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ORIGINAL PAPER

Improved yielding and reduced puffiness under extreme temperatures induced by fruit-specific expression of *rolB* in processing tomatoes

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Abstract Tomato fruit production is severely hampered by both extremely high and low temperatures, mainly due to impaired microsporogenesis and pollination under these conditions. Even mild temperature stress, leading to partial damage to pollen viability can result in the production of under-fertilized puffy fruits of poor quality, while severe stress can abolish fruit set completely. Genetic or transgenic parthenocarpy that enables fertilization-independent fruit development offers a solution for tomato yielding under conditions unfavorable for pollen production and/or fertilization. A transgenic processing tomato UC82 line, expressing *rolB* specifically during early stages of fruit development was compared to the parental line with respect to yield and fruit quality under extreme temperatures. Under both high and low temperatures the transgenic line performed significantly better than the parental line. Its yield was significantly higher mainly due to a higher number of fruits that did develop, and also because of increased fruit weight. While the UC82 fruits developed under high temperatures were very puffy and severely malformed, the transgenic fruits maintained improved jelly fill and were of smooth and regular shape. Interestingly, under high temperatures the improved jelly fill in the transgenic line was accompanied by a higher number of seeds, suggesting that not only the developing seeds promote development of the placental tissue but also that proliferation of this tissue supports better seed development.

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Introduction

Fruit set and development normally depend on successful fertilization (Gillaspy et al. 1993). In tomato (*Solanum lycopersicum*), 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). Hence, any method enabling fruit set to be independent of pollination may circumvent the environmental constraints on tomato fruit production. 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 under-fertilized fruits. However, the auxin may cause severe morphological malformations if not applied to each truss separately, it also suppresses further flowering and frequently yields puffy fruits of poor quality (reviewed by Abad and Monteiro 1989). An alternative solution is genetically controlled facultative parthenocarpy, which enables seeded fruit set if fertilization occurs, and seedless fruit set under pollination-restrictive conditions. Yet even the best available genetic sources for parthenocarpy in tomato suffer from shortcomings that hindered their incorporation into elite tomato cultivars (see Gorguet et al. 2005, and references therein).

Based on the phenomenon of auxin-induced parthenocarpy, two different approaches for construction of transgenes for parthenocarpy were followed: The first is based on ovule or ovary-specific elevation of the auxin content due to organ-specific expression of the *Agrobacterium tumefaciens* derived gene *iaaM* which together with the *iaaH* gene enable IAA

biosynthesis from tryptophan via the non-auxin intermediate indoleacetamide (IAM). Placenta and ovule-specific expression of *iaaM* led to parthenocarpy in tomato, eggplant and cucumber (Rotino et al. 1997; Ficcadenti et al. 1999; Yin et al. 2006). Similarly, we have shown that expressing the *iaaH* gene under the ovary and early fruit-specific promoter *TPRP-F1* enables parthenocarpic fruit set from emasculated tomato flowers treated with NAM; a synthetic analogue of IAM (Szechtman et al. 1997). Following a second approach, we have demonstrated that ovary-specific expression of the *Agrobacterium rhizogenes* derived gene *rolB* induces parthenocarpy in both indeterminate and determinate tomato cultivars (Carmi et al. 1997, 2003; Barg et al. 2001). The evidence that *rolB* and no other sequence within the T-DNA is responsible for the parthenocarpic phenotype, came from the fact that parthenocarpy was not observed in any of 14 independent transgenic tomato lines transformed with a similar T-DNA, where *GUS* rather than *rolB* was fused to the *TPRP-F1* promoter (Carmi et al. 2003, and our unpublished data). *rolB* does not affect auxin metabolism (Delbarre et al. 1994; Nilsson et al. 1993), but imitates some of the auxin effects, by as yet only partly elucidated mechanism involving nuclear localization of RolB, which is dependent on interaction with a 14-3-3 protein (Moriuchi et al. 2004).

Preliminary analysis (at R_2) of three transgenic (*TPRP-F1*::*rolB*) lines derived from the processing tomato cultivar UC82 indicated for improved jelly fill in their fruits compared to the parental line in the summer (Barg et al. 2001). Here, we report a detailed analysis of line UC82B-4, the most promising among the three established lines, showing that under extreme high or low temperatures this line performs significantly better than the parental line both in terms of yield and fruit quality.

