Behaviour of Tomato Plants Genetically-Manipulated to Have a Lower Level of Ethylene

by

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ABSTRACT

Compartmentalization of enzymes involved in ethylene synthesis plays an important role in a plant's response to flooding and other stresses. Heterologous gene expression, plant growth-promoting bacteria with ACC deaminase activity and selective inhibitors were used to ameliorate the effects of stress ethylene. In this study, the upstream region of the *Enterobacter cloacae* UW4 ACC deaminase gene was found to contain FNR-, CRP- and LRP-binding sites and an novel LRP-like protein named Bacca that appear to be involved in the transcriptional regulation of the *acd* gene. To determine the effect of ACC deaminase activity on the phenotype of tomato plant, transgenic plants were compared to non-transformed plants in a number of traits including root and shoot growth, leaf chlorophyll content, fruit weight, lycopene and β-carotene content, and the effect of Ag^{+}. The *acd* gene was under the transcriptional control of either the 35*S* cauliflower mosaic virus promoter, the *rolD* promoter from *Agrobacterium rhizogenes* or the PRB-1*b* promoter from tobacco. The 35*S* and *rolD* plants behaved similarly to one another and differently from the non-transformed plants. Transgenic tomato plants were also compared to the non-transformed plants in the ability to accumulate Cd, Co, Cu, Mg, Ni, Pb, or Zn. Parameters that were studied include metal concentration, ACC deaminase activity, root and shoot growth, and leaf chlorophyll content. Transgenic tomato plants acquired a greater amount of metals and were less affected by the metals than were non-transformed plants. Both transgenic tomato plants and plants grown from seeds bacterized with bacteria expressing ACC deaminase were compared to non-transformed plants in their response to flooding based on the shoot height, fresh and dry weight,

epinasty, ACC deaminase activity, ethylene production, and chlorophyll and protein content. All of the transgenic tomato plants as well as the bacterized plants showed a significantly increased tolerance to flooding stress.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic-acid
acd	ACC deaminase gene
Amp	ampicillin
AOA	aminooxyacetic acid
AVG	L-α-(aminoethoxyvinyl)-glycine
BACCA	putative activator of *acd* transcription in *E. cloacae* UW4
cAMP	cyclic adenosine monophosphate
CRP	catabolite repressor protein
DW	dry weight
FNR	fumarate-nitrate reduction regulatory protein
FW	fresh weight
GC	gas chromatography
IAA	indole-3-acetic acid
kb	kilobase
kD	kilodalton
LRP	leucine responsive regulatory protein
ORF	open reading frame

1. INTRODUCTION

Ethylene which is produced by almost all plants, mediates a range of different plant responses and developmental steps (Yang and Hoffman, 1984; Mattoo and Suttle, 1991; Abeles et al., 1992). Ethylene plays an active role in the stimulation of seed germination, tissue differentiation, the formation of root and shoot primordia, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and senescence, pollination, fruit ripening and degreening, the production of volatile organic compounds responsible for aroma formation in fruits, storage product hydrolysis, leaf and fruit abscission, and the response of plants to both biotic and abiotic stress (Neljubow, 1901; Mattoo and Suttle, 1991; Abeles et al., 1992; Frankenberger and Arshad, 1995). Ethylene is also involved in plant-microbial symbiotic interactions that are important in plant nutrition and growth and are essential for establishment of legume-*Rhizobium* association (Lynch and Brown, 1997; Glick et al., 1999).

With various stresses in plants, the increase in ethylene synthesis serves as the only known common step in the complex chain of events leading to a variety of responses. Not only ethylene biosynthesis but even the emission of ethylene by a plant is strictly regulated, varying from one organ to another and among different species (De Vries et al., 1996). The inability of a stressed plant to initiate ethylene-related responses decreases its chance for survival (O'Donnell et al., 1996; Saab and Sachs, 1996; Morgan and Drew, 1997; Nilsen and Orcutt, 1997).

1.1. Ethylene in flooding

Deprivation of oxygen to the roots of plants is the main consequence of flooding (Kozlowski, 1984; Jackson, 1985). Under normal conditions, water dissolves about 230 mmol m^{-3} of oxygen and hypoxia occurs when the oxygen level falls below 50 mmol m^{-3} (Nilsen and Orcutt, 1997). Within one hour of flooding, the oxygen partial pressure declines from 20.8 to 7.9 kPa, and further decreases to 1 kPa after one day of flooding (Nilsen and Orcutt, 1997). Shortly after the onset of flooding, soil microorganisms consume all of the available oxygen, and different toxic compounds begin to accumulate in the soil (Fukuda et al. 1993; Frankenberger and Arshad, 1995).

Plants respond to flooding with a reduced root permeability, water absorption and mineral uptake; closure of stomata followed by a decrease in photosynthesis; alterations in hormone balance; inhibition of stem and root growth; hypertrophy of lenticels; development of aerenchyma and adventitious roots; epinasty, chlorosis, leaf abscission and premature fruit drop, which is accelerated by increased production of ethylene (Vartapetian and Jackson, 1997).

Aquatic plants with slow root ethylene synthesis (e.g., rice) show a positive adaptive response to ethylene which decreases the level of abscisic acid (ABA), enhances tissue sensitivity to gibberellin and stimulates growth of submerged internodes (Knaap et al., 1996). Low humidity and treatment with ethylene lower ABA levels in rice as well, but without an increase in the internode length (Azuma et al., 1995). Resistance of *Rumex palustris* plants to flooding is a consequence of both the rapid shoot elongation promoted by ethylene and the low sensitivity of the senescence process in *Rumex palustris* to ethylene (Banga et al., 1997).

Adventitious root formation along the submerged stem portion, reduction in stem growth, wilting, chlorosis, epinasty, and abscission are the responses of *Lycopersicon esculentum* to flooding (Jackson, 1956). Both the epinasty and stomata closure decrease the rate of plant transpiration (Jackson, 1997). Stem hypertrophy facilitates the aeration of submerged roots (Vartapetian and Jackson, 1997). Hypoxia and ethylene affect both the transport within a plant and perception of indole-3-acetic acid (IAA), and lead to IAA accumulation at the stem base (Wample and Reid, 1979; Suttle, 1988; Liu and Reid, 1992). The development of adventitious roots can be prevented by the exogenous application of IAA transport inhibitors. Tryptophan from root exudates may be utilized by soil bacteria for IAA synthesis (Patten and Glick, 1996) and exogenous IAA of bacterial origin may act synergistically with plant-synthesized IAA, increasing the amount of stress ethylene produced in response to a flooding. In flooded *Rumex palustris* plants, a sharp increase in ethylene levels precedes the development of adventitious roots while IAA levels remain constant; the high level of ethylene stimulates the formation of adventitious roots by increasing plant sensitivity to IAA, a hormone which stimulates the formation of root primordia (Visser et al., 1995; Visser et al., 1996; Visser et al., 1997). IAA and ethylene act together to stimulate stem hypertrophy, which is often observed in flooded tomatoes and is associated with the enlargement of the cortical cell layer.

1.2. The Yang cycle

In higher plants, the immediate precursor of ethylene is 1-amino-cyclopropane-1-carboxylic acid (ACC) which is produced as a result of the Yang cycle (Adams and Yang, 1979). In the initial step catalyzed by the 'house-keeping' enzyme S-adenosyl-L-

methionine (SAM) synthetase (i.e., methionine adenosyltransferase) the addition of an adenosyl moiety to L-methionine results in SAM formation (Adams and Yang, 1977). While plant methionine adenosyltransferase has not been thoroughly studied, it is similar to the enzyme from non-plant sources and has a K_m for methionine of approximately 0.4 mM (McKeon and Yang, 1990). SAM is the major methyl group donor in the transmethylation of proteins, nucleic acids, polysaccharides and fatty acids (John, 1997). The activity of SAM synthetase is highly regulated in higher plants, and using selenomethionine and other inhibitors of SAM synthetase, it was shown that SAM could regulate ethylene production (Espartero et al., 1994; Fluhr and Matoo, 1996).

The second step of the Yang cycle, the conversion of SAM to ACC and 5'-methylthioadenosine, is catalyzed by the enzyme ACC synthase in one of the rate-limiting steps of ethylene synthesis (Yang and Hoffman, 1984). This reaction is not limited to plants. Thus, the fungus *Penicillium citrinum* can synthesize ACC from SAM (Jia et al., 1999). The 5'-methylthioadenosine that is produced in this reaction is recycled so that in the following four enzymatic steps methionine is regenerated (Miyazaki and Yang, 1987).

