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Root and Hypocotyl Growth in Transgenic Tomatoes That Express the Bacterial Enzyme ACC Deaminase

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The seeds of tomatoes transformed with a bacterial ACC deaminase gene under the transcriptional control of either the cauliflower mosiac virus *35S* promoter, the *rol*D (root specific) promoter or the *prb-1b* (pathogenesis related) promoter, and the seeds of nontransformed (NT) tomato plants were germinated in the presence or absence of ACC in the dark, in the light, or in the presence of UV-B light for 12 days. Roots grew to a greater length under UV-B than in the light or dark, in the absence of ACC for all of the plant lines tested. In contrast, root growth was inhibited by ACC under all conditions tested. The *35S*/ACC deaminase transgenic seedlings had the longest hypocotyl, the *rol*D/ACC deaminase had intermediate sized hypocotyls and the NT and the *prb-1b*/ACC deaminase had the shortest hypocotyl in the presence of ACC in the dark. Hypocotyl growth was inhibited by both light and UV-B in both the absence and presence of ACC in NT and transformed plants. Taken together these results indicate that root growth is more sensitive to applied ACC (the precursor of ethylene) than is hypocotyl growth under all of the light regimes tested.

Keywords: ACC deaminase, Ethylene, Hypocotyls, Root, Tomato, UV-B

The plant hormone ethylene elicits a wide range of plant responses during various developmental stages such as seed germination, root and hypocotyl growth, hook opening, flowering, fruiting, and leaf and flower senescence (Yang and Hoffman, 1984; Kieber, 1997). Ethylene exhibits the triple response: inhibition of root and hypocotyls elongation; swelling of hypocotyls; and exaggeration in the curvature of apical hook by inhibiting cell elongation and increasing lateral expansion in Arabidopsis and other plants when germinated in presence of ethylene (Ecker, 1995). Although the metabolic pathway of ethylene biosynthesis has been dissected, the relationship between ethylene, light and UV-B on hypocotyl and root growth is poorly understood.

Ethylene inhibits rooting in *Vigna radiata* (Geneva and Heuser, 1983) and root elongation in crop plants (Coleman et al., 1980). On the other hand, considerable research has demonstrated the stimulatory role of ethylene on root growth in mung bean (Mudge and Swanson, 1978; Robbins et al., 1983; Jusaitis 1986) and in tomato (Pathak et al., 1981), adventitious rooting in sunflower (Liu and Reid, 1992) and root hair formation in Arabidopsis (Tanimoto et al., 1995). Clark et al. (1999) observed more adventitious roots in wild type than in ethylene-insensitive tomato, suggesting a key role for ethylene in adventitious root initiation. Loss of root penetration capacity of tomato seedlings through compressed media was observed when ethylene biosynthesis was inhibited (Zacarias and Reid, 1992). Despite extensive research on the role of ethylene on root development, it is not clearly understood whether ethylene stimulates lateral roots, root hairs or main root initiation and growth.

Canola and tomato seeds treated with certain plant growth promoting rhizobacteria produced seedlings with longer roots (Glick, 1995; Hall et al., 1996; Mayak et al., 1999). This effect was attributed to the activity of the endogenous bacterial ACC deaminase enzyme (Glick et al., 1998) which converts ACC the immediate precursor of ethylene in plants into α-ketobutyrate and ammonia. In addition, a lowering of ethylene levels was found in tomatoes expressing the ACC deaminase gene driven by the cauliflower mosaic virus *35S* promoter (Klee et al., 1991; Sheehy et al., 1993; Robison et al., 2001) or the *rol*D (root-specific) promoter (Robison et al., 2001).

UV-B treated plants exhibit short hypocotyls and expanded cotyledons with Arabidopsis seedlings (McNellis and Deng, 1995). Although the role of UV-B on hypocotyl growth is known, its role on root development is poorly understood. Recently it was found that the *prb-1b*/ACC deaminase gene construct was expressed

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Abbreviations: ACC, 1-Aminocyclopropane-1-carboxylic acid.

only in transgenic tomatoes that was treated with UV-B (Tamot et al., 2003). Moreover, characterization of transgenic plants with *35S*/ACC deaminase, *rol*D (root specific promoter)/ACC deaminase and *prb-1b*/ACC deaminase (UV-B inducible promoter) should facilitate an understanding of the precise role of ethylene and its interaction with UV-B in root and hypocotyl growth.