Materials and methods

Transformation and molecular analysis of the *TPRP-F1*::*rolB* transgene

Construction of the binary vector pGBrolB (harboring the transgene *TPRP-F1*::*rolB* and the selectable marker *nptI*, presented in Fig. 1a), and analysis of the ovary and young fruit-specific promoter *TPRP-F1* exploited to drive the expression of *rolB* were reported earlier (Carmi et al. 2003). Transformation to the tomato processing cultivar UC82B was done by co-cultivation as described earlier (Barg et al. 1997, 2001).

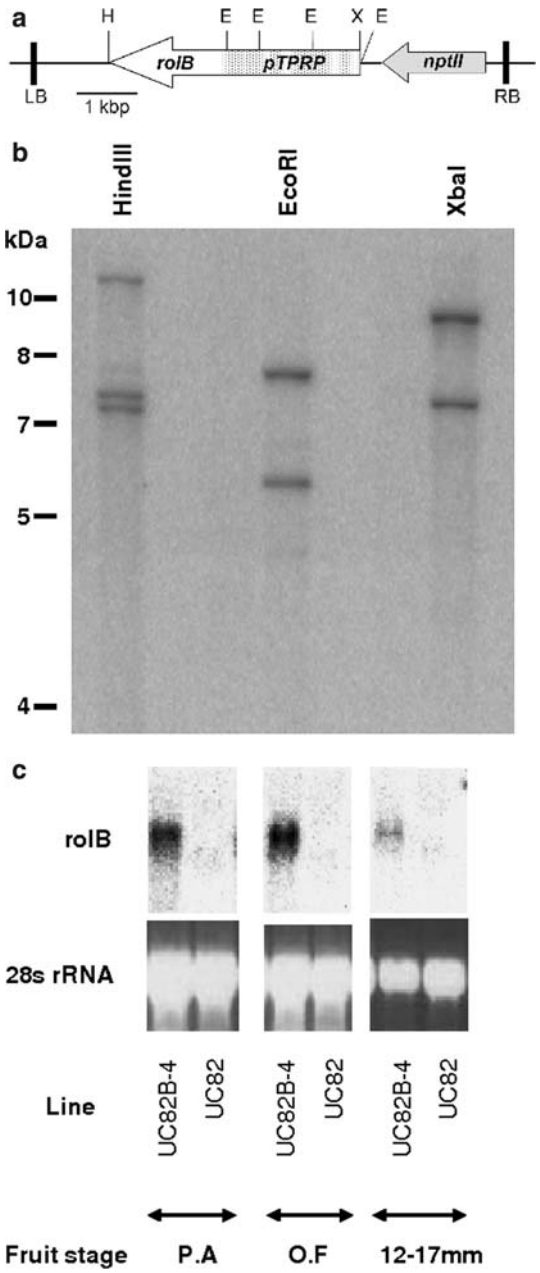

Fig. 1 Molecular analysis of line UC82B-4. **a** A chart presenting the T-DNA including the pTPRP::rolB transgene and the selective gene nptII between the right and left borders (*BR* and *BL*, respectively) of the binary vector pGBrolB. The restriction sites for EcoRI (*E*), XbaI (*X*) and HindIII (*H*) are presented. **b** RFLP analysis performed on DNA samples (ca. 10 μg) restricted with either HindIII, EcoRI or XbaI. **c** Northern analysis of *rolB* expression in developing fruits from the stages of: ovaries 1–2 days before anthesis (*P.A*); ovaries collected from open flowers (*O.F*); fruits of 12–17 mm in diameter. ^{32}P-ATP-labeled *rolB* probe served in both the Southern and Northern analyses, and visualized by phosphor-imager, 28S rRNA was visualized by ethidium bromide staining

Southern and Northern analyses were done following established procedures, and as previously described (Carmi et al. 2003).