ACC synthase requires pyridoxal phosphate as a co-factor (PLP), and the reaction is susceptible to competitive inhibition by either L-α-(aminoethoxyvinyl)-glycine (AVG) or aminooxyacetic acid (AOA) (Yu and Yang, 1979; Amrhein and Schneebeck, 1980). With the enzyme from tomato plants, AVG and AOA have K_i values of 200 nM and 800 nM, respectively (Yang and Hoffman, 1984).

ACC synthase is a dimer with a molecular mass of 94 kDa (Hohenester et al., 1994). ACC synthase is unstable enzyme which exists in low quantities in plants except

for fruit. In wounded tomato pericarp tissue, ACC synthase comprises 0.001% of the total protein (Abeles et al., 1992).

Genes for about fifty different ACC synthases have been isolated (Sato and Theologis, 1989; Liang et al., 1992; Lincoln et al., 1993; Fluhr and Mattoo, 1996; Rosenfield et al., 1996; Shiu et al., 1998). In tomato plants, nine separate genes encode different ACC synthases which are induced by a multiplicity of factors in various organs (Van Kan et al., 1995); six of them are inducible by IAA. In tomato fruit, only *ACS2* and *ACS4* are expressed at a high level (Theologis, 1986; Yip et al., 1992; Zarembinski and Theologis, 1994). Based on an analysis of PLP binding sites, ACC synthase has been linked evolutionarily to aminotransferases (Rottmann et al., 1991). In fact, the appearance of ACC synthase may have occurred earlier than the divergence of monocots and dicots (Theologis, 1995) because ACC synthase genes are often more similar to ACC synthase genes of other plant species than to the genes from the same species (Liang et al., 1992).

Protein phosphorylation and dephosphorylation is important for the induction of ACC synthase (Raz and Fluhr, 1993). Regulatory phosphorylation was observed in tomato cell cultures, where ACC synthase activity was stimulated by phosphatase inhibitors and suppressed by kinase inhibitors (Spanu et al., 1994). In this way, ACC synthase gene expression is regulated at a post-translation level. Thus, rapid stress-induced ethylene synthesis may take place by changes in the activity of pre-existing ACC synthase enzyme (Spanu et al., 1994). ACC synthase activity can be increased substantially by treatment with a fungal elicitor in the presence of the transcriptional inhibitors cordycepin and actinomycin D (Chappell et al., 1984).

Not all of the ACC that is synthesized by plants is converted to ethylene. ACC may also be enzymatically converted into either N-malonyl ACC that is stored in vacuoles (Amrhein et al., 1981; Hoffman et al., 1982) or 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid (Martin et al., 1995). The enzyme responsible for the formation of N-malonyl ACC, i.e., ACC N-malonyltransferase, has a K_m for ACC of 0.5 mM and represents approximately 0.006% of the total soluble protein in the pericarp tissue of tomato fruit (Martin and Saftner, 1995). In tomato, ACC N-malonyltransferase is found mainly in pericarp and seeds; its activity is enhanced by ethylene treatment (Liu et al., 1985a). Prolonged incubation and high concentrations of N-malonyl ACC with ACC N-malonyltransferase results in some N-malonyl ACC hydrolysis to ACC; however, since the plant N-malonyl ACC level is far below the K_m, the probability of this reaction occurring in nature is negligible (Jiao et al., 1986; Hanley et al., 1989; Fluhr and Mattoo, 1996).

1.3. Oxidation of ACC to ethylene

ACC is converted to ethylene (Adams and Yang, 1979; Amrhein et al., 1981), water, carbon dioxide and hydrogen cyanide by enzyme ACC oxidase which is relatively unstable (Yang and Hoffman, 1984) and loses activity at 35°C (Yu et al., 1980). The hydrogen cyanide is detoxified to either asparagine or γ-glutamyl-β-cyanoalanine (Kende, 1993). Inhibitors of ACC oxidase (a non-heme Fe^{2+}-ascorbate dioxygenase) include Co(II) (Yu and Yang, 1979), sodium benzoate and CO_2, under certain conditions (John, 1997). Similar to ACC synthase, ACC oxidase exists as a multi-gene family (John, 1997). Tomato plants have at least three ACC oxidase genes (Holdsworth et al., 1988).

Expression of ACC oxidase genes, accumulation of ACC oxidase and its activity are all inducible (Barry et al., 1996). Since in many plants, ACC oxidase must be induced prior to ethylene synthesis the oxidation of ACC to ethylene is also considered as a rate-limiting step of the Yang cycle (Barry et al., 1996). A short exposure to ethylene induces ACC oxidase without a concomitant increase in ethylene production because induction of ACC synthase requires longer exposure (Liu et al., 1985b; Dong et al., 1992). ACC oxidase genes are manipulated through the feedback in autoinhibition of ethylene production (Yoshii and Imaseki, 1982; Yang and Hoffman, 1984). In ripening tomato fruits, reduction in the amount of E8 protein which is homologous to a family of enzymes known as 2-oxoglutarate-dependent dioxygenases similar to ACC oxidase, causes an increase in the levels of ACC oxidase mRNA and ethylene (Kneissl and Deikman, 1996). In apple fruit, the values of k_{cat} for ACC synthase and ACC oxidase are 9.2 s^{-1} and 0.013 s^{-1}, respectively, while each of these enzymes comprises 0.002% and 0.56%, respectively of total cellular protein (Li et al., 1996).

1.4. Exogenous ethylene

Ethylene is readily available from air and soil where its level depends on soil composition and bacterial activity (Frankenberger and Arshad, 1995). In some soils, approximately 30% of the soil microorganisms can produce ethylene at a rate of at least 100 nL L^{-1} h^{-1} (Fukuda et al., 1993). However, unlike higher plants, bacteria do not synthesize ethylene from ACC (Abeles et al., 1992). In most soil microorganisms, production of ethylene from either 2-keto-4-methylthiobutyrate (e.g., *E. coli*, *Cryptococcus albidus*) or 2-ketoglutarate (e.g., *Pseudomonas syringae*, *P. digitatum*) is

catalyzed by an NADH:Fe(III)-EDTA oxidoreductase. Similar to the plant pathway the bacterial pathway requires oxygen and is arrested by anaerobiosis. Because the relative density of ethylene in air is 0.978, ethylene that is produced by soil bacteria, normally does not accumulate in soil. However, in waterlogged root zones, ethylene is present at elevated levels. Most of the ethylene, which is found in submerged soils, is of abiotic origin (Abeles et al., 1992). The low solubility of ethylene in water results in the trapping of ethylene in submerged plant organs. When labeled exogenous ethylene was applied to the roots of tomato plants, both ethylene uptake and its movement inside the plant was rapid and produced substantial physiological effects (Jackson and Campbell, 1975a). Elongation of barley roots is slightly reduced by 0.1 ppm of ethylene, is reduced by 50% by 1 ppm, while 10 ppm of ethylene is completely inhibitory (Smith and Russel, 1969). In *Zea mays*, 100 nL L^{-1} of ethylene induces development of a gas space (aerenchyma) with concomitant lysis of 47% of the root cortical cells (Drew et al., 1981). Ethylene concentrations of 25-100 ppb cause epinasty in sunflower, tomato, buckwheat and pear (Burg, 1962).

1.5. Ethylene perception

The cyanobacterium *Synechocystis* wild-type strain PCC6803 expresses an ethylene binding protein (Kaneko et al., 1996). No data were reported on ethylene production by this bacterium while another cyanobacterium *Synechocystis* wild-type strain PCC7942 was not reported to produce ethylene (Sakai et al., 1997). In higher plants, prokaryotic- and eukaryotic-like components coexist in ethylene perception and signaling. A protein kinase cascade followed by a cascade of transcription factors, which

are encoded by developmentally regulated multigene families serves as an ethylene sensor (Theologis, 1998). For example, in *Arabidopsis*, the action order of genes involved in ethylene perception is: *ETR1, EIN4, ERS, ETR2* (ethylene-resistant, ethylene-insensitive) → *CTR1* (constitutive triple response) → *EIN2*, *EIN5*, *EIN6*, *EIN7* → *EIN3*, *EIL1*, *EIL2*, *EIL3* → *ERF1* (Ecker, 1995; Chang, 1996; Kieber, 1997). Ethylene binds to the hydrophobic domain of membrane-bound ETR1 protein (Schaller and Bleecker, 1995) followed by autophosphorylation of ETR1 at histidine-353 (Bleecker and Schaller, 1996; Gamble et al., 1998). Without a functional ETR1 protein plants do not bind ethylene (Bleecker et al., 1988; Chang et al., 1993). ETR1 requires Mn^{2+} for autophosphorylation and is homologous to a prokaryotic two-component histidine kinase with a sensory hydrophobic domain located near the amino terminus and a histidine kinase domain located in the carboxyl terminus of the protein (Gamble et al., 1998). Similar two-component environmental sensors allow bacteria to respond to diverse stimuli. ETR1 dimerization is not essential for ethylene binding (Schaller and Bleecker, 1995) and mutations within the sequence in the second transmembrane domain can abolish ethylene binding (Rodriguez et al., 1999). The altered conformation of ETR1 after ethylene binding shuts off the kinase activity of CTR1, a putative Raf type serine/threonine protein kinase (Chang and Meyerowitz, 1995). The signaling mechanism involves specific interaction between the CTR1 amino-terminal domain and the histidine kinase domain of ETR1 (Clark et al., 1998). CTR1 negatively regulates the activity of EIN2 and EIN3 by mitogen-activated protein kinase (MAP kinase) cascade; protein phosphorylation is essential for ethylene signaling (Raz and Fluhr, 1993).

ERS which encodes another ethylene receptor in *Arabidopsis* was discovered by cross-hybridization with the *ETR1* gene (Hua et al., 1995); the ERS protein is 67% identical to ETR1 but lacks the receiver domain of the response regulator. In tomato plants, two ETR1 homologues have been identified. The eTAE1 protein contains all of the structural elements found in ETR1 (Wilkinson et al., 1995; Zhou et al., 1996). The NR gene is strongly induced by ethylene during tomato fruit ripening and is responsible for the susceptibility of ripening fruit to ethylene (Wilkinson et al., 1995). In *Never-ripe* tomatoes, a dominant mutation in the trans-membrane response regulator domain of the NR protein is responsible for the plant's insensitivity to ethylene (Lanahan et al., 1994).

The EIN3 protein is both a DNA-binding protein and a transcriptional regulator; the promoter of the tomato early ethylene-response ACC oxidase gene *LE-ACO1* contains an EIN3 binding site (Chao et al., 1997). ERF1 is a DNA-binding protein as well; it binds to the GCC cis-acting ethylene-response box which is present in the late ethylene-inducible genes expressed during fruit ripening, senescence, the triple response and wounding (Theologis, 1998). The GCC-box is localized in the promoter of the ethylene-inducible PRB-1*b* gene which is induced relatively quickly following pathogen infection (Eyal et al., 1993; Shinshi et al., 1995). The phosphorylation of transcription factors that interact with the PRB-1*b* promoter may be a part of the mechanism of regulation of this promoter (Sessa et al., 1995). Recently "cross-talk" between the glucose and ethylene signal transduction pathways in plants has been observed (Zhou et al., 1998).

Transgenic yeast expressing ETR1 contain 40 pmol g^{-1} of binding sites compared to 1 pmol g^{-1} in *Arabidopsis* leaves (Schaller and Bleecker, 1995) and 3.5 pmol g^{-1} in

tobacco leaves (Sisler, 1979). In transgenic yeast, the dissociation constant for the ETR1-ethylene complex is 2.4 nM and the half-life of ethylene dissociation is about 12 h (Schaller and Bleecker, 1995). A copper(I) cofactor was proposed for the ETR1 receptor despite the fact that the dissociation constant of the native ethylene-receptor complex is significantly lower than those of known copper(I) complexes (Rodriguez et al., 1999).

1.6. Flooding stress at the plant level

The similarity between the specific reactions of plants to excessive watering and their response to treatment with ethylene is striking (Turkova, 1944). The connection between root hypoxia and the effect of ethylene was established when the roots of tomato plants were made anaerobic by the application of gaseous nitrogen, and an increased ethylene level was detected in plant shoots. When the stems of these plants were split at the base to initiate two parallel root systems the ethylene level increased only in those leaves that were vascularly connected to the anaerobic roots (Jackson and Campbell, 1975b; Jackson and Campbell, 1976; Bradford and Dilley, 1978, Jackson et al., 1978). Treatment with nitrogen for 12 h resulted in an 11-fold increase in the amount of ACC in roots which has been identified as a positive message in root-to-shoot communication during flooding stress (Bradford and Yang, 1980). Low oxygen levels arrest ACC oxidation to ethylene in waterlogged plant roots. In addition, flooding is accompanied by a substantial increase in the soil CO_2 level from a partial pressure of 35 Pa to 12400 Pa within one day of root submergence (Else et al., 1995a), a condition which may further inhibit ACC oxidase in waterlogged roots. ACC synthesized in anaerobic tomato plant roots is transported in the xylem to the shoots where it is rapidly oxidized to ethylene

(Bradford and Yang, 1980; Bradford et al., 1982). It was estimated that the delivery rate of ACC from roots to shoots increased by 3.1-fold in tomato plants flooded for one day compared to well-drained plants, while under the same conditions the transport of ABA is inhibited (Else et al., 1995b). In addition to moving between the anaerobic roots and aerial shoots, ACC promotes expression of ACC oxidase genes in plant shoots, stabilizes ACC oxidase mRNAs and increases the activity of pre-existing ACC oxidase (English et al., 1995). In flooded tomato plant roots, ACC serves as a positive message from the oxygen-deficient roots that is transported in xylem and subsequently induces epinastic leaf curvature following its conversion to ethylene (Jackson, 1997).

Despite the increased delivery of ACC from flooded roots, ethylene synthesis in shoots is unaffected within the first photoperiod following the flooding (Else and Jackson, 1998). The twelve hour lag from the appearance of ACC at the stem base until the ethylene burst in the upper leaves is a hallmark of flooding stress (Bradford and Yang, 1980) which induces both the expansion of the adaxial cells of petioles and the development of epinasty within only six hours of flooding onset. The ACC oxidase activity in the petioles of flooded plants also transiently increases within 6 to 12 hours following flooding of tomato plants (English et al., 1995). The delay between increased delivery of ACC and an increase in ethylene synthesis in the plant's shoot is not well understood although it might be partly explained by the formation of N-malonyl ACC since the N-malonyl ACC level increases in both roots and shoots of flooded tomato plants (Amrheim et al., 1981) as does the conjugation of ACC to glutamic acid (Banga et al., 1996). The time which is required for the expression of the *ACO1* gene in shoots may contribute to this lag as well (Else and Jackson, 1998). Ethylene derived from ACC

transported from the roots could promote induction of early ethylene-inducible *ACO1* (English et al., 1995; Theologis, 1998).

1.7. Flooding stress at the molecular level

Hypoxic roots have a higher activity of ACC synthase and show a higher concentration of ACC than do well aerated roots (Drew, 1997). Hypoxia induces ACC synthase genes in a tissue- and organ-specific manner (Zarembinski and Theologis, 1993) resulting in an increase in ACC synthase activity in roots within five hours (Wang and Arteca, 1992). Once induced, ACC synthase activity in roots fluctuates in a diurnal rhythm (Shiu et al., 1998) resulting in diurnal production of ethylene (Machckova et al., 1997). Consequently, the ACC content decreases during dark periods despite the fact that transpiration is low at night. Flooding-induced ethylene emission by leaves peaks during daytime when transpiration is high (Jackson et al., 1978).

Consecutive induction of *ACS7* and *ACS3* genes, which directly sense root hypoxia, is followed by *ACS2* gene induction in roots of flooded tomato plants (Olson et al., 1995; Shiu et al., 1998). *LE-ACS7* is a primary gene in the root-to-shoot signaling, in flooded plants, which is induced early and transiently in flooded roots (Shiu et al., 1998). The promoter of *LE-ACS7* includes a Sol3 transposon, which is apparently involved in the root-to-shoot communication in waterlogged tomato plants (Shiu et al., 1998). The promoter of *LE-ACS3* contains several putative cis-acting response elements similar to the root-specific expression element from the cauliflower mosaic virus 35*S* promoter and an anaerobic response element from the promoter of maize *adh1* gene which is unaffected by ethylene (Olson et al., 1995). *LE-ACS3* is induced early after flooding

similar to *LE-ACS7*, although about half of its transcript remains unspliced (Shiu et al., 1998). In tomato plants, *Nr*-like gene is induced by flooding similarly to *ETR2*, *ERS1* and *ERS2* which are induced by ethylene in *Arabidopsis* (Shiu et al., 1998; Hua et al., 1998). *LE-ACS2* is induced by ethylene in a dose-dependent manner in green tomato fruits (Rottmann et al., 1991). The induction is responsible for the autocatalysed ethylene biosynthesis which occurs during fruit ripening, abscission, and senescence (Yang and Hoffman, 1984). The *LE-ACS2* gene was identified as a secondary gene, which may account for the burst of ethylene production in hypoxic roots (Shiu et al., 1998). A similar phenomenon may take place following plant wounding. Accumulation of *LE-ACS7* mRNA precedes the production of ethylene in wounded leaves (Shiu et al., 1998). It was shown that wounding induces *de novo* synthesis of ACC synthase (Acaster and Kende, 1983; Yang and Hoffman, 1984) and results in localized two-phase emission of ethylene (De Vries et al., 1996). A peak of approximately 15 pmol g^{-1} min^{-1} of stress ethylene is synthesized 1-2 hours following wounding (Bowles, 1998). Similar two-phase kinetics are observed for both H_2O_2 (Lamb and Dixon, 1997) and ethylene (Woltering and Harren, 1989). With flooding conditions, the amount of ATP which is produced by plant roots decreases and can eventually limit ACC synthesis.

Not only flooded roots, but also ripening fruits and germinating seeds are affected by a low oxygen level. Ethylene is readily soluble in lipids (Abeles et al., 1992) which accumulate in mitochondria, chloroplasts and liposomes during low oxygen stress (Nilsen and Orcutt, 1997). Similar to the ethylene trapped inside submerged roots, ethylene is concentrated inside attached tomato fruit as a consequence of the very low emission of

ethylene through the fruit skin. In tomato fruit, 82.5-95% of the ethylene is released through the pedicel (De Vries et al., 1996).

Ethylene synthesis exhibits autoinhibition due to the E8 gene, which is both regulated by ethylene and encodes a protein that negatively regulates ethylene synthesis in tomato fruits (Abeles et al., 1992). Reduction in the amount of E8 protein results in an increase in the levels of both *ACS2* mRNA and ACC, and does not affect the level of *ACS4* mRNA (Kneissl and Deikman, 1996).

1.8. Modulating plant ethylene levels

Ethylene production in plants can be controlled by genetic suppression of the key enzymes of the Yang cycle (Theologis et al., 1992). For example, in tomato plants transformed with antisense ACC synthase, ethylene production was lowered by 99.5% and fruit ripening was suppressed; antisense ACC synthase transgenic fruits never turn soft and never develop an aroma (Oeller et al., 1991). In addition, leaf senescence and fruit ripening were delayed and ethylene production in transgenic tomatoes in which a high level of antisense ACC oxidase mRNA lowered levels of ethylene (Hamilton et al., 1990; John et al., 1995). Transgenic tomato plants containing an antisense *ACO1* gene have the same ACC flux as the non-transformed plants and a similar response to exogenous ethylene; however, they exhibit less epinasty and a lower level of ethylene synthesis following root submergence (English et al., 1995).

The antisense phenotype is reversible by ethylene in the case of both ACC synthase and ACC oxidase antisense expression. The disadvantage of using the above mentioned approaches to lower plant ethylene levels is the complexity of gene

suppression technology and the possiblity of unexpected outcomes similar to what has been observed with ETR1-1 transgenic tobacco plants. ETRI-1 ethylene-insensitive transgenic tobacco plants, which rely on the constitutive production of ethylene, overproduce ethylene, do not slow growth when contacting neighboring plants and develop spontaneous stem browning (Knoester et al., 1998).

Expression of a microbial ACC deaminase gene, the product of which cleaves ACC to ammonia and α-ketobutyric acid, has been proposed as a possible cure for both ethylene-related negative responses in plants and for the control of fruit ripening (Klee et al., 1991; Sheehy et al., 1991; Klee and Kishore, 1992). In plants, ACC is uniquely involved in the ethylene synthetic pathway, and α-ketobutyric acid is a naturally occurring metabolite that may be converted to branched-chain amino acids. ACC deaminase is comprised of three identical subunits of 36.5 kDa (Sheehy et al. 1991); enzyme temperature optimum is 30°C, and optimum pH is 8.5 (Honma and Shimomura 1978). The K_ms for ACC deaminases are 1.5-17.4 mM (Yang and Hiatt, 1991; Klee and Kishore, 1992) compared to K_ms for ACC oxidases of 8-120 μM (Smith et al., 1992).

Despite its weaker affinity for ACC compared with ACC oxidase, ACC deaminase gene expressed under the transcriptional control of the 35*S* promoter from cauliflower mosaic virus in tomato plants can lower ethylene levels by 90-95% (Klee et al., 1991). In this instance, expression of ACC deaminase was used to delay ripening in tomato fruits (Klee et al., 1991; Sheehy et al., 1991). In these plants, ethylene production was greatly reduced (but not abolished), fruit ripening was delayed, and ACC treatment did not cause an increase in either ethylene production or epinasty in petioles (Klee et al., 1991; Sheehy et al., 1991; Klee and Kishore, 1992). In fact, when transgenic plants were

infiltrated with ACC, only a low level of ethylene production occurred and no epinastic response (Klee et al., 1991). The *E8* promoter can also be used to direct fruit-specific and ethylene-dependent ACC deaminase expression in transgenic tomatoes with similar results (Klee and Kishore, 1992).

ACC deaminase transgenic tomato plants that respond to flooding may be constructed by using root-specific promoters which are also anaerobically inducible (Robison et al., in preparation; Grichko and Glick, in preparation). For example, the promoter of a maize glyceraldehyde-3-phosphate dehydrogenase gene was shown to efficiently direct the expression of a β-glucuronidase reporter gene in tobacco plants during root exposure to anaerobiosis (Kohler et al., 1996). In a variety of tissues, transcription from this promoter was anaerobically induced within 12 h of the onset of anaerobiosis and was turned off within 3 hours after commencement of aerobic conditions.

Transgenic tomato plants that express ACC deaminase under the control of the 35*S* promoter from cauliflower mosaic virus are less susceptible to damage from several different phytopathogens (Lund et al., 1998). These observations are consistent with the suggestion that plants with decreased ethylene levels should be less susceptible to various types of stress (Klee, 1992). In addition, the growth of canola and tomato seedlings treated with an ACC deaminase-containing plant growth-promoting bacterium was partially protected from inhibition by nickel (Burd et al., 1998). Recently, considerable attention has been directed toward the possibility of using plants to remove heavy metals from the environment (Raskin et al., 1997; Salt et al., 1998). Phytoremediation, which may be defined as the use of plants to remove, destroy or sequester hazardous substances

from the environment, is considered to be an attractive, although unproven, alternative to the approaches that are currently in use for dealing with heavy metal contamination. While considerable effort has been directed toward identifying plants that are metal tolerant and accumulate large amounts of metal from the soil and might therefore be useful in phytoremediation, an attractive alternative might include the genetic engineering of metal tolerance in plants in an effort to specifically increase their usefulness in phytoremediation. Therefore, the ACC deaminase transgenic tomato plants were also in this thesis examined for their ability to tolerate heavy metals.

In flooding, roots release a high amount of ACC into the surrounding water (Else et al., 1995a; Else et al., 1995b). Soil bacteria capable of degrading ACC are relatively common (Glick et al., 1995; Jacobson et al., 1994). For example, when 103 soil microorganisms from various regions in South Africa were screened, 81 of them were able to utilize ACC (Campbell and Thomson, 1996). To control flooding damage in crop plants the establishment of useful microbial populations may be an effective alternative to chemical fertilizers and antisense technology. This noninvasive approach to ameliorating the damage to plants caused by flooding utilizes the beneficial relationships between a plant and ACC deaminase-containing plant growth-promoting bacteria that can significantly decrease ACC levels in plants (Glick et al., 1994; Hall et al., 1996; Penrose et al., submitted for publication). In plant growth-promoting bacteria, the regulation of ACC deaminase gene expression by the oxygen concentration in the soil helps to lower the level of endogenous ACC and hence, the concentration of ethylene in a plant (Grichko and Glick, in preparation). In flooded soils, the growth of fungi and actinomycetes is suppressed and bacteria predominate, with strict anaerobes constituting

10% of facultative anaerobes (Ponnamperuma, 1984). Mechanisms responding to the decrease in oxygen concentration are well characterized in *E. coli*, *Rhizobium meliloti*, and *Pseudomonas* spp.; however, the low level of oxygen in flooded soils may limit the ability of a plant growth-promoting bacterium to proliferate.

Plant growth-promoting bacteria can affect plant growth in a variety of ways (Glick, 1995a). Plant growth-promoting bacteria increase seed germination and viability, root respiration and formation. They stimulate root growth and whole plant growth by accelerating cell division; and increase plant membrane permeability for more efficient nutrient uptake. Inoculants of these beneficial bacteria may solubilize phosphorus and other minerals thereby reducing dependence on chemical fertilizers (Lynch and Brown, 1997). In addition, many bacteria associated with plant roots synthesize auxins such as indole-3-acetic acid (IAA) which in low doses stimulate plant growth (Patten and Glick, 1996). In plants, induction of ethylene by IAA takes place via the accumulation of ACC oxidase mRNAs (Fluhr and Mattoo, 1996) and stimulation of ACC oxidase enzyme activity (Peck and Kende, 1995). Auxins cause the plasma membrane to transport H^+ into the cell wall (Arms and Camp, 1982) and the auxin molecule could affect the stability of the ethylene-metal bond in ethylene receptors due to a ligand trans-effect (Powell, 1988).

In summary, the thesis objectives are (i) elucidation of the regulation of the ACC deaminase gene from *E. cloacae* UW4; (ii) assessment of possibility of using of wild-type *E. cloacae* UW4 and CAL2 to ameliorate flooding stress in tomato plants; (iii) elaboration of the physiological effects of genetically transforming tomato plants with a bacterial ACC deaminase gene; (iv) evaluation of the degree of protection afforded against abiotic stresses in transgenic tomato plants by ACC deminase.

2. MATERIALS AND METHODS

2.1. Plant material

Lycopersicon esculentum Mill cv Heinz 902, *Lycopersicon esculentum* Mill cv Heinz 1439VF 402A (Stokes Seeds Ltd., Canada) and ACC deaminase transgenic plants (Robison, M., Shah, S., Tamot, B., Griffith, M., Moffatt, B.A., Pauls, K.P. and Glick, B.R., in preparation) were grown in Pro-Mix 'BX' general-purpose growth medium (General Horticulture, Inc., Red Hill, PA, U.S.A.) in a greenhouse at 25 ± 5°C (day) and 20 ± 5°C (night) at a minimum day length of 16 h. Seeds were germinated in 30 x 60 cm^2 flats. After two weeks seedlings were transferred to 5 inch diameter pots and after one month to 10 inch diameter pots. Plants were watered daily with tap water until the onset of the experimental treatment.

2.2. Transgenic plants

The transgenic tomato plants are all homozygous transformants of *L. esculentum* Mill cv. Heinz 902, each with a single ACC deaminase gene per haploid genome (Robison, M., Shah, S., Tamot, B., Griffith, M., Moffatt, B.A., Pauls, K.P. and Glick, B.R., in preparation). The promoters that were utilized include (i) the constitutive two tandem 35*S* cauliflower mosaic virus promoters (Christopher et al., 1987), (ii) the root-specific *rolD* promoter from *Agrobacterium rhizogenes* (Elmayan and Tepfer, 1995) and the pathogenesis related promoter PRB-1*b* from tobacco (Eyal et al., 1993). The ACC deaminase gene was isolated from the soil bacterium *Enterobacter cloacae* UW4 (Shah et al., 1998).

2.3. Flooding experiment

Plants were used for flooding experiments in 55 days after sowing. Plants were flooded by placing each 10-inch pot in a tightly fitting polyethylene bag and then in an additional 5-inch pot; plants were flooded with de-ionized water which was maintained at the level of the cotyledonary node throughout the experiment.

2.4. Epinasty

Epinasty was measured as described (English et al., 1995). Angles between the stem and the adaxial surface of the petiole were traced onto a white card using a fine pencil, taking care not to wound the plant. A tangent was drawn to the petiole curve using points at 20, 30, and 40 mm from the stem. The angle between this tangent and the stem was then measured using a protractor. Epinastic curvature was defined as any change in angle (°) after the initial reading.

2.5. Pouch assay in the presence of Cd, Co, Cu, Mg, Ni, Pb, or Zn

The 125 x 157 mm growth pouches (Mega International, Minneapolis, MN, USA) were filled with 10 mL of 1 mM $3CdSO_4 \cdot 8H_2O$, $CoCl_2$, $Cu(NO_3)_2$, $NiSO_4$, or $Pb(NO_3)_2$ and 10 mL of 10 mM $MgSO_4$ or $ZnSO_4$ and were sterilized by autoclaving. Control pouches were filled with 10 mL of the corresponding acid with the pH and concentration adjusted to that of the metal solution, 5.5 - 5.8. Seeds were sterilized in 70% ethanol for 1 minute, in a 1% sodium hypochlorite solution for 10 minutes and were then rinsed thoroughly with sterilized distilled water. Five seeds were placed aseptically in each growth pouch. Trays containing the upright growth pouches were incubated in a

Conviron model CMP 32444 growth chamber (Controlled Environment Co., Winnipeg, Manitoba, Canada) for either 7 or 9 days with a photo period of 12 h and a photosynthetic photon flux of 12.9 $\mu E\ m^{-2}\ s^{-1}$ at the bottom pouch level, at a temperature of 20°C.

2.6. Treatment of pot-grown plants with metals

With Co, Cu, Ni, and Pb 34-day-old tomato plants were watered with 100 mL of a 1 mM salt solution for each of two days and were then treated daily with 50 mL of a 1 mM salt solution for five days up to a cumulative dosage of a 0.45 mmole per 5-inch pot. At the end of the treatment, the plants were 40 days old and are subsequently referred to as 40-day-old plants. With Cd and Zn 42-day-old tomato plants were treated with either a) 10 mL of 10 mM $3CdSO_4 \bullet 8H_2O$ for ten consecutive days up to a cumulative dosage of 1 mmole per 5-inch pot or b) 10 ml of 100 mM Zn salt for ten consecutive days up to a cumulative dosage of 10 mmole per 5-inch pot. At the end of the treatment, the plants were 51 day old and are referred to as 51-day-old plants. In both cases, controls were done with the acid (either HCl, H_2SO_4 or HNO_3) with the pH adjusted to a value of 5.5 with the addition of NH_4OH and were carried over in the same way. During the experiment plants were kept at 25 ± 5°C (day) and 20 ± 5°C (night) at a minimum day length of 16 h.

2.7. Pouch assay in the presence of Ag

The 125 x 157 mm growth pouches (Mega International, Minneapolis, MN, USA) were filled either with 10 mL of 1 mM nitric acid with the pH adjusted to 5.7 using concentrated NH_4OH or 10 mL of 1 mM $AgNO_3$, pH 5.7, and were sterilized using an

autoclave at 121°C for 20 minutes. Four hundred seeds of each plant type were immersed in 70% ethanol for 1 minute followed by a 10 minute immersion in a 1% bleach solution. The seeds were then rinsed thoroughly with sterilized distilled water. Five seeds were aseptically placed in each growth pouch that was subsequently kept upright in a plastic tray. The tray was covered with plastic wrap and incubated in a Conviron model CMP 32444 growth chamber (Controlled Environment Co., Winnipeg, Manitoba, Canada) for 7 days with a photo period of 12 h and a photosynthetic photon flux of 12.9 $\mu E\ m^{-2}\ s^{-1}$ at the bottom pouch level, at a temperature of 20 ± 2°C. The number of seeds germinated was monitored daily. At the end of the experiment the root length was measured along with the root and shoot fresh weights.

2.8. ACC deaminase and protein assay of plant

Samples (the third leaf from the top from five-month-old tomato plants grown in the greenhouse) were immediately frozen in liquid nitrogen and powdered for cell disintegration. Protein was extracted in cold buffer containing 0.2 M Tris-HCl, pH 7.2, 2 mM EDTA, 2% polyvinylpyrrolidone, 1 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Dumbroff and Gepstein, 1993). Insoluble material was removed by centrifugation at 4 C°. The solubilized protein content was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976). Total protein extracted from fresh plant tissue was analyzed for ACC deaminase activity as described (Honma and Shimomura, 1978). Blanks consisted of tissue extracts added to reagent in the absence of the substrate.

2.9. Leaf chlorophyll determination

Chlorophyll was extracted from leaf tissue (the third leaf from the top from five-month-old tomato plants grown in the greenhouse) by incubation in dimethyl sulphoxide at 65°C (Hiscox and Israelstam, 1978). The concentrations of chlorophyll a and b were calculated as described by Arnon (1949).

2.10. Carotenoid determination

Lycopene and β-carotene in tomato fruit was determined spectrophotometrically (Tereshina et al., 1994). Carotenoids were extracted in acetone until the disappearance of the tissue color. Solutions were diluted with hexane in a 1:50 ratio. The absorbance was measured at 451 and 502 nm in 1-cm cells. The lycopene and β-carotene concentrations were calculated according to the following equations: $\mu M_{lycopene} = 7.16\ A_{451} - 1.49\ A_{502}$, and $\mu M_{\beta\text{-carotene}} = 8.25\ A_{451} - 5.81\ A_{502}$ (Tereshina et al., 1994).

2.11. Atomic absorption analysis

Samples were digested with 7*N* HNO_3 (Soon, 1998) and the concentration of metals was measured by platform electrothermal atomic absorption spectroscopy (AAS). A Varian model Spectra AA-600 atomic-absorption spectrophotometer equipped with a graphite furnace and an automated sampler was used as recommended by the manufacturer. The device was zeroed using 1% HNO_3 (ACS grade). The samples were dried at room temperature, ashed and digested for 1 hour with 1 mL 1:1 HNO_3 in 1.5 mL Eppendorf tubes at 65°C as described (Soon, 1998). The solutions were diluted with

distilled deionized water when required. Results are reported as ng g^{-1} fresh weight. Calibration standards were prepared from commercially available standards.

2.12. Leaf fluorescence measurements

Five-week-old plants were treated with 10 mL of 10 mM $AgNO_3$ on a daily basis for ten consecutive days. The measurement of the efficiency of Photosystem II in control and treated plant leaves was performed with a PAM Chlorophyll Fluorometer (Walz, Germany) kindly made available by Dr. B. Greenberg in this department using a DA 100 (Walz, Germany) data acquisition system. Immediately prior to taking a measurement, all plants were dark-adapted for 30 minutes. For each plant, fluorescence in the third oldest leaf was nondestructively measured at the same location using a four-armed fiberoptic probe at the same intervals throughout the experiment. The measurements were done in triplicate at an actinic light intensity of 50 $\mu E\ m^{-2}\ s^{-1}$ and an effective pulse duration of 600 ms at a repetition rate of 1 min, until the steady-state was reached. The F_v/F_m ratio, which is proportional to the quantum yield of photochemistry (Agati et al., 1996), was measured at an intensity of 3000 $\mu E\ m^{-2}\ s^{-1}$.

2.13. Ethylene and CO_2 measurements

For ethylene measurement by GC the samples were kept in 12-ml vials in the dark for 30 min at 25°C. Ethylene was determined on a GC-17A gas chromatograph (Shimadzu, Japan) with an AT-1 capillary column (Alltech, USA) and a flame ionization detector. IR spectra were recorded on a FT-IR spectrometer MB100 (Bomem, Quebec,

Canada) with a resolution of 1 cm^{-1}. Fruit were placed in a 260 mL glass vial attached to a measuring cell with a length of 10-cm with KCl windows.

2.14. Fruit and seed collection

The plants were pollinated naturally in the greenhouse, and were not manipulated manually to achieve pollination. Seeds obtained from ripe fruits were extracted and put in a container with some fruit juice for 3 days at 37°C until the mold formed. The ensuing fermentation was used to remove gelatinous material that contains compounds that can inhibit seed germination. Seeds were then rinsed with distilled water and air-dried on petri dishes to preserve seed vigor. Seeds were stored in paper envelopes at 4°C.

2.15. Scanning electron microscopy

Roots were fixed for 2 h in 2.5% gluteraldehyde in 0.2 M phosphate buffer, pH 7.2 at room temperature, washed in the same buffer for 1 h at 4°C and dehydrated by passage through increasing acetone concentrations (50, 70, 95 and 100%) at 4°C. The samples were then dried in a critical-point dryer, affixed to stubs with carbon cement, coated with gold (30 nm thickness) and examined using a Hitachi S570 scanning electron microscope at 15 kV.

2.16. Statistics

Whenever it was possible the sampling procedures were performed accordingly to Benton (1998) who suggested sampling 20-25 of greenhouse tomato plants per treatment. All tests for significance were performed with ANOVA, Microsoft Excel 97 for

Windows, for $P < 0.05$. The data are given as means ± SE. The curves were generated using Microsoft Excel 97 for Windows.

2.17. Flooding experiments using plant growth promoting bacteria

The gnotobiotic assay procedures are based on methods described by Lifshitz et al. (1987) and Tang et al. (1995). Bacterial suspensions, inoculated from a single colony, were grown overnight in 300 mL of TSB medium at 30°C in a shaking water bath. Bacterial cells were separated from the culture medium by centrifugation at 3,000-5,000 x *g* for 10 min at room temperature and then washed twice by suspension in 5 mL sterile distilled water followed by centrifugation for 10 min. Resuspended cells were adjusted to an absorbance of 0.5 at 600 nm. Tomato seeds, previously stored at 4°C, were surface sterilized in 70% ethanol for 1 min and then 1% bleach, sodium hypochlorite, for 10 min. Seeds were washed five times with sterilized distilled water to remove residual bleach. Fifty seeds were then transferred aseptically to sterile polystyrene Petri dishes and incubated with 5 mL suspended bacteria or water for 1 h at room temperature to allow bacteria to bind to the seed coat. The treated seeds were aseptically planted in 25.5 x 52.5 x 5 cm growth trays containing 1000 g of sterilized Pro-Mix 'BX' general purpose growth medium (General Horticulture, Inc., Red Hill, PA).

For flooding experiments using free bacterial suspensions, five days prior to the onset of flooding 240 mL of bacterial suspension with an absorbance of 0.5 at 600 nm was added per 1000 g of soil (Glick et al., 1997). For gnotobiotic pouch assays, five seeds were placed aseptically in each growth pouch. Trays containing the upright growth pouches were incubated in a Conviron model CMP 32444 growth chamber (Controlled

Environment Co., Winnipeg, Manitoba, Canada) for 9 days with a photo period of 12 h and a photon flux of 12.9 $\mu E\ m^{-2}\ s^{-1}$ at a temperature of $20 \pm 2°C$.

2.18. Bacterial strains and plasmids

Strains and plasmids used in this work are shown in Table 1. *Enterobacter cloacae* UW4 and *E. cloacae* CAL2 were isolated from the rhizospheres of reeds and tomato plants, respectively (Glick et al., 1995; Shah et al., 1997). Both of these strains contain a sufficient level of ACC deaminase activity to facilitate the elongation of the roots of canola seedlings. *Enterobacter cloacae* strains CAL2 and UW4 were isolated from soil samples based on their ability to utilize ACC as a sole source of nitrogen and were originally designated as *Pseudomonas* sp. CAL2 and UW4 (Glick et al., 1995); however, a more complete characterization revealed that both of these strains were actually *E. cloacae* (Shah et al., 1997). Both *Enterobacter cloacae* strains CAL2 and UW4 were deemed to plant growth-promoting bacteria based on their ability to promote the elongation of canola roots under gnotobiotic conditions (Glick et al., 1995).

Pseudomonas putida ATCC 17399 was obtained from the American Type Culture Collection. It is a psychrophilic bacterium with a maximal growth rate in TSB medium at 25°C. *P. putida* ATCC 17399 has neither ACC deaminase nor plant growth-promoting activity. *P. putida* ATCC 17399/pRK415 and *P. putida* ATCC 17399/pRKACC are transformants carrying plasmids pRK415 and pRKACC, respectively. Plasmid pRK415 is a low copy number broad-host-range plasmid with a tetracycline resistance gene, and a multiple cloning site within an *E. coli lacZ* gene (Keen et al., 1988). Plasmid pRKACC

was constructed by inserting the ACC deaminase gene (*acd*) and its promoter from *E. cloacae* UW4 into the multiple cloning site of pRK415 (Shah et al., 1998).

Escherichia coli strains were grown in LB (Luria broth, Difco Laboratories, Detroit, MI, U.S.A.) medium or on LB agar plates supplemented with the appropriate antibiotics (ampicillin: 100 $\mu g\ mL^{-1}$; tetracycline: 15 $\mu g\ mL^{-1}$) at 37°C unless otherwise stated. The *Enterobacter cloacae* UW4 and CAL2 strains were grown on either solid or in liquid Tryptic Soybean broth (TSB) medium (Difco Laboratories, Detroit, MI, U. S. A.) at 30°C. Czapex Dox liquid medium (Oxo Ltd., London, England) containing 24 mM $NaNO_3$, 7 mM KCl, 2.6 mM magnesium glycerophosphate, 66 μM $FeSO_4$, 2 mM K_2SO_4, and 88 mM sucrose was used for anaerobic growth.

2.19. DNA manipulations and plasmid construction

A large scale isolation of plasmids was carried out according to the alkaline/ polyethylene glycol purification protocol outlined by Sambrook et al. (1989). Restriction enzyme digestions, DNA fragment isolation from low-melting-point agarose gels, ligation, and agarose gel electrophoresis were performed according to standard procedures (Sambrook et al., 1989).

The shuttle vector pME6030 was kindly provided by Dr. D. Haas, Universitė de Lausanne, Switzerland.

Table 1. Bacterial strains and plasmids used in this work.

Strain or plasmid	Genes and relevant phenotypes	Reference or source
Bacterial strain		
Enterobacter cloacae	Wild-type (CAL2)	Shah et al., 1997
Enterobacter cloacae	Wild-type (UW4)	Shah et al., 1997
E. coli DH5α	*supE*44 Δ*lac*U169 (∅80 *lacZ*ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 1 *rel*A1	Sambrook et al., 1989
E. coli JRG465	*fnr*$^{+}$ *sucA8 gal-25* λ- *trpA9767 IN(rrnD-rrnE)1 iclR7*(Const) *trpR47*	CGSC #4456
E. coli SD7	*gal-25* λ- *topA10 pyrF287 fnr*$^{-}$ *rpsL195(strR) gyrB226 iclR7*(Const) *trpR72*(Am)	CGSC #7078
E. coli JC1552	*leuB6 fhuA2 lacY1 tsx-1 glnV44*(AS) *gal-6* λ- *trp-31 hisG1*(Fs) *argG6 rpsL104 malT1*(λ^R) *xylA7 mtlA2 metB1*	CGSC #4273
E. coli AE84064	*fhuA2 lacY1 glnV44*(AS) *lky-501::Mu cts gal-6* λ- *serC3 lrp-1 trp-31 hisG1*(Fs) *gyrA260*(NalR) *thyA746 argG6 rpsL104 malT1*(λ^R) *xylA7 mtlA2*	CGSC #6575

Plasmid		
p4U2	pUC18 with a 6.8-kb *Sau*3AI fragment containing the ACC deaminase *(acd)* gene from *E. cloacae* UW4 and its upstream region	Shah et al., 1998
pME6030	8.0-kb PVS1-pACYC177 shuttle vector (p15A replicon)	NCBI, accession #AF133830
pRK2501*fnr*	pRK2501 with 1.64-kb *Bam*HI-*Hind*III fragment carrying the *E. coli fnr* gene (Tc^r, RK2 replicon, 11.7 kb).	Winteler and Haas, 1996
p53997	1,71-kb *Sph*I fragment with *acd* gene from p4U2 inserted in the *Sph*I site of pME6030	this work
pEag	1,13-kb *Eag*I fragment with *bacca* gene from p4U2 inserted in the *Xma*III site of pBR322	this work
pEco	Circular 8.4-kb *Eco*RV fragment carrying *bacca* gene excised from p4U2	this work

2.20. Computer analysis

Deduced protein sequences were compared with the sequences in the GenBank, Swiss Prot and PIR databases available at the NCBI server (http://www.ncbi.nlm.nih.gov) using the programs ORF finder, FASTA and BLAST. Sequence aligments were obtained with the ALIGN program available at the GeneStream server (http://vega.igh.cnrs.fr; IGH Montpellier, France). Protein properties were deduced using PepTool for Windows (BioTools Inc., Edmonton, AB, Canada); PHDsec, PHDacc, PHDhtm, TOPITS, MaxHom, EvalSec, Blast, ProSite, and ProDom programs available at the PredictProtein server (http://dodo.cpmc.columbia.edu/predictprotein/). Putative promoters and regulatory region were deduced with the program Promoter Predictions for PC, available at Lawrence Berkeley National Laboratory server (http://www-hgc.lbl.gov/cgi-bin/promoter.pl).

2.21. ACC deaminase enzyme assays and protein determination in bacteria

Bacterial liquid cultures were grown either aerobically with shaking, or in an anaerobic GasPak jar (Becton Dickinson, Cockeysville, MD, U.S.A.) without shaking in the presence of ACC. GasPakPlus disposable hydrogen and carbon dioxide gas generator envelopes (Becton Dickinson, Cockeysville, MD, U.S.A.) which are activated by the addition of water were used to achieve anaerobiosis. Hydrogen, generated from a sodium borohydride tablet following the addition of water, combined with the oxygen in the jar in the presence of the palladium catalyst to form water. BBL© dry anaerobic indicator strips (Becton Dickinson, Cockeysville, MD, U.S.A.) were used for monitoring oxygen limiting conditions in the anaerobic jar.

For the preparation of protein extracts for the measurement of ACC deaminase activity, 5-mL cultures were harvested by centrifugation and washed twice in 100 mM Tris•HCl buffer, pH 8.0. The bacterial cells were then resuspended in M9 minimum medium of the same volume (Sambrook et al., 1989) without NH_4Cl but supplemented with ACC (Honma and Shimomura, 1978), and incubated at 30°C (*E. cloacae*) or 37°C (*E. coli*) for 4-24 hours under aerobic or anaerobic conditions, as indicated above. Cells were harvested by centrifugation, suspended in 0.3 mL of 100 mM Tris•HCl buffer pH 8.5 supplemented with 3 μL of toluene and mixed vigorously on a vortex mixer. The resultant suspension was distributed evenly among the three 1.5 mL Eppendorf tubes before being assayed for protein content (Bradford, 1972) and ACC deaminase activity (Honma and Shimomura, 1978). All analyses were performed with fresh cell extracts.

3. RESULTS

3.1. Regulation of the ACC deaminase gene in *Enterobacter cloacae* UW4

3.1.1. Preface

A number of free-living soil bacteria can facilitate the growth of plants (Glick, 1995; Glick et al., 1999). To promote plant growth, bacteria employ a variety of mechanisms including utilizing ACC (Glick et al., 1995) which can cleave ACC the immediate precursor of ethylene in plants (Abeles et al., 1992) to decrease ethylene levels in plants (Glick et al., 1998). ACC deaminase-containing plant growth-promoting bacteria can also protect plants from a variety of abiotic stresses (Glick et al., 1997; Burd et al., 1998; Grichko and Glick, submitted for publication). Thus, for example, since the accumulation of ACC in the roots of flooded tomato plants is responsible for the burst in ethylene production observed in the shoots following the flooding (Jackson, 1997), those bacteria that express ACC deaminase under the transcriptional control of an anaerobic promoter might act as a sink for a plant's ACC and hence, ameliorate some of the damage imposed by flooding (Nilsen and Orcutt, 1997; Grichko and Glick, submitted for publication).

While most of the organisms with ACC deaminase activity are prokaryotes (Honma and Shimomura, 1978), ACC deaminase has also been found in fungi (Jia et al., 1999) and yeast (Minami et al., 1998). Until recently, both ACC and the enzyme ACC synthase, which catalyzes the synthesis of ACC from S-adenosylmethionine had not been found to occur in microorganisms (Abeles et al., 1992). However, Zhang et al. (1998) reported that the ACC moiety can be synthesized from L-methionine by the soil

bacterium *Streptomyces* sd. RK95-74, and Jia et al. (1999) observed that ACC synthase and ACC deaminase coexist in *Penicillium citrinum*. These observations suggest that the current view regarding what types of organisms contain ACC deaminase and ACC synthase may be an oversimplification.

Both ACC synthase and ACC deaminase are cytosolic, pyridoxal phosphate-requiring enzymes with pH optima of approximately 8.5. These two enzymes share a number of additional common features i.e., stereospecificity, homologous active sites, and reaction mechanism (Penrose and Glick, 1997). It is tempting to speculate that ACC deaminase and ACC synthase have similar features at the regulatory level. In fact, although a number of ACC deaminase genes have been isolated (Penrose and Glick, 1997), at present there are no published reports dealing with the mechanism of transcriptional regulation of ACC deaminase gene expression.

In this study, the DNA sequence upstream of the *Enterobacter cloacae* UW4 ACC deaminase gene cloned by Shah et al. (1998) was examined and found to contain a number of features that appear to be involved in the transcriptional regulation of this gene. These features include a half-site of the CRP-binding site (cAMP receptor protein), an FNR-like (fumarate-nitrate reduction regulatory protein) box, an LRP-like (leucine responsive regulatory protein) binding site and an LRP-like protein coding region. The evidence presented suggests that all of these features are involved in the transcriptional regulation of ACC deaminase activity in this bacterium.

3.1.2. DNA sequence analysis

Upon examination of the DNA sequence of the 5' upstream region of the *E. cloacae* ACC deaminase gene (*acd*) cloned by Shah et al. (1998) it became apparent that

there was an open reading frame (ORF) 165 base pairs upstream of *acd*. The ORF consists of 510 bp and potentially encodes a protein of 169 amino acids with a molecular weight of 18,772 kDa and a pI of 9.6. The protein encoded by the ORF shares a high similarity (E value $1 \cdot 10^{27}$) with LRP, a DNA-binding leucine-responsive transcription regulatory protein found in a number of different gram negative bacteria. LRP is a regulatory protein that mediates a global response to exogenous leucine (Haney et al., 1992) which can affect the expression of a number of different operons. The amino acid sequence of LRP is highly conserved (Friedberg et al., 1995). For example, the LRP protein of *Enterobacter aerogenes* is 99% identical with the LRP protein of *Klebsiella pneumoniae*, *Klebsiella aerogenes*, *Proteus mirabilis*, *Serratia marcescens*, and is 98% of identical with the LRP protein of *Salmonella typhimurium*.

Results of PIR pairwise alignment of the *E. cloacae* UW4 ORF and *E. aerogenes* LRP amino acid sequences made with a Smith-Waterman function are shown in Fig. 1. The amino acid composition of the *E. cloacae* UW4 ORF encoded protein revealed a high content of leucine (13.6%), alanine (11.8%) and arginine (7.1%). Periodicity analysis revealed the presence of seven copies of a leucine 6-aa-periodic pattern within a 42-83 amino acid domain (Fig. 1). A plot of the theoretical charge of the ORF encoded protein versus pH indicates that the second plateau in this figure corresponds to the optimum pH of ACC deaminase activity of *E. cloacae* UW4 (Fig. 2). The estimated half-life of the LRP-like protein is approximately 10 h in *E. coli*.

Analysis of the DNA sequence between the *E. cloacae* UW4 *acd* gene and the LRP-like ORF (NCBI, accession #AF047710) revealed the presence of a palindromic FNR-binding site, TTGAT--ATCgA which shows high identity with the consensus FNR-

binding site TTGAT----ATCAA (Guest, 1992) provided that the 2 bp in the center of the consensus sequence were ignored. In *E. cloacae* UW4, the FNR binding site is located at position -67.5 from the deduced transcription start site of the ORF from the strong promoter 1p (Fig. 3). Typically, the promoters of FNR-activated genes have FNR-site centered at -39 to -49 relative to the transcription start-point and do not contain a -35 sequence (Guest, 1992). The sequence data are compatible with the possibility that the ORF is transcribed in an FNR-independent manner from the promoter 1p (Fig. 3), although DNA sequence analysis revealed the presence of an additional, weaker, promoter located between the two genes. A number of potential promoters were also identified in the 3' region of the *acd* gene; however, it is unlikely that the ORF is transcribed from any of these potential promoters. A sequence showing a high percent similarity to the consensus A/T-rich binding site for bacterial LRP proteins, (c/t)AG(a/t/c)A(a/t)ATT(a/t)T(a/c/t)CT(a/g) (Napoli et al., 1999) was identified in the 5' upstream region of the ORF (Fig. 3). This LRP binding site overlaps the -10 region of the strong promoter 1p, suggesting that LRP-like protein if bound could repress the transcription of ORF.

The FNR binding site is located at position -39.5 relative to the deduced transcription initiation site of the *acd* gene and could direct anaerobic FNR-dependent transcription of the *acd* gene from the $2p^a$ promoter (Fig. 3). The FNR-binding site should be centered at –41.5 bp relative to the transcription initiation site to direct transcription effectively (Kaiser and Sawers, 1997).

Figure 1. Full length alignments of *E. cloacae* ORF and LRP of *Enterobacter aerogenes* (NCBI, accession #P19494) sequences. Smith-Waterman score: 409; 42.6% identity in 141 aa overlap. Leucine in a 6-amino acid periodic pattern is shown in bold in positions 42, 48, 54, 60, 65, 72, 78. Two dots indicate the presence of the identical amino acid. One dot indicates the presence of a similar amino acid.

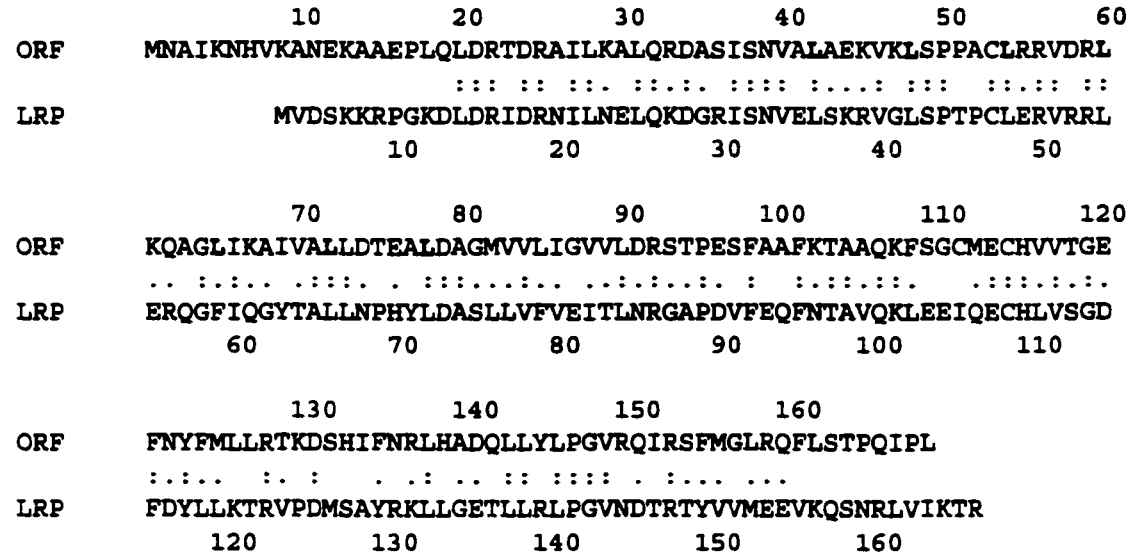

10 20 30 40 50 60
ORF MNAIKNHVKANEKAAEPLQLDRTDRAILKALQRDASISNVALAEKVKLSPPACLRRVDRL
LRP MVDSKKRPGKDLDRIDRNILNELQKDGRISNVELSKRVGLSPTPCLERVRRL
10 20 30 40 50
70 80 90 100 110 120
ORF KQAGLIKAIVALLDTEALDAGMVVLIGVVLDRSTPESFAAFKTAAQKFSGCMECHVVTGE
LRP ERQGFIQGYTALLNPHYLDASLLVFVEITLNRGAPDVFEQFNTAVQKLEEIQECHLVSGD
60 70 80 90 100 110
130 140 150 160
ORF FNYFMLLRTKDSHIFNRLHADQLLYLPGVRQIRSFMGLRQFLSTPQIPL
LRP FDYLLKTRVPDMