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Evaluation of Tomato Plants with Constitutive, Root-Specific, and Stress-Induced ACC Deaminase Gene Expression[1]

V. P. Grichko*, B. R. Glick, V. I. Grishko**, and K. P. Pauls*****

**North Carolina State University, Campus Box 7622, Raleigh, NC 27695, United States;*
fax: 919-515-2047, e-mail: vpgrichk@ncsu.edu
***University of Waterloo, Waterloo, ON, Canada N2L 3G1*
****University of Guelph, Guelph, ON, Canada N1G 2W1*

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Abstract—Transgenic tomato (*Lycopersicon esculentum* Mill, cv. Heinz 902) plants expressing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase were compared with nontransformed plants in a number of traits that are thought to be affected by ACC and ethylene in plant tissues. In the transgenic plants, the ACC deaminase gene was under the transcriptional control of either two tandem 35S cauliflower mosaic virus promoters (constitutive expression), the *rolD* promoter from *Agrobacterium rhizogenes* (root-specific expression), or the PRB-1*b* promoter from tobacco (stress-induced expression). The parameters that were examined included plant growth, leaf fluorescence, protein and chlorophyll content, fruit weight, and also lycopene and β-carotene fruit content. Expression of ACC deaminase affected a number of these characteristics with the 35S and *rolD* promoters generally behaving similarly to one another and differently from either the nontransformed or the PRB-1*b* plants.

Key words: Lycopersicon esculentum cv. Heinz 902 - transgenic tomato plant - ethylene - ACC deaminase - growth - fruit

INTRODUCTION

Metabolizing ethylene immediate precursor, ACC, is the well-established approach to reduce ethylene levels in plants. Bacterium that contains ACC deaminase, which converts ACC to α-ketobutyrate and ammonia [1], and binds to plant roots acts as a sink for ACC, thereby lowering plant ethylene levels [2]. Treatment of plants with ACC deaminase-containing rhizobacteria promotes root elongation and decreases stress ethylene levels [3, 4]. In tomato plants, ACC deaminase expression, under the control of the cauliflower mosaic virus 35S promoter, reduced ethylene levels, and with the exception of a delay in tomato fruit ripening, the plants developed normally [5]. Transgenic tomato plants with ACC deaminase activity showed enhanced resistance to pathogens [6, 7].

In this study, transgenic tomato plants [7] transformed with an ACC deaminase gene from *Enterobacter cloacae* UW4 [8] under the control of a 2 × 35S promoter that is expressed constitutively [9], a *rolD* promoter that is root-specific [10] or a PRB-1*b* promoter that is stress-induced [11] were grown, and a range of properties were compared. Other studies have examined ripening, senescence, and pathogen resistance in transgenic plants [5–7, 12–14]. As far as we are aware, this is the first report that evaluates the effect of genetically modified ACC and ethylene levels on the transgenic tomato fruit overall quality and plant growth responses that might be essential for agriculture.

MATERIALS AND METHODS

Plant material. Seeds of *Lycopersicon esculentum* Mill, cv. Heinz 902, were purchased from Stokes Seeds Ltd. (Canada). Construction of transgenic plants, that were homozygous for a single copy of the ACC deaminase gene, was described in [7]. In these plants, the ACC deaminase gene was under the control of either the two tandem 35S cauliflower mosaic virus promoters (constitutive expression), the *rolD* promoter from *Agrobacterium rhizogenes* (root specific expression), or the PRB-1*b* promoter from tobacco (stress-induced expression). Western Blot analyses indicated that with the 35S promoter the ACC deaminase was found in both shoots and roots, with the *rolD* promoter it was detected only

[1] This article was submitted by the authors in English.

Abbreviations: ACC—1-aminocyclopropane-1-carboxylic acid; IR—infrared; PSII—photosystem II; WT—wild type.