Experimental procedures

To identify the transgenic progenies of line UC82B-4 at generations R_3 and R_4, seeds were surface-sterilized and germinated on solidified 1/2 MSO medium containing 100 mg/l kanamycin, where only the transgenic seedlings developed branched root system. Control (untransformed) UC82 seeds were similarly germinated on the same medium without kanamycin (protocol detailed in Barg et al. 1997). For hardening, plants were transferred to soil mixture, and grown in a walk-in growth chamber. Established plants were transferred to 10 l pots and grown in a net-house in Bet-Dagan (located at the central part of Israel). At generation R_5, seedlings were germinated in a soil mixture and PCR-tested for the presence of the transgene *rolB* (with the primers: tgact atagcaaacccctcctg and caagctacctctctcccgtaaa), before transfer to the greenhouse. In this generation plants were grown in the soil in a plastic-covered net house during the summer and in a net house in the winter experiment. ^{0}Brix was measured on squeezed juice, pooled from at least three red fruits per plant.

Statistical analysis

All the presented data (in Table 1) was subjected to one-way Anova analysis, performed using SigmaStat2.0 program. When Normality test passed, *t* test was performed, and Mann–Whitney rank sum test was done when it failed. Unless otherwise specified, the analyses were performed only on the yield of big red fruits (visually estimated as larger than 30 g), so as to present the agriculturally meaningful yield.

Results

Molecular characterization of line UC82B-4

Up to the sixth generation (i.e. R_5) we could not establish a homozygote UC82B-4 line. This indicates that homozygous embryos are not viable, and most probably it also reflects reduced viability of the *rolB* male gametes. The PCR results for *rolB* segregation tested at R_5 were subjected to Chi-square analysis. In some seed batches the H_0 hypothesis of 2:1 ratio of transgenic to WT progenies was found to be highly probable ($0.70 < P < 0.90$), but the H_0 hypothesis 1:1 ratio was also accepted ($0.2 < P < 0.3$). In other seed batches the H_0 hypothesis of 1:1 was highly likely ($P > 0.95$).

To determine the uniformity of the transgenic UC82B-4 plants, Southern analyses were performed at R_2, R_3 and R_4, on several groups of plants, each group representing progenies of a single self-pollinated plant. In all the three generations, all the analyzed plants gave the same patterns when the RFLP was tested using *rolB* as the probe, on DNA digested with three different restriction enzymes (XbaI, EcoRI and HindIII). As shown in Fig. 1a, the T-DNA region of the binary vector pGBrolB contains a single XbaI immediately upstream, and a single HindIII site immediately downstream to the transgene. And there are four EcoRI sites, one in the MCS upstream to the transgene and three within the pTPRP-F1 promoter, but none in the *rolB* gene. Hence, when probing with radio-labeled *rolB*, restriction with each of these enzymes is expected to give a single band if one copy of the transgene is incorporated to the tomato genome. A representative RFLP pattern for each restriction enzyme is shown in Fig. 1b. Restriction with either EcoRI or XbaI gave two bands, one twice the intensity of the other. The intensity was determined by quantifying the phosphorimages, using the ImageGauge3.12 program. The measured intensities were 1,240 and 2,260 psl for the two EcoRI, and 1,800 and 3,800 psl for the two XbaI restricted fragments. Three copies of *rolB* were visualized when restricting with HindIII. The intensity measured was 500 psl for the upper band and 1,130 and 1,140 psl for the two shorter ones. The lower intensity of the ca. 20 kDa band obtained in the HindIII digest, most likely results from higher frequency of breakage of such a long DNA fragment, and possibly also because of its lesser efficient trans-blotting to the membrane. The same restriction patterns shown in Fig. 1b were observed in all the tested progenies, in three successive generations, indicating that the three copies do not segregate, i.e. they are integrated into a single locus. Both the fact that there are three bands of different sizes following HindIII digest, and the fact that there is no common size band for the XbaI and EcoRI digests indicate that rearrangement(s) occurred while the T-DNA was transformed to the plant. Lack of rearrangements in the vector itself was confirmed (data not shown).

Northern analysis performed on developing fruits collected in the summer of 2003 (Fig. 1c) indicates that *rolB* is expressed already at pre-anthesis (1–2 days before anthesis) and expression persists in the young developing fruits.

Analysis of yield and fruit quality

To test the performance of the transgenic line under extreme temperatures, line UC82B-4 was compared to the parental line UC82 during several growing seasons and under diverse environmental conditions.