MATERIALS AND METHODS

Untransformed (*Lycopersicon esculentum* Mill cv. Heinz 902) and T_2 homozygous seeds of transgenic tomato lines (Robison et al., 2001) carrying the ACC deaminase gene from *Enterobacter cloacae* UW4 (Glick et al., 1995; Shah et al., 1998) under the control of the cauliflower mosaic virus *35S* promoter, (i.e., independent lines C22, C27, C211, C223), the *rol*D root specific promoter (i.e., independent lines C31, C39, C317, C319) or the *prb-1b* pathogenesis-related protein promoter (i.e., independent lines C41, C44 and C49) were selected for further study. T_2 seeds of transgenic and nontransgenic siblines, selected after selfing the primary transgenics, were used for the present investigation.

The seeds were surface sterilized with 25% bleach and then rinsed thoroughly with distilled water. Unless otherwise mentioned, the seeds were plated on Murasige and Scoog (1962) medium, 2% sucrose and 0.6% (w/v) agar. Filter sterilized ACC was added to the plate before planting seeds. The seeds were germinated in a growth chamber set at 22 ± 2°C. The average photosynthetic photon flux density in the growth chamber was 300 µmol $m^{-2}s^{-1}$ at the top of the plants.

UVB light was provided by a UVB 313 (Q-Panel, Cleveland, OH) fluorescent bulb along with a white cool fluorescent bulb for 24 h/day. The total output of the UVB 313 fluorescent bulb was measured with a calibrated Spectroradiometer (Oriel Corp., Stratford, CT). A 0.076-mm thick cellulose diacetate film was used to absorb all radiation below 290 nm (Middleton and Teramura, 1993). One µmol $m^{-2}s^{-1}$ of UVB was provided on the surface of plant leaves by adjusting the distance of the plants from the source of light.

Total soluble protein from leaves and roots were separated by 9% (w/v) SDS-PAGE and then Western blotted as described previously (Tamot et al., 2003). The blot was probed using a rabbit antibody directed against purified ACC deaminase with the second antibody being goat anti-rabbit IgG-conjugated to alkaline phosphate (Sigma, St. Louis, MO). The ACC deaminase antibody bound to membrane was detected colorimetrically as described by the supplier (Boehringer Mannheim, Colorometric Western Blotting Kit).

RESULTS

To determine the expression level of ACC deaminase under the control of various promoters, proteins from leaves and roots of all transgenic and NT plants were characterized immunologically. Figure 1 is a Western blot showing that as expected, ACC deaminase protein was present in the leaf and root samples of all C2 lines (carrying the *35S* promoter) as well as in root samples of the C3 lines (which have the *rol*D promoter). However, no ACC deaminase was detected in either leaf or root samples of the C4 transgenic lines (which carry the *prb-1b* promoter). Previous measurement (Robison et al., 2001) of ACC deaminase enzyme activity in dark-grown seedlings yielded expression patterns that were similar to what was observed here by immunological assays. In particular, the enzyme levels in C2 hypocotyls were high but were absent in hypocotyls of C3 and C4 plants. C2 and C3 plants had high levels of ACC deaminase activity in their roots. Moreover a lower level of ethylene was found in *35S*/ACC deami-

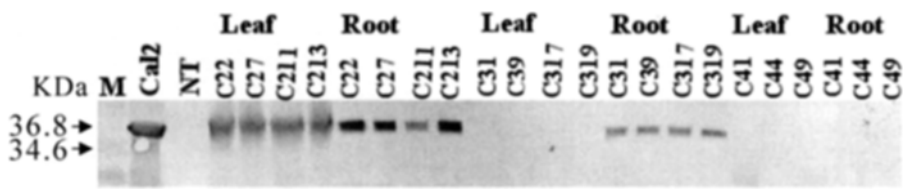

Figure 1. A Western blot of ACC deaminase protein in leaf and root homogenates from nontransformed, *E. cloacae* CAL2 and transgenic tomato plants with ACC deaminase under the control of the *35S* promoter (C2), *rol*D promoter (C3), and *prb-1b* promoter (C4). All sample lanes contained 80µg of soluble proteins. M, Molecular Weight Markers; B, *E. cloacae* CAL2 extract, NT, Nontransformed, Leaf of root transgenic tomato plants C2 (35S/ACC deaminase; Lane 4, 5, 6, 7, 8, 9, 10, 11), Leaf and root o C3 (*rol*D/ACC deaminase; Lane 12, 13 , 14, 15, 16, 17, 18, 19), Leaf and root of C4 (*prb-1b*/ACC deaminase; Lane 20 , 21, 22, 23, 24, 25).

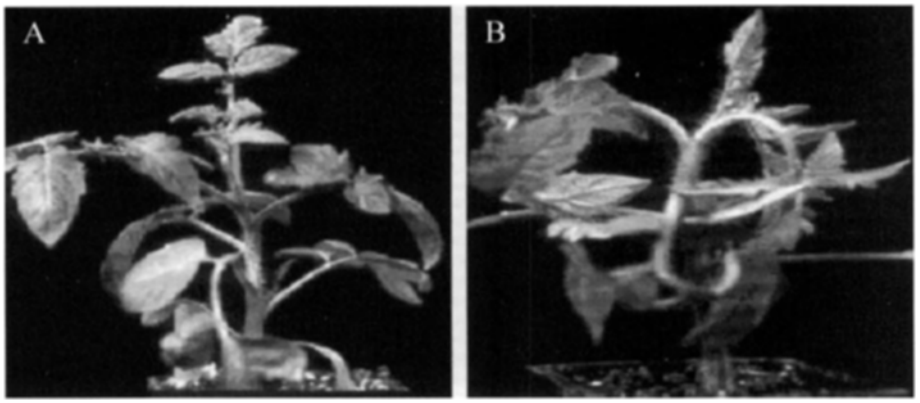

Figure 2. (A-B). ACC infiltered transgenic tomato plant with the ACC deaminase gene with the *35S* promoter (A) and non-trangenic tomato (B). 20 mM ACC (50 µL) was infiltered into four week old 35S/ACC deaminase transgenic and nontransgenic tomato plants. The picture was taken after two hours of ACC infilteration.

nase transgenic tomatoes than in *rol*D or *prb-1b*/ACC deaminase and nontransformed tomatoes (Robison et al., 2001).

The effect of the transgene expression in tomatoes was tested by injecting ACC into the stem apex of NT or C2, C3, and C4 plants. In the NT, ACC caused severe epinasty in the shoots (Fig. 2B). Similar observation was also made in C3 and C4 plants (data not shown). By contrast, the C2 transgenic plants were resistant to the ACC injection (Fig. 2A).

Response of hypocotyl and root growth to various doses of ACC in nontransformed (NT) tomato is shown in Table 1. Tomato seedlings that were germinated on an agar surface containing up to 20 µM ACC had shorter hypocotyls and roots in comparison to seedlings grown without ACC. In addition, seeds germinated in 20 µM ACC exhibited a typical triple response, namely extremely short, swollen hypocotyls and closed hooks.

To elaborate the role of ethylene on root development, the seeds of C2 and C3 transgenic tomatoes which have a reduced level of ethylene were germinated under light. Table 2 shows the root regrowth of tomato plants grown in water for nine days in light after the original roots were excised. The data indicate that the roots of C2 lines were apparently 25% longer than the C4 and NT plants. The C3 lines had 15% longer root length between the C4 and NT lines. There was no significant difference in root regrowth between C4 lines and NT plants.

When the NT and transgenic (C2, C3, and C4) lines were germinated in the dark on the surface of an agar plate without ACC, C2 lines had the longest roots (approximately 10%) greater than the C4 NT lines while C3 lines had 5% longer roots (Fig. 3B). In all instances, the root growth was inhibited by 20 µM ACC although the C2 and C3 lines continued to have the longest roots. In the absence of added ACC in the seed-germinating medium, there was no difference in the phenotype of hypocotyl growth between the transgenic and NT lines. The NT and C4 lines exhibited a triple response when they were grown on the ACC (20 µM) containing media in the dark (Fig. 3A). Under the same conditions, the hypocotyls of the C2 lines (which were approximately 75% longer than the NT and C4 lines) were nearly identical (elongated, closed hook and not swollen hypocotyl) to hypocotyls grown in the absence of ACC while the C3 hypocotyls were an intermediate length (i.e., 25% longer than the hypocotyls from the NT and C4 lines) (Fig. 3A).

When the seeds of the NT, and C2, C3, and C4 transgenic tomato lines were germinated in the light, the seedlings of the NT and C2, C3 and C4 plants

Table 1. Hypocotyl and root length of NT Tomato grown in various doses of ACC. For more details see legend of Figure 3.

ACC (µM)	Hypocotyl (cm)	Root (cm)
0	13.1 ± 0.8	3.2 ± 0.4
1	12.7 ± 0.9	2.9 ± 0.3
5	9.7 ± 0.8	2.2 ± 0.3
10	7.8 ± 0.6	1.7 ± 0.4
20	3.9 ± 0.3	1.2 ± 0.2

Table 2. Root growth in NT, C2, C3, and C4 transgenic tomatoes in water. Seeds were germinated in a growth pouch in water vertically for 9 days under white light.

	Root length (cm)	± SE
Nontransformed	5.8	± 1.3
C2	8.1	± 1.4
C3	7.2	± 1.7
C4	5.9	± 1.6

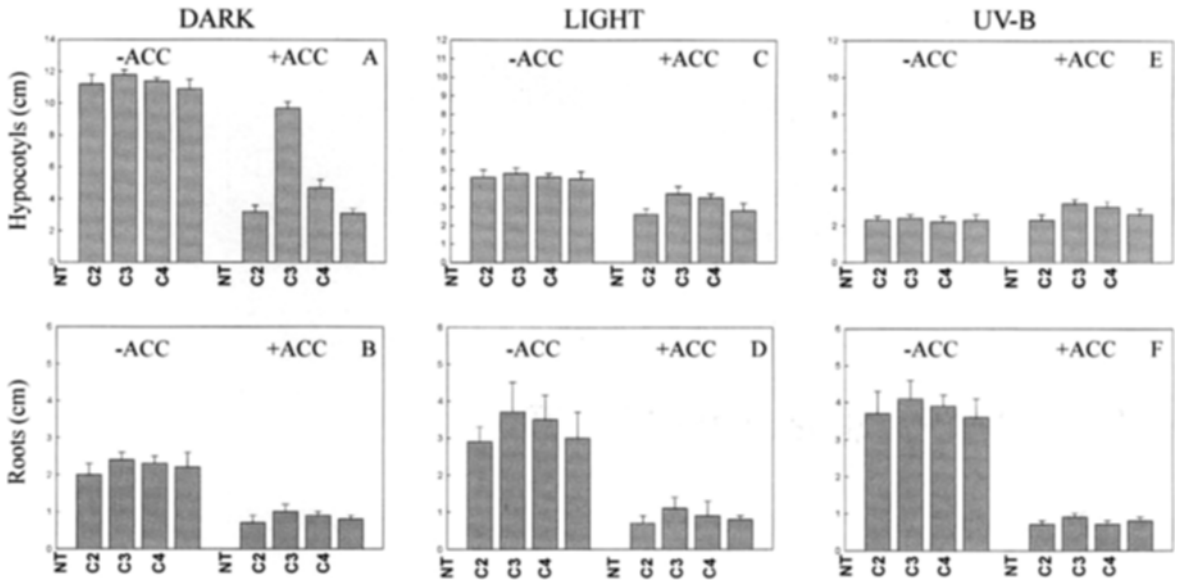

Figure 3. (A-F). Surface sterilized seeds of NT and transgenic tomato plants with the ACC deaminase gene with a *35S* (C22, C27, C211, C223), *rol*D (C31, C 39, C317, C319) or *prb-1b* (C41, C44 and C49) promoter germinated in the dark, light and UV-B with and without 20 μM ACC for 9 days in a vertically oriented plate. The value of each vertical bar in each figure with ± SEM is the pooled mean value of 4 lines of C2 (80 seedlings) and C3 (80 seedlings) and 3 lines of C4 (60 seedlings) with at least two replications. The experiments were repeated minimum twice.

grown without ACC, all had a similar phenotype green expanded cotyledons, short hypocotyls and short roots (Fig. 3, C and D). The length of the hypocotyls of the C2 lines grown with ACC under light, were slightly longer than those of the C3, C4 and or NT lines (Fig. 3C). Root growth in C2 (>15%) and C3 (>10%) lines was moderately longer than in the NT and C4 lines grown in light without ACC. However, the root growth of all transgenic lines (C2, C3, and C4) and the NT line was inhibited by 20 μM ACC when seedlings were grown in the light (Fig. 3D).

In both transgenic lines and the NT line, UV-B significantly enhanced root growth (approximately 20%) in seedlings grown under UV-B without ACC (Fig. 3F) compared to seedlings grown in the dark (Fig. 3B). Transgenic C2 lines had 10% longer roots than the NT and C4 plants whereas C3 lines exhibited 5% longer roots compared to the NT and C4 lines grown without ACC under UV-B. Root growth was severely suppressed by ACC (20 μM) in all of the seedlings grown under UV-B (Fig. 3F). UV-B drastically suppressed hypocotyl and cotyledon (data of cotyledon not shown) growth in all transgenic lines as well as in the NT line grown with or without ACC in the medium (Fig. 3E). C2 transgenic lines grown under UV-B without ACC had much expanded and greener cotyledons than the C3, C4, or NT lines. However, no difference was observed in the seedlings grown under UV-B with ACC.

DISCUSSION

The results presented here suggest that UV-B stimulates root growth in transgenic tomatoes that express a bacterial ACC deaminase to a greater extent than in NT lines. The longer taproot growth in the *35S*/ and *rol*D/ ACC deaminase transgenic tomato is presumably the result of the lower level of ethylene in these plants. The lower level of ethylene in *35S*/ACC deaminase transgenic tomato has been demonstrated previously (Klee et al., 1991; Sheehy et al., 1993). The observations of greater root growth in the transgenic tomatoes with the *35S*/ACC deaminase and *rol*D/ACC deaminase than in the NT tomatoes are in agreement with results with various mutants of ethylene biosynthesis and perception (Ecker, 1995; Hau et al., 1995; Roman et al., 1995; Clark et al., 1999; Barry et al., 2001). Ethylene changes the growth pattern of primary roots by reducing primary cell division (Schiefelbein et al., 1997). When ACC was added to those transgenic lines, root growth was sharply inhibited in the dark, in the light and in UV-B (Fig. 2, B, D, and F). The inhibition of root growth by ACC could not be overcome by an ACC deaminase gene driven by a *35S* or *rol*D promoter under any conditions.

A possible explanation for longer root growth under

UV-B and light may relate to the fact that both stimulate short hypocotyls and young leaves. In expanding leaves, auxin is synthesized and transported to the root where it enhances root elongation (Hobbie and Estell, 1995). Auxin is also used to induce rooting in plantlets developed by in vitro culture. However, if auxin is the primary hormone that induces root growth, there should not be a difference in root length between transgenic (C2 and C3) and NT lines. The results presented here and the studies of Liu and Reid (1992) and Clark et al. (1999) indicates that auxin may not be the sole root growth inducer, but rather that the level of ethylene is also involved in regulating root growth in plants. It is likely that ethylene, in conjuction with auxin, is required for root devolvement. In this regard, in a study of adventitious rooting in mung bean cuttings, using plant growth promoting bacteria with and without ACC deaminase activity and producing different levels of IAA, it was concluded that while ethylene was responsible for the formation of root primordia, root length was a consequence of both the ethylene and IAA concentration (Mayak et al., 1999).

Our results indicate that more inhibition of hypocotyl elongation in transgenic tomatoes under UV-B than in the light. This is inconsistent with similar observations in tomato (Ballaré et al., 1995; McNellis and Deng, 1995) and Arabidopsis (Boccalandro et al., 2001). Kim et al. (1998) showed that UV-B inhibition of hypocotyl growth was mediated by phytochrome A and B. The *35S*/ACC deaminase gene cassette-transformed tomato had the same seedling phenotype when grown in ACC in the dark as did ethylene insensitive tomato mutants (Guzman and Ecker, 1990) and ethylene insensitive Arabidopsis mutants (Roman et al., 1995). Transgenic tomato plants in which the ACC deaminase gene was driven by the *rolD* promoter had slightly longer hypocotyls than the *prb-1b* promoter transgenic and the NT, when grown in the dark in ACC. Interestingly, no such drastic change in seedling growth (hypocotyls) was observed either in C2 or C3 transgenic tomatoes in light and UV-B.

One possible reason for not finding a difference in hypocotyl growth between transgenic ACC deaminase gene with *35S/ rolD* promoter and non-transgenic tomatoes may be that these promoters are sensitive to light. Hypocotyl growth in light is a complex phenomena which is controlled by photoreceptors such as phytochromes (Quil et al., 1995), cryptochrome (Ahmad and Cashmore, 1997) and UV receptors (Boccalandro et al., 2001); ethylene (Ecker,1995); and auxin (Wightman and Thimann, 1980). While auxin has a more important role in hypocotyl development in the light than in the dark (Jensen et al., 1998), our results suggest that ethylene has a greater effect on hypocotyl growth in the dark than in the light. Although light induces ethylene biosynthesis in plants (Goeschl et al., 1967; Janes and Loercher, 1976), ethylene biosynthesis under UV-B is poorly understood. To our great surprise the *prb-1b*/ACC deaminase gene construct was only expressed in transgenic tomatoes exposed to 48 h of UV-B (Tamot et al., 2003). Transgenic plants like this may be useful in the future to monitor the level of UV-B.

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