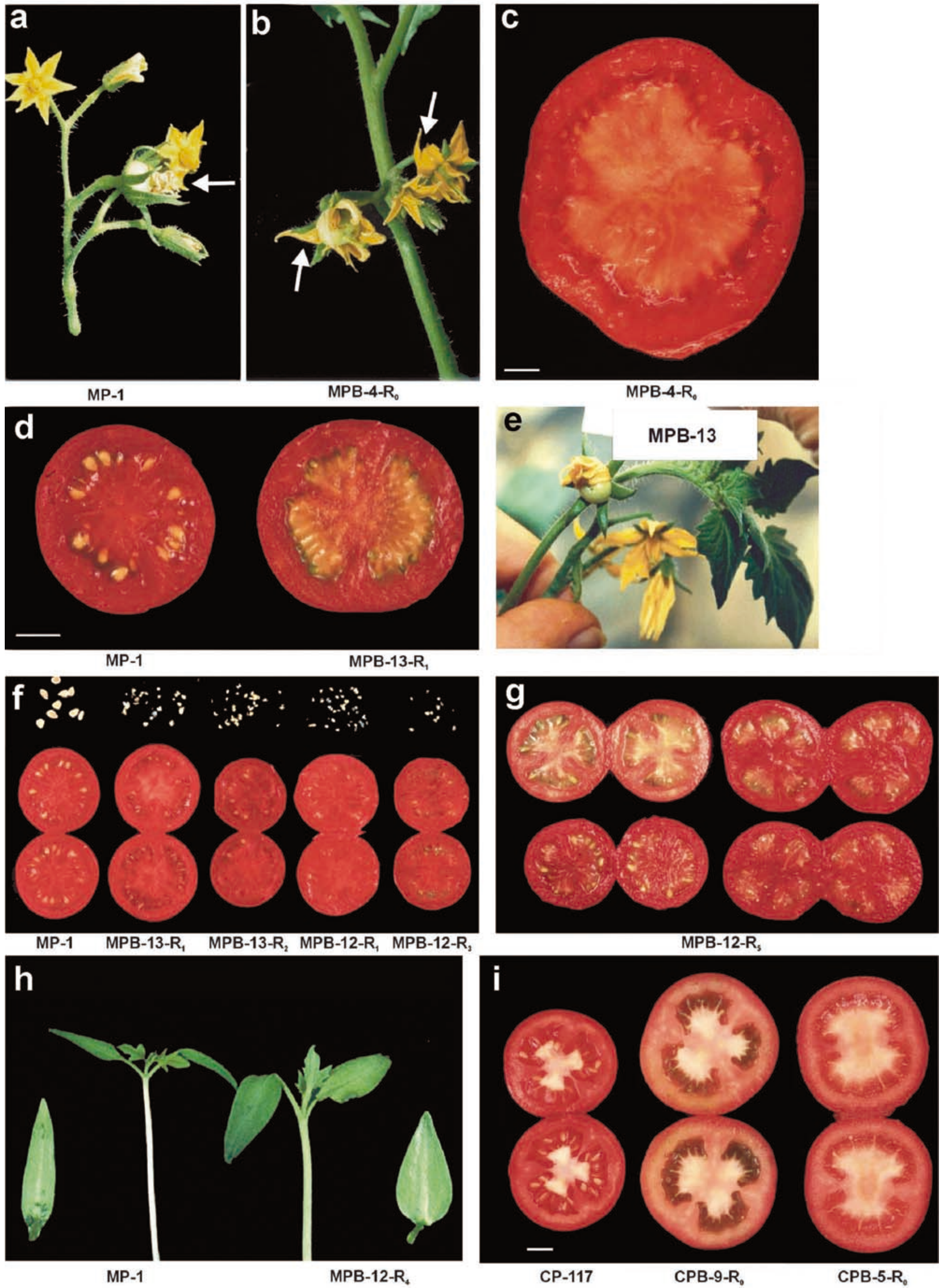
from cuttings) were completely seedless, with an enlarged columella (the central core of the pericarp), and were full of jelly (Fig. 2c). This plant was also male-sterile: anther morphology was normal, but its pollen was non-viable according to vital staining, and pollen germination tests (data not shown). Male sterility due to *rolB* expression in the pollen has been previously reported (An et al. 1994). Enforced cross-pollination with viable pollen derived from MP-1 failed to yield seed development. Unlike in the parental line, in this particular plant the strong parthenocarpy was characterized by very early enlargement of the ovary, well prior to anthesis (Fig. 2b compared with 2a). Thus, its seedlessness may have resulted from a physical barrier, created upon early closure or rupture of the stylar tube. Alternatively, the seedlessness may be reflecting female sterility, due to residual expression of the transgene in the ovules. It should be noted that similar to the *pat-2*-induced parthenocarpy, and in contrast to fertilization-induced fruit set in wild-type (WT) tomato, ovary development into fruit was not accompanied by rapid wilting and abscission of the petals, stamens or pistil. The two former organs remained viable until they were detached from the receptacle by the growing ovary (Fig. 2a vs. Fig. 2b).