Table 1 Analyses of yield parameters of UC82B-4 compared to UC82 during four experiments, performed in the specified years and growing seasons

Experiment no., growth season and plant numbers (*n*)	Parameter	UC82	UC82B-4
1. Summer 2000	Number of fruits/plant	19.9 ± 2.26 a	30.2 ± 1.69 b
UC82 (*n* = 12)	Fruit weight (g/fruit)	32.4 ± 2.11 a	38.9 ± 0.83 b
UC82B-4 (*n* = 17)	Yield big red fruit (kg/plant)	0.64 ± 0.082 a	1.18 ± 0.076 b
	Big red fruit yield/total yield	0.49 ± 0.016 a	0.63 ± 0.019 b
	^{0}Brix	6.75 ± 0.173 b	5.58 ± 0.086 a
	^{0}Brix yield [^{0}Brix × yield (kg/plant)]	4.26 ± 0.483 a	6.54 ± 0.406 b
2. Spring 2001	Yield big red fruit (kg/plant)	1.36 ± 0.104 a	1.56 ± 0.108 a
UC82 (*n* = 7)	^{0}Brix	3.91 ± 0.074 a	3.74 ± 0.067 a
UC82B-4 (*n* = 14)	^{0}Brix yield [^{0}Brix × yield (kg/plant)]	5.30 ± 0.361 a	5.89 ± 0.431 a
3. Summer 2003	Number of fruits/plant	24 ± 3.70 a	45.8 ± 5.73 b
First harvest	Fruit weight (g/fruit)	40.9 ± 2.78 a	57.28 ± 2.10 b
UC82 (*n* = 5)	Yield big red fruit (kg/plant)	0.97 ± 0.160 a	2.54 ± 0.271 b
UC82B-4 (*n* = 10)	Big red fruit yield/total yield	0.28 ± 0.036 a	0.50 ± 0.047 b
3. Summer 2003	Number of fruits/plant	28.4 ± 2.64 a	49.3 ± 2.72 b
Second harvest	Fruit weight (g/fruit)	49.7 ± 3.43 a	61.5 ± 5.06 a
UC82 (*n* = 5)	Yield big red fruit (kg/plant)	1.40 ± 0.142 a	2.90 ± 0.124 b
UC82B-4 (*n* = 15)	Big red fruit yield/total yield	0.39 ± 0.035 a	0.67 ± 0.035 b
	^{0}Brix	4.0 ± 0.20 a	3.1 ± 0.12 b
	^{0}Brix yield [^{0}Brix × yield (kg/plant)]	5.9 ± 0.71 b	9.0 ± 0.55 a
4. Winter 2004	Number of fruits/plant	13.6 ± 1.15 a	35.7 ± 5.33 b
UC82 (*n* = 7)	Fruit weight (g/fruit)	43.3 ± 2.42 a	47.4 ± 1.13 a
UC82B-4 (*n* = 12)	Yield big red fruit (kg/plant)	0.59 ± 0.067 a	1.67 ± 0.243 b
	Big red fruit yield/total yield	0.17 ± 0.019 a	0.29 ± 0.033 b

The presented analyses were performed on red fruits larger than 30 g (also defined as big red fruit yield). Total yield included red fruits larger than 15 g and green fruits larger than 10 g. In Experiment 3, the first harvest was on July 8, 2003, and the second on July 15, 2003

For each analyzed parameter (i.e. within rows), values accompanied by different letters differ significantly (Anova analysis, Student–Newman–Keuls Method, *t* test, $P < 0.05$)

Improved yielding of line UC82B-4 under high temperatures

At generation R_3 plants were grown in 10 l pots in a 50-mesh net-house located at the Volcani center in Bet-Dagan (located at the central part of Israel). Plants were transferred to the net-house relatively late in the season on May 25, 2000 and fruits were collected on August 28, 2000. Flowering and fruit set occurred during late June and early July when the daily temperatures in the net house exceeded 35°C for several hours. Analysis of yield parameters is presented in Table 1 (Experiment 1), only red fruits larger than 30 g were included. The significant increase in the yield of red fruits in line UC82B-4 compared to the parental line UC82 reflects a significant increase both in the number of fruits and their average weight.