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Table 1. ACC deaminase activity, mmol/(h g protein)

Plant organ	Wild type	Transgenic plants: promoter fused to ACC deaminase gene		
		35S	*rolD*	PRB-1*b*
Leaf	0.022 ± 0.009	0.805 ± 0.243*	0.029 ± 0.007	0.061 ± 0.030
Root	0.067 ± 0.016	0.641 ± 0.130*	0.830 ± 0.144*	0.151 ± 0.045
Green fruit	ns	0.781 ± 0.122	0.126 ± 0.036	ns
Red fruit	ns	0.170 ± 0.050	0.142 ± 0.026	0.084 ± 0.018

Note: (*) Significantly different from nontransformed plants; ns—not significant.

in roots, and with the PRB-1*b* promoter it was detectable in both shoots and roots only after appropriate elicitation, e.g., UV light or inoculation with *Verticillium dahliae* [7]. Plants were grown in Pro-Mix growth medium (General Horticulture, United States) in a greenhouse at 25°C (day) and 20°C (night). Seeds were germinated in 30 × 60 cm^2 flats. After two weeks of growth, seedlings were transferred to 13-cm diameter pots and after one month to 25-cm diameter pots. Plants were watered daily.

Measurement of ACC deaminase activity. Samples were ground in liquid nitrogen. Protein was extracted in a cold buffer containing 0.2 M Tris–HCl, pH 7.2, 2 mM EDTA, 2% polyvinylpyrrolidone, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride [15]. Insoluble material was removed by centrifugation at 4°C. Total protein was determined as described in [16] using BSA as a standard. ACC deaminase activity was measured as described in [1]. Blanks consisted of tissue extracts added to reagent in the absence of ACC.

Ethylene and CO_2 measurement. Concentration of ethylene was determined with a GC-17A gas chromatograph (Shimadzu, Japan) equipped with flame ionization detector and AT-1 column (Alltech, United States). Plant samples were kept in the closed vials in the dark at 25°C to allow ethylene accumulation. For on-line monitoring, IR spectra were recorded with a resolution of 1 cm^{-1} on an FT-IR spectrometer MB100 (Bomem, Canada). Fruits were kept in a 250-ml glass vessel attached to a 10-cm measuring cell equipped with KCl windows.

Chlorophyll, lycopene, and β-carotene determination. Chlorophyll was extracted from the third top leaf from five-month-old plants [17]. Lycopene and β-carotene were determined as described in [18].

Leaf fluorescence measurements. The efficiency of PSII was measured with a PAM-102 chlorophyll fluorometer (Walz, Germany). Prior to measurement, plants were dark-adapted for 30 min, and the third oldest leaf was probed at the same location by four-armed fiber-optics at the same intervals throughout the experiment. The measurements were made at an actinic light intensity of 50 μE/(m^2 s) and effective pulse duration of 600 ms at a repetition rate of 1 min, until the steady-state was reached. F_v/F_m ratio was measured at 3000 μE/(m^2 s).

Seed collection. The plants were pollinated naturally. Seeds obtained from ripe fruits were kept in a container with some fruit juice for 3 days at 37°C to remove gelatinous material that can inhibit germination. Seeds were then rinsed with water, air-dried, and stored at 4°C.

Growth assays. Growth pouches (13 × 16 cm) (Mega International, United States) were each filled with 10 ml of either water, 1 mM $AgNO_3$, or 1 mM HNO_3, at pH 5.7 adjusted with alkali, and autoclaved at 120°C for 20 min. Seeds were soaked in 70% ethanol for 1 min, immersed in a 1% bleach for 10 min, and rinsed with sterilized distilled water. Five seeds were aseptically placed in each pouch that was kept upright in a plastic tray. The tray was covered with plastic wrap and incubated in a Conviron CMP 32444 growth chamber (Controlled Environment, Canada) for 7 days with a photoperiod of 12 h and a photosynthetic photon flux of 12.9 μE/(m^2 s) at 20°C. The number of seeds germinated was monitored daily. At the end of the study, the root length, root and shoot fresh weights were measured.

Scanning electron microscopy. Roots were fixed for 2 h in 2.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2, at 20°C, washed in the same buffer for 1 h at 4°C, and dehydrated with acetone (50, 70, 95, and 100%) at 4°C. Samples were then dried in a critical-point dryer, affixed to stubs, coated with 30-nm gold layer, and examined with a Hitachi S570 scanning electron microscope at 15 kV.

Statistics. Tests for significance were performed with ANOVA using Microsoft Excel.