The vegetative growth habit of the original MPB-4 plant, and the cuttings propagated from it, was not notably different from that of the parental line (data not shown). This sterile plant was eventually lost due to viral infection.

Facultative parthenocarpy of plants MPB-12 and MPB-13

The MPB-12 and MPB-13 R_0 plants manifested conditional (i.e. facultative) parthenocarpy, namely, both completely seedless fruits (e.g. Fig. 2d), as well as seeded fruits developed from non-emasculated flowers. This parthenocarpic manifestation was maintained in successive generations (Fig. 2f, g). The parthenocarpic fruits were similar in size and shape to those of the parental line and full of jelly, even when completely seedless. Smaller fruits, such as the one shown in Fig. 2f, developed at higher trusses in MP-1 and in the transgenic lines MPB-12 and MPB-13. In both transgenic lines, in most of the fruits the columella was larger than in the parental-line fruits. The seedless fruits contained remnants of unfertilized ovules and integuments

Fig. 2a–i Characteristics of *TPRP-F1::rolB* transgenic plants derived from lines MP-1 and CP-117. **a** Flowers and young fruit of the parental line MP-1; the remains of the detached petal seen on top of the developing fruits are completely shriveled (*arrowhead*). **b** Flowers and young fruits of the obligate parthenocarpic plant MPB-4; the petals surrounding the developing fruit are viable (*arrowheads*). **c** Mature seedless fruit of plant MPB-4 with enlarged columella. **d** Seeded fruit of line MP-1 (*left*) compared with seedless fruit of line MPB-13 (*right*) in R_1. **e** Flowers and young developing fruit of MPB-13 at R_0; the petals surrounding the developing fruit are still viable. **f** Seedless fruits of lines MPB-13 at R_1 and R_2 and MPB-12 at R_1 and R_3, containing ovules and integuments only, vs. seeded fruit of MP-1. The content of each of the photographed fruits was squeezed out, fermented overnight, washed, collected on a net, photographed and is presented *above* the respective fruit. **g** Facultative parthenocarpy in homozygous MPB-12-R_5 plants; both seeded (the two fruits on the *left*) and seedless (the two on the *right*) fruit developed on the same plant under pollination-permissive conditions, without enforced pollination (i.e. no flower vibrating). **h** Seedling of MP-1 (*left*) with horizontal narrow blade-like cotyledons and of MPB-12 (*right*) in R_4, with wide heart-shape downward-bending cotyledons. **i** Seedless fruit of independent transgenic R_0 plants CPB-9 and CPB-5 derived from line CP-117. Bars = 2 cm (**c**), 1 cm (**d, i**)


a
b
c
MP-1
MPB-4-R0
MPB-4-R0
d
e
MPB-13
MP-1
MPB-13-R1
f
g
MP-1
MPB-13-R1
MPB-13-R2
MPB-12-R1
MPB-12-R3
MPB-12-R5
h
i
MP-1
MPB-12-R4
CP-117
CPB-9-R0
CPB-5-R0

(Fig. 2f). In some of the MPB-13 fruits, the jelly had a greenish hue (e.g. Fig. 2d). This phenomenon has been previously reported for auxin-induced fruits (Abad and Monteiro 1989). The fasciation typical of auxin-induced parthenocarpic fruits was not observed in the seedless fruits of MPB-12 and MPB-13.