^{0}Brix was determined on juice that was hand-squeezed from the red fruits. Since line UC82 did bear less fruits than the transgenic line, not surprisingly the ^{0}Brix measured in its red fruits was significantly higher (Table 1, Experiment 1). Nonetheless, the ^{0}Brix yield [i.e. ^{0}Brix * Yield (kg) of big red fruit/plant] was still significantly higher in line UC82B-4 because of its significantly higher yield under the given environmental conditions.

Comparable yielding of UC82B-4 and UC82 under ambient temperatures

To test whether the transgene, which enabled better yielding of line UC82B-4 under high temperatures, bears a penalty on yielding under ambient conditions, the performance of this line was compared to that of UC82 earlier in the season. On March 1, 2001, R_4 seedlings of UC82B-4 and UC82 were transferred to 10 l pots and grown in a net-house (located in Bet-Dagan). Flowers and fruits developed in the spring under moderate temperatures. Daily temperatures did not exceed 32°C until 4 weeks before fruit harvest on June 6, 2001. As shown in Table 1 (Experiment 2), the two lines did not differ in their yield, neither did they differ in their ^{0}Brix or ^{0}Brix yield values.

Improved yielding under extremely high temperatures

To further evaluate the effect of the transgene on fruit development under even higher temperatures, at R_5,

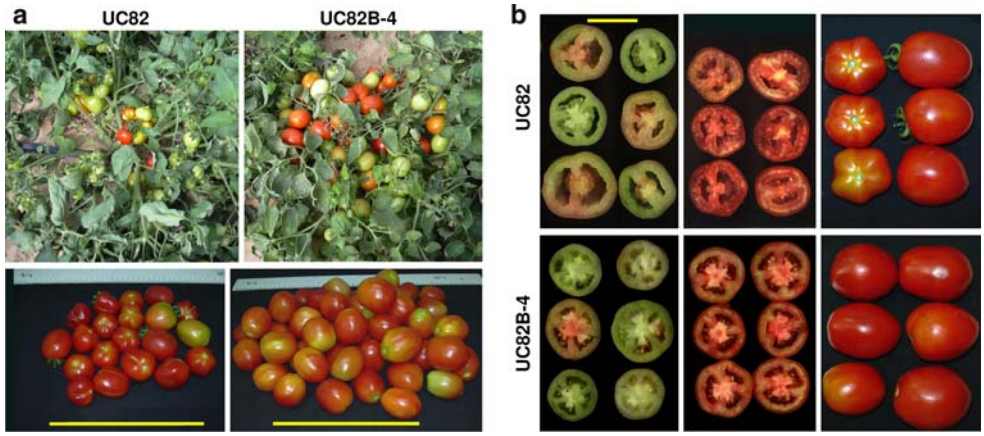

Fig. 2 Improved yielding of better quality fruits in line UC82B-4 plants grown in the summer of 2003. **a** UC82B-4 plant (*upper right panel*) grown in the summer bears much more fruits than UC82 plant (*upper left panel*). Plants were photographed on June 26, 2003. The fruits harvested from these plants were photographed on July 8, 2003 (*lower right and left panels*). *Bars* = 30 cm. **b** The fruits of UC82 are puffy and fasciated, whereas the UC82B-4 fruits are of smooth regular shape with improved jelly fill. *Bar* = 4 cm (in all the photographs). The photographed fruits were harvested on July 8, 2003

plants were grown in a plastic-covered net-house located in the southern (and warmer) part of Israel (situated in the village Netiv Haasara). The plants were transferred to the greenhouse on May 15, 2003. Five plants of UC82 and 10 plants of UC82B-4 were harvested on July 8, 2003, and other 5 plants of UC82 and 15 plants of UC82B-4 were harvested on July 15, 2003. During the period of flowering, fruits set and development, the temperatures within the plastic-covered greenhouse exceeded 38°C for at least 4 h a day.