RESULTS

Significant levels of ACC deaminase activity were found in 35S plants in both leaves and roots, in *rolD* plants only in roots, while the PRB-1*b* plants did not display a significant enzyme activity (Table 1). The apparent activity seen in nontransformed (WT) plants may be the consequence of the nonspecific cleavage of ACC. Although ACC deaminase was found in yeast and fungi [19, 20] there is no evidence for the presence of this enzyme in tomato plants.

Table 2. Root length of one-week-old seedlings grown in growth pouches, mm

Medium	Wild type	Transgenic plants: promoter fused to ACC deaminase gene	
		35S	*rolD*
Water	75 ± 2	80 ± 2*	82 ± 2*
1 mM HNO_3, pH 5.7	58 ± 2	72 ± 2*	73 ± 2*

* Significantly different from nontransformed plants.

With the exception of PRB-1*b* seeds, which germinated slowly, there was no any consistent difference in the rate or percentage of germination of the transformed and nontransformed seeds (data not shown). As expected, germination of nontransformed seeds was delayed by silver. Surprisingly, germination of 35S and *rolD* seeds was accelerated in the presence of silver. Since the PRB-1*b* seeds germinated slowly, they were not included in this study. One-week-old seedlings grown in growth pouches had significantly longer roots than the nontransformed seedlings in different media (Table 2). The root weights exhibited the same behavior. For example, in nitrate medium, *rolD* and 35S seedlings had similar average root weights of 7.87 and 8.04 mg, respectively, while WT seedlings exhibited a significantly lower average root weight of 6.54 mg. In addition, the *rolD* plants had significantly higher shoot fresh weights. Treatment of seedlings with silver resulted in similar levels of silver accumulated by the roots of WT, 35S, and *rolD* plants and a significant increase in root measurements in 35S plants. The fresh shoot weights were approximately inversely correlated with the amounts of silver they accumulated. The shoot/root ratios of fresh weights, which were similar before the treatment with silver in all plants, did not change in the *rolD* transgenic plants while it dropped by 17% in both WT and 35S plants.

The transgenic plants grown in a greenhouse had fewer root hairs than the WT plants as observed by scanning electron microscopy (data not shown). Transgenic *rolD* and 35S plants had significantly higher shoot fresh and dry weights than nontransformed plants. All of the transgenic tomato plants appeared greener and more vigorous than the WT plants. A similar observation was made by Knoester *et al.* in [21] who reported that ethylene-insensitive tobacco mutants appeared to be greener than control plants. The chlorophyll and leaf protein content was the highest in *rolD* plants (Table 3). The ratio F_v/F_m, a measure of the quantum yield of PSII *in vivo*, was about the same for nontransformed and transgenic plants. When plants were treated with 10 ml of a 10 mM solution of $AgNO_3$, pH 5.7, on a daily basis for ten days, the F_v/F_m ratio was higher for transgenic plants despite the fact that average WT plants only accumulated 1.1 μg/g fr wt of silver in their shoots, while 35S, *rolD*, and PRB-1*b* plants accumulated as much as 1.5, 1.7, and 2.7 μg/g, respectively (Fig. 1).

The transgenic plants did not exhibit a visible delay in the onset of flowering or ripening of attached fruits. In 35S plants, green fruits had a higher level of ACC deaminase activity than ripening fruits (Table 1). Ethylene production in 35S fruits was lower as compared to that of nontransformed plants (Fig. 2). The increases in respiration were equal in nontransformed and 35S plants. The average fruit weight from *rolD* plants was found to be significantly higher than that from nontransformed, 35S and PRB-1*b* plants. With the exception of *rolD* plants, which behave like a determinate cultivar, both nontransformed and 35S plants exhibited a prolonged ripening period. Both 35S and *rolD* plants had a significantly higher content of lycopene and β-carotene in pericarp tissue of fully ripened fruit than nontransformed plants (Table 3).