The flowers of MPB-13 (at R_0) manifested a moderate mode of 'parthenocarpic' development, i.e. early enlargement of the ovary and postponed wilting of the petals (Fig. 2e), and their pollen was viable. The flowers of MPB-12 manifested an even milder parthenocarpic phenotype: their ovaries were slightly enlarged and wilting of the petals was somewhat postponed. Their pollen was viable but the percentage of pollen germination and the rate of pollen-tube growth in vitro were lower than in the parental line (data not shown).

In the vegetative growth phase, the only profound difference between the parental line and both MPB-12 and MPB-13 was the shape of the cotyledons: they were substantially broader than in MP-1 and rolled downwards (Fig. 2h); later in vegetative development the transgenic plants were only somewhat more robust.

mRNA levels of the transgene

Based on the steady-state level of *rolB* mRNA in ovaries collected 7 days post-anthesis, there appears to be an association between the abundance of transcripts of the transgene, and the strength of parthenocarpy (Fig. 1b). The level of *rolB* expression in MPB-4, which manifested obligate parthenocarpy, was approximately 6-fold higher than in facultative parthenocarpic lines MPB-12 and MPB-13. The steady-state level of *rolB* mRNA in developing fruits of line MPB-12 was analyzed in homozygous plants at R_5. As shown in Fig. 1c, the transcript peaks in the very early stages of ovary development, i.e. in ovaries at the open-flower stage, and declines shortly thereafter (in fruits of 4–6 mm in diameter). This mode of expression is expected of a gene driven by the *TPRP-F1* promoter, which is highly specific to ovaries and very young fruit (Salts et al. 1991, see also the Electronic Supplementary Material). Further insight into the distribution of the *rolB* transcripts within the pericarp/embryos requires RNA in situ analysis.

Quantitative analysis of fruit characteristics in line MPB-12

Fruit characteristics of line MPB-12 were analyzed on homozygotic R_5 progeny and compared to those of the parental line MP-1. Plants (20 MP-1 and 18 MPB-12) were potted in 10-l containers and grown in a net-house during the spring and the summer. To evaluate the strength of the parthenocarpy under pollination-permissive conditions, the flowers were neither emasculated nor vibrated. Because line MP-1 is indeterminate, fully ripened fruits were collected over a period of 10 weeks between mid-July and mid-September. The results are summarized in Table 3.

Yield components As shown in Table 3, the average fruit weight of line MPB-12 was significantly higher than that of the parental line, but the total yield was lower. In line MP-1, seedless or nearly seedless fruits were significantly smaller than seeded fruits, and there was a significant correlation ($r^2=0.515$, $F=453.6$, $P<0.001$, $n=429$ fruits) between seed number and fruit weight (Fig. 3). This correlation is a well-established phenomenon (Imanshi and Hiura 1975). However, in line MBP-12 this correlation was totally abolished ($r^2=0.0046$, $F=0.818$,

Table 3 Effects of *rolB* on yield and fruit characteristics of MPB-12 tomato plants. Experiment included 20 plants of MP-1 and 18 homozygous (*rolB*/*rolB*) plants of MPB-12. Values followed by different capital letters differ significantly according to Tukey–Kramer multiple comparison test (a stringent test). Values followed by different lower case letters (in parenthesis) differ significantly only according to Student–Newman–Keuls comparison test (a less stringent test)

Parameters	MP-1	MPB-12	Effect of the transgene
Fruit weight (g)			
All fruits	19.75 ± 0.46 D	25.65 ± 0.90 B	Increase (+30%)
Fruits >10 g	23.07 ± 0.43 C	28.40 ± 0.87 A	Increase (+23%)
Yield per plant (g)			
All fruits	427.6 ± 27.1 A (a)	292.9 ± 18.8 BC (b)	Decrease (−31%)
Fruits >10 g	387.3 ± 27.2 AB (a)	283.2 ± 19.1 BC (b)	Decrease (−27%)
Fruits >10 g, S-5[a]	372.0 ± 26.3 AB (a)	278.8 ± 18.1 C (b)	Decrease (−25%)
Days to full red ripening			
All fruits on truss	48.0 ± 0.6 A	43.2 ± 0.9 B	Decrease (−10%)
First four fruits on truss	46.5 ± 0.6 A	42.7 ± 0.9 B	Decrease (−8%)
Locule number			
All fruits	3.08 ± 0.045 A	3.08 ± 0.08 A	None
Seedless fruits	–	3.15 ± 0.098 A (b)[b]	–
Brix			
All fruits	6.94 ± 0.021 B	7.32 ± 0.021 A	Increase (+5.5%)
Seedless fruits	6.75 ± 0.108 B (c)	7.43 ± 0.051 A (a)	Increase (+10%)
Seeded fruits	6.97 ± 0.056 B (b)	7.11 ± 0.067 B (b)	Not significant
Seed number			
All fruits	34.8 ± 1.4 ($n=430$) B	4.7 ± 0.7 ($n=157$) D	Decrease (−86%)
Seeded fruits	47.0 ± 1.5 ($n=319$) A	14.5 ± 1.4 ($n=50$) C	Decrease (−70%)
Jelly fill			
All fruits	Complete	Complete	None

[a] S-5 refers to yield up to the 5th inflorescence
[b] The significance of the Student–Newman–Keuls test for this value was only $0.05<P<0.1$

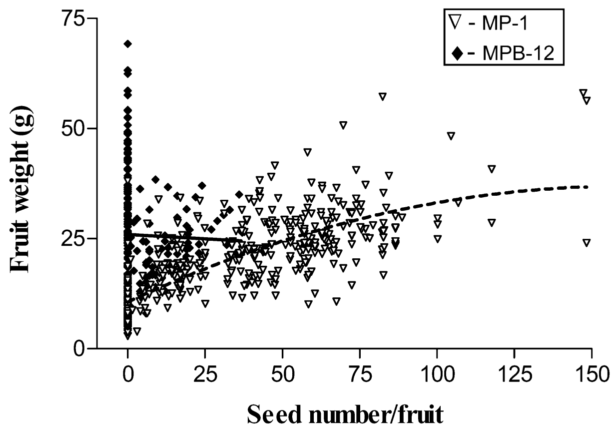


Fig. 3 Correlation between fruit weight (g) and the number of seeds in the fruit. For fruits of the transgenic line MPB-12 (*solid line*) the regression is insignificant ($P<0.0001$), while for the parental line MP-1 (*broken line*) it is significant ($P>0.05$) in the range of 0–70 seeds per fruit

$P=0.367$, $n=180$ fruits) indicating that *rolB* expression compensated for the contribution of the seeds to the final fruit weight. Average fruit weight and yield were analyzed both for all fruits and for fruits greater than 10 g only (Table 3). The latter represent the agronomically significant yield, because small (less than one-third of the average fruit weight), seedless pseudo-fruits (also termed 'nuts') are not considered part of the marketable crop. It was not reported whether the transgenic *DefH-9::iaaM*-based parthenocarpy (Ficcadenti et al. 1999), or its modified version under a weaker promoter (*DefH9-RI-iaaM*), affect the total yield in tomato. However reduced weight of the fruit (by ca. 20%) was found for the latter (Pandolfini et al. 2002).

Earliness The transgenic fruits ripened 4–5 days earlier than the WT (Table 3). This significant hastening of fruit maturation resembles that reported for auxin-induced parthenocarpic fruit development (reviewed by Abad and Monteiro 1989).

Seed number One of the most profound differences between the transgenic and WT fruits was in the number of seeds per fruit. As shown in Table 3, only 30% of the transgenic fruits were seeded, vs. 75% in the WT. Moreover, under these fertilization-permissive conditions, the average seed number in the seeded transgenic fruits was significantly lower than in the WT fruits.

Fruit structure The completely seedless fruits of MPB-12 were always full of jelly (Table 3, Fig. 2g, h) regardless of their size.

Brix value A significant increase in the Brix value, which is an index of the total soluble solids (TSS) content in the fruit juice, was found in the transgenic fruits (Table 3). This increase cannot be simply attributed to the decreased yield of the transgenic plants (Stevens and Rudich 1978). First, as demonstrated in Fig. 4a, in line

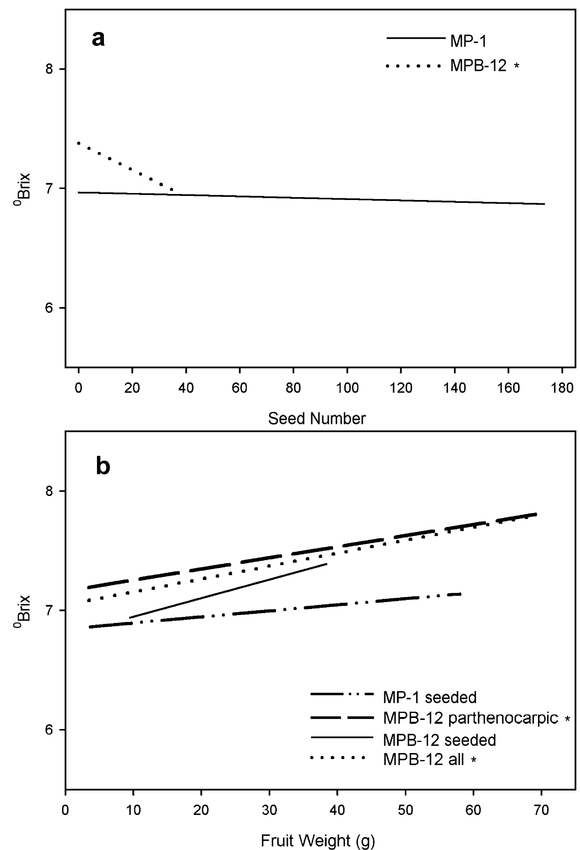


Fig. 4a, b Effect of seedlessness on Brix value. **a** Regression of Brix value over seed number per fruit in line MPB-12 and in MP-1. **b** Regression of Brix value over fruit weight of seeded fruits of MP-1, of all fruits of MPB-12, of seeded fruits of MPB-12 and of parthenocarpic fruits of MPB-12. An *asterisk* (*) indicates significant regression ($P<0.05$)

MPB-12 there was a significant negative correlation ($r^2=0.0373$, $P=0.015$, $n=157$ fruits) between the seed content and the Brix value, but not for MP-1 ($r^2=0.00085$, $P=0.632$, $n=271$ fruits). Moreover, as shown in Fig. 4b, for line MP-1, no relationship was found between Brix value and fruit weight ($P=0.171$), whereas for MPB-12 there seemed to be a slight correlation between the two ($P=0.002$ and $P=0.094$ for parthenocarpic and seeded fruit, respectively). The parthenocarpic fruits of MPB-12 seemed to have a higher Brix value than the seeded ones for a given fruit weight. To test whether this difference was significant, seeded and parthenocarpic fruits of MPB-12 were subjected to analysis of covariance with the fruit weight as the covariate: the two slopes were not significantly different ($P=0.55$). Therefore, a model with a common significant ($P=0.0018$) slope was fitted, and the regression line for the parthenocarpic fruits was found to be significantly higher than that for the seeded fruits ($P=0.03$). Since both seeded and parthenocarpic fruits of MPB-12 developed on each of the plants, it is clear that seedlessness per se contributed to the increased Brix value, beyond a possible effect of the reduced total yield.

Fruit set at low temperatures

In a small experiment, plants were grown during the wintertime in a non-heated glass-house, where night temperatures declined well below 8 °C for several hours. Under these conditions, where pollen viability was completely lost, the parental line MP-1 set only a few very small fruits ('nuts') averaging 5.1 g, whereas MPB-12 set seedless fruits of 22.1 g, indicating maintenance of the parthenocarpic potential under these pollination-restrictive conditions.

Discussion

In this study, we demonstrated that expression of *rolB* under the control of an ovary- and young-fruit-specific promoter induces parthenocarpic fruit set and development in two tomato lines differing in their horticultural make-up, including fruit size (Tables 1, 2, and Fig. 2). Presently, it is not clear how ROLB substitutes for fertilization in triggering fruit development. In fact, the molecular basis underlying the other phenotypic changes imposed by *rolB* was not elucidated either. Although most ROLB effects resemble those of auxin, comparisons of IAA uptake, metabolism, content, conjugation, biosynthesis and degradation in WT and *rolB*-transformed tobacco plants did not reveal any significant difference in any of these parameters (Nilsson et al. 1993; Schmulling et al. 1993; Delbarre et al. 1994). Filippini et al. (1996) suggested that ROLB is a tyrosine phosphatase operating in auxin signaling. Taken together, it was postulated that ROLB causes changes in the auxin signal perception/transduction pathway. However, despite extensive research concerning *rolB*, the actual function of its product is still unknown, as it was in 1997 (when reviewed by Nilsson and Olsson).

The strength of the parthenocarpy (i.e. facultative or obligate) seems to be related to the transgene mRNA level in the developing fruit (Fig. 1b). We could not establish a correlation between ROLB abundance and the strength of parthenocarpy since quality antibodies were not available to us.

The developmental mode of expression of *rolB* (Fig. 1c) indicates that the highest level of expression is during the early stages of fruit development, as expected of a *TPRP-F1* promoter-driven gene (Salts et al. 1991, and Electronic Supplementary Material). Thus the ROLB protein synthesized at these stages is sufficient to fully substitute for the contribution of the seeds to fruit shape and final size.

Analysis of yield components in line MPB-12 indicated significant increase in fruit weight together with decrease in total yield per plant (Table 3). The reduction in yield resulted from both reduction in flower development at inflorescence 5–7 and a substantial decrease in the number of fruits developed per truss. Interestingly, in subsequent inflorescences developing late in the season, plants of line MPB-12 developed flowers that set fruits, whereas in the parental line, scarcely any flowers developed into fruits from the corresponding inflorescence. The altered pattern of fruit set along the stem is a consistent phenomenon in this particular line.

The reduction in the number of fruits per truss seems to result from enhanced dominance or strength of the early developing sinks over the late ones along a given inflorescence. Bangerth (1989) suggested that dominance among fruits at the same truss results from the polar IAA export from earlier developed sinks, which inhibits IAA export from the later developed ones. A reduction in the percentage of fruit set has also been reported for auxin-induced fruit development (reviewed by Abad and Monteiro 1989). However, since ROLB does not affect IAA levels or metabolism (reviewed by Nilsson and Olsson 1997), this hypothesis seems unlikely to apply to *TPRP-F1::rolB*-based parthenocarpy.

The transgenic fruits bear significantly lower seed numbers (Table 3, Fig. 3). The reduction in seed number cannot be simply attributed to the somewhat reduced pollen viability in MPB-12, since cross-pollination of this line with viable non-transgenic pollen also yielded fruits with only a few seeds, and in many cases with no seeds at all (data not shown). A high tendency for seedlessness has also been reported for auxin-induced fruit set under pollination-permissive conditions. For those latter cases, it was suggested that sensitivity of the ovules to high auxin concentrations leads to lack of fertilization and even abortion of fertilized ovules (reviewed by Abad and Monteiro 1989). In agreement, reduced seed set was reported also for the *DefH9-RI-iaaM* parthenocarpic tomatoes (Pandolfini et al. 2002). The fact that self- and cross-pollinated MPB-12 flowers occasionally set seeded fruits indicates incomplete expression of the damaging effect of *rolB* on ovule/embryo development. Moreover, in successive experiments it was found that very late in the summer, there is a higher tendency for production of seeded fruits on homozygous MPB-12 plants. This phenomenon indicates for reduced phenotypic expression of parthenocarpy under varying conditions, e.g. fluctuating temperatures, shortening day length, or aging of the plant.

The fact that the transgenic fruits were all full of jelly indicates that expression of *rolB* in the pericarp stimulates the development of the jelly tissue similar to the effect of seeds. This is in contrast to auxin-induced fruits, which are frequently 'puffy' (hollow) (Abad and Monteiro 1989). Interestingly, the columella of the transgenic fruits is usually larger than that of the WT (e.g. Fig. 2c, f). There was a slight change in the structure of the transgenic fruits as shown by a tendency toward increased locule number, especially among the seedless fruits, although the increase in locule number was not highly significant ($0.05 < P < 0.1$). This phenomenon was more prevalent among the largest seedless fruits.

An important positive effect of the transgene is the increase in Brix value (Table 3) which is a desirable trait in tomato, especially in processing cultivars. As shown in Fig. 4a, b, beyond a possible effect of decrease in yield

on the Brix value (Stevens and Rudich 1978), it is also directly affected by the lack of seeds. An increased Brix value of seedless parthenocarpic fruits relative to seeded fruits has been reported for facultative parthenocarpic tomato lines based either on *pat*, *pat-2* or the multigenic system derived from line 75/59 (Falavigna et al. 1978; Casas Diaz et al. 1987), as well as for the *DefH-9::iaaM*-based parthenocarpy (Ficcadenti et al. 1999). An increased Brix value has been suggested to result from lack of re-allocation of assimilates from the pericarp to the seeds, thus maintaining higher TSS content in the fruit (Late Prof. R. Frankel, personal communication).

The *rolB*-based parthenocarpy differs from the *iaaM*-controlled one in two aspects. First, in the latter, biosynthesis of optimal levels of IAA depends on adequate spatial and temporal expression of endogenous hydrolases for which indoleacetamide is a substrate, while the effect of ROLB does not seem to depend on any special endogenous gene-expression. Second, because ROLB acts autonomously in the cells in which it is expressed (Chilton et al. 1982; Bercetche et al. 1987; Cardarelli et al. 1987) there is less risk of spreading of the auxin-like effects to neighboring organs. Indeed high expression of *iaaM* in the ovules led to development of malformed fruit (Pandolfini et al. 2002).

An apparent problem concerns the sexual propagation of the parental lines, in all cases where obligate parthenocarpy is the preferred phenotype (e.g. processing cultivars or raisin tomatoes). Maintenance of such transgenic lines can be accomplished following several alternative molecular approaches: For example, the transgene can be transiently silenced in the parental line, via RNAi, e.g. using a viral vector bearing antisense sequence of *rolB* (Liu et al. 2002). Alternatively, transcription of the *rolB* gene can be blocked in the parental line by insertion of a DNA fragment (flanked by recombination target sites), between the promoter and the gene. In the hybrid cultivar *rolB* transcription will be activated via removal of the blocking fragment by site-specific recombination (Dale and Ow 1991).

If *rolB* imitates auxin-induced parthenocarpy, it is anticipated to induce parthenocarpy in other species where application of synthetic auxin leads to fruit set. Yet it might induce parthenocarpy via activation of an auxin-independent signaling route. Now that microarray representing thousands tomato genes is available (http://www.ted.bti.cornell.edu/public.asp), the profiles of gene expression during fruit development in response to fertilization, auxin and *rolB* expression can be readily compared.

Acknowledgements This research was supported by the USA–Israel BARD foundation, and by the Chief Scientist of the Israeli MOAG Fund. It is contribution No. 137-2002, from the Agricultural Research Organization, The Volcani Center, Bet-Dagan. The plasmid pUC19-B26 (*rolB*) was kindly provided by Prof. J. Schell, Max Planck Institute, Koln, Germany, and plasmid TR-3 (*28S rRNA*) was kindly provided by Prof. E. Lifschitz, The Technion, Haifa, Israel. The assistance of Dr. A. Gnizi, The Volcani Center, A.R.O., with the statistical analysis is deeply appreciated.

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