Fruits harvested on July 8, 2003, were visually subdivided to three groups: big red fruits (> 30 g), small red fruits (only fruits > 15 g were included), and green fruits (only fruits > 15 g were included). As shown in Table 1 (Experiment 3), UC82B-4 yielded significantly more big red fruits than the parental line, and their average fruit weight was also significantly higher. The higher weight apparently reflects the fact that their jelly fill was substantially better. Moreover, the fruits of UC82B-4 were smooth and of regular shape, while the UC82 fruits were puffy and severely fasciated (Fig. 2). However, at the given harvest date the UC82 plants still did bear a substantial portion of green fruits. Thus, a similar analysis was performed on plants harvested a week later. Fruits were visually subdivided to four groups: big red fruits (> 30 g), small red fruits (only fruits larger than 15 g were included), big green fruits (> 30 g), and small green fruits (only fruits > 10 g were included). As shown in Table 1 (Experiment 3, second harvest), line UC82B-4 yielded significantly more than the parental cultivar, and that due to significantly greater number of big red fruits. The average fruit weight was also higher (but *t* test significance was only $P = 0.096$). The concentrated fruit production and its abundance in line UC82B-4 compared to the parental line UC82, is demonstrated in Fig. 2a. The promoting effect of the transgene on jelly fill and fruit shape regularity is shown in Fig. 2b.

The ^{0}Brix value was significantly higher in the parental. Nonetheless the calculated ^{0}Brix yield was significantly higher in the transgenic line (Table 1, experiment 3, second harvest).

Improved yielding under low temperatures

To examine whether the transgene supports better yielding also under low temperatures, seedlings (at R_5) from UC82 ($n = 7$ plants) and the transgenic line UC82B-4 ($n = 12$ plants) were planted in the winter (February 4, 2004) in a non-heated net house located in Bet-Dagan. The plants were harvested on May 20, 2004. During the month of March when fruit set occurred night temperature dropped below 10°C for more than 4 h in 21 out of 31 nights. As shown in Table 1 (Experiment 4), the yield of big red fruits of the transgenic line UC82B-4 was significantly higher than that of the parental line, and that due to significantly greater number of fruits reaching at least 30 g, thus indicating both enhanced fruit set and better fruit development in the transgenic line, which is expressed also as a significantly larger ratio of big red fruits from the total harvested yield (Table 1, Experiment 4). However, the few big red fruits of UC82 that did develop contained seeds, and were similar in weight (Table 1, Experiment 4) and shape (data not shown) to the transgenic fruits, and their jelly fill was complete, apparently because enough seeds developed.

Discussion

The performance of the transgenic line UC82B-4 was compared to that of the parental line UC82 in successive generations, during several growing seasons and under diverse environmental conditions. Line UC82B-4 is still maintained as hemizygous for a single locus containing three copies of the transgene (Fig. 1b). The fact that the three restriction products do not share a common size band indicates that rearrangements occurred while integrating the three copies to a single integration site, a phenomenon previously documented for T-DNA integration (e.g., Ohba et al. 1995; Tzfira et al. 2004). It is not clear as yet whether homozygosity could not be established due to reduced gametic viability, zygotic lethality, or both. That is because Chi-square analyses did not clearly distinguish between these possibilities. Moreover, the results of the analyses varied among different seed batches, suggesting that environmental parameters differently affected the *rolB*-related lethality. Reduced pollen viability due to *rolB* expression was previously reported by An et al. (1994). The fact that transient expression of the *TPRP-F1* promoter occurs in early stages of embryo development (Carmi et al. 2003, electronic supplement), could explain homozygous lethality. However, the fact that in another genetic background (in line MP-1) we could establish a homozygote line (Carmi et al. 2003), suggests that the lethality might be related to leakiness of *rolB* expression in line UC82B-4.

Improved yielding of UC82B-4 under extreme temperatures

A significantly superior performance of the transgenic line was repeatedly manifested in successive generations, and during two summer growing seasons (Table 1, Experiments 1 and 3), hence supporting our preliminary observation (Barg et al. 2001). In tomato, positive relation between seed number and fruit set and weight, and conversely, an adverse effect of under-fertilization on fruit set, fruit weight and its quality are well documented (e.g. Imanshi and Hiura1975; Picken 1984; Varga and Bruinsma 1976). Two parameters contribute to the higher yielding of the transgenic line under high temperatures. The first and major one is the significantly higher number of red fruits reaching at least 30 g by the time of harvest (Table 1, Experiments 1 and 3). This finding indicates that the transgene compensated for the adverse effect of under-fertilization, which severely reduced the number of fruits reaching 30 g in the parental line. The second parameter is the significantly higher average weight of the transgenic fruits [Table 1, Experiments 1 and 3 first harvest, a higher weight was found also in transgenic fruits harvested on July 15, 2003, although the difference was not significant ($P = 0.096$)]. The higher weight of the fruits apparently reflects the fact that the jelly fill in the transgenic fruits was substantially more complete than in the UC82 fruits. Most importantly, this difference was manifested also by development of smooth and regular fruits in line UC82B-4, unlike the puffy and fasciated fruits developed from under-fertilized UC82 ovaries as demonstrated in Fig. 2b. Thus unlike in the UC82 parthenocarpic lines generated by introducing the transgene *DefH9-RI-iaaM*, where fruit weight was significantly reduced without improving the yielding compared to the parental line in the summer (Rotino et al. 2005), in line UC82B-4, both fruit weight and yield were significantly higher than in the parental line.

A significantly higher yield of the transgenic line was observed under cold stress as well, and that due to development of a significantly higher number of fruits reaching at least 30 g (Table 1, Experiment 4). This indicates that the promoting effect of the transgene is effective also at low temperatures.

How is the expression of *rolB* in the ovary translated to parthenocarpic fruit development is still not clear. Yet it was elegantly shown that interaction of RolB with a 14-3-3 protein is essential for its nuclear localization and induction of hairy root initiation (Moriuchi et al. 2004). Thus one may speculate that once shuttled into the nucleus RolB modulates the expression of auxin-responsive gene(s), leading to fertilization-independent fruit development, similarly to auxin-induced parthenocarpy.

Interestingly, under the summer conditions, most of the fruits of the transgenic line reaching at least 30 g did bear more seeds than those of UC82 (Fig. 2b). At present there is no satisfactory explanation for this observation. Actually the widely accepted notion is that the fertilized ovules, i.e., the young embryos, promote the development of the parenchymatic placenta tissue to engulf the developing seeds (Ho and Hewitt 1986). We would like to propose that the opposite is also true. Namely, that profuse development of this engulfing tissue, in this case due to early *rolB* expression in the parenchymatic placenta, provides better contact with, and nourishment to the few developing embryos, resulting in a higher number of fully developed seeds. In line UC82, abortion of under-nourished embryos could explain the development of fairly large puffy fruits containing less than handful of seeds (see in Fig. 2b the transversely cut UC82 fruits). Thus it cannot be ruled out that in this particular line, the transgene actually improves the viability of the embryos,

and that in return, results in development of better quality fruits. Nonetheless, the fact that we got also large transgenic fruits completely seedless, that maintained a better fill of the jelly tissue compared to WT fruits, and the fact that under extreme temperatures, significantly more transgenic fruits reached weight above 30 g (Table 1, Experiments 1 and 3), indicate that the transgene induces parthenocarpic fruit development also in this genetic background, similar to its effect when introduced to other tomato cultivars (Barg et al. 2001; Carmi et al. 2003).

In processing tomato the ^{0}Brix yield is an economically important parameter. While under ambient conditions the two lines did not differ in their ^{0}Brix values or yield (Table 1, Experiment 2), in the summer the ^{0}Brix values in the hand-squeezed juice from the puffy UC82B fruits was significantly higher than in the jelly-filled transgenic fruits (Table 1, Experiments 1 and 3 second harvest). Since under high temperatures, the yield of line UC82 was significantly lower than that of the transgenic line, this finding agrees with the repeatedly observed negative relation between fresh yield and ^{0}Brix (e.g. Fulton et al. 1997 and references therein). Still, the differences in yielding were high enough to result in a significantly higher ^{0}Brix Yield per plant (Table 1, Experiments 1 and 3 second harvest) in the transgenic line.

To summarize, without reducing the yielding potential of this line under ambient environmental conditions, the transgene *TPRP-F1*::*rolB* significantly improves yielding of better quality tomato fruits both under extremely high and low temperatures.

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