DISCUSSION

The results were consistent with the supposition that ACC and endogenous ethylene was the main cause of the observed behavior of the transgenic plants. In agreement with the earlier observation [5], the ACC deaminase transgenic tomato plants exhibited more or less normal behavior in their growth and development. Thus, it appears that tomato plants can tolerate a wide

Table 3. Chlorophyll, protein, lycopene, and β-carotene contents

Index	Wild type	Transgenic plants: promoter fused to ACC deaminase gene		
		35S	*rolD*	PRB-1*b*
Leaf protein, mg/g fr wt	39.6 ± 2.0	43.2 ± 0.7*	53.9 ± 0.4*	28.8 ± 0.3*
Root protein, mg/g fr wt	11.4 ± 1.2	8.1 ± 0.3*	10.8 ± 0.5	10.5 ± 1.0
Leaf chlorophyll, mg/g fr wt	1.5 ± 0.1	1.6 ± 0.1	1.9 ± 0.1*	1.7 ± 0.1
Fruit lycopene, μg/g fr wt	8.7 ± 0.9	13.8 ± 1.0*	13.7 ± 1.1*	nd
Fruit β-carotene, μg/g fr wt	7.5 ± 0.7	11.7 ± 0.8*	11.2 ± 0.8*	nd

Note: (*) Significantly different from nontransformed plants; nd—not determined.

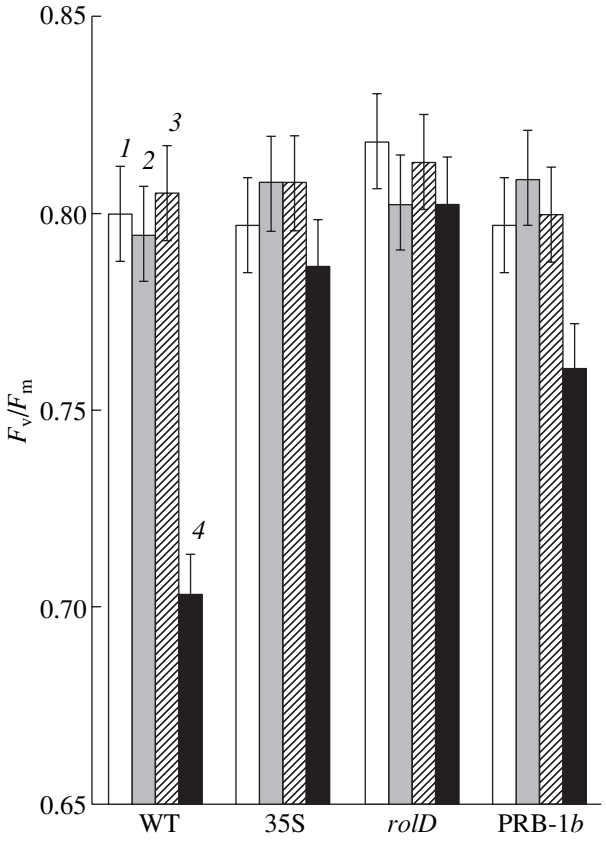

Fig. 1. Quantum efficiency of photosynthesis (F_v/F_m) in transgenic tomato plants and its temporal change with silver nitrate treatment.
(*1*) Initial value; (*2*) 2 days; (*3*) 6 days; (*4*) 10 days. Error bars represent standard errors.

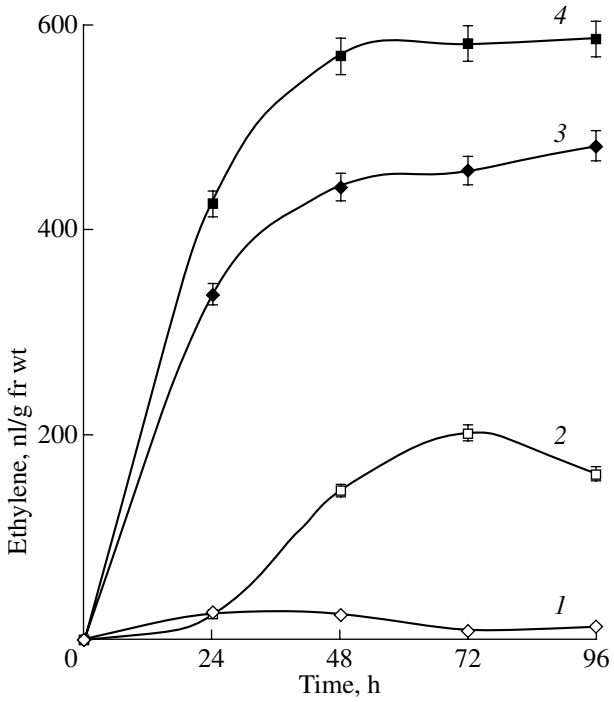

Fig. 2. Ethylene emission by fruits of wild-type and 35S transgenic tomato plants.
(*1*) 35S, green; (*2*) WT, green; (*3*) 35S, red; (*4*) WT, red. Error bars represent standard errors.

range of ethylene levels for many physiological processes. In some cases, the behavior of *rolD* plants was more like that of 35S plants than that of WT plants, suggesting an important role of both root-derived ACC and its transport in tomato plant growth and development. For example, 35S and *rolD* seeds germinated normally, but responded to silver in a different way than WT seeds. Silver is an inhibitor of ethylene receptors, and treatment of plants with low doses of silver should make them insensitive to ethylene [22]. Note that silver is a heavy metal, and can be toxic to a plant when accumulated at a high level [22]. The increase in germination of silver-treated transgenic seeds can be the result of activation of an ethylene biosynthetic pathway followed by the elevated production of (α-ketobutyrate that could accelerate plant growth. Also, 35S and *rolD* plants developed significantly longer roots than nontransformed plants. The similar effect was observed in the experiments with the ACC deaminase containing rhizobacteria [3]. In *Arabidopsis thaliana*, roots of *eri-1* (ethylene root insensitive) and *etr1-3* (ethylene insensitive) mutants were longer than roots of the WT plant [23].

Laser fluorescence spectroscopy and the pump-and-probe chlorophyll fluorescence measurement *in situ* are used to detect stress in plants, select mutant, and study the photosynthesis [24]. In this study, ACC deaminase transgenic plants (especially *rolD* plants) showed a higher chlorophyll content and a similar fluorescence ratio when compared to WT plants. PSII activity in the transgenic plants was barely affected by the high level of silver accumulated by plant organs. This is consistent with the earlier report on the increased tolerance of ACC deaminase transgenic plants to the heavy metal stress [25] and other observations. For example, under stress conditions, the photosynthetic capability of antisense ACC oxidase transgenic tomato plants was found to exceed that of WT plants [14]. A 50% decrease in leaf chlorophyll content in WT tomato plants and a small increase in chlorophyll content were observed in antisense ACC oxidase plants subjected to a severe light stress [26].

Ethylene initiates the events that lead to fruit ripening. During ripening, the color of tomato fruit changes due to the transformation of chloroplasts into chromoplasts. It was found that the lycopene and β-carotene content in ripe transgenic fruits was higher than in WT fruits. That is in a good agreement with the data on the increased chlorophyll content in transgenic plants. Moreover, it has been reported that tomato fruits, from transgenic lines expressing S-adenosylmethionine decarboxylase under the control of the ripening and

ethylene regulated E-8 promoter, accumulate several-fold higher levels of lycopene and ripened more slowly than WT plants [27]. In this study, it was observed that the specific activity of ACC deaminase was low in ripening tomato 35S fruits, probably reflecting only a moderate level of transgene expression. It is likely that, in the previously described transgenic tomato plants, ACC deaminase was expressed to a higher level and that the ethylene concentration was lowered to a greater extent than in the present study [5]. Since ACC oxidase binds ACC about 100-fold more tightly than does ACC deaminase, ACC deaminase must be present in a significantly greater amount than ACC oxidase to be effective at lowering ACC and ethylene levels in plants [2]. Thus, a very high level of ACC deaminase may prevent ACC oxidase from being induced thereby slowing fruit ripening and consequently reducing lycopene level in ripening fruits. In attached, fully ripened transgenic fruits, lycopene content may be higher due to the increased supply of α-ketobutyrate, which can be utilized in many biosynthetic pathways.

The results presented in this study suggest that the ACC deaminase transgenic tomato plants exhibit a very healthy and more productive phenotype compared to the WT plants. For most of the characteristics tested, *rolD* and 35S plants showed similar responses and phenotypes. They had larger roots and shoots, higher chlorophyll leaf and protein content, increased average fruit weight, and increased lycopene and β-carotene content in mature fruit. These ACC deaminase transgenic tomato plants also displayed increased resistance to phytopathogens, heavy metal stress, and flooding stress [4, 6, 7, 25]. Taking into account numerous issues associated with the introduction of a genetically modified food on a market, *rolD* plants look as very promising candidates. With the transgene expressed in the no edible part of the plant, these plants have a significantly improved phenotype and a much better fruit quality.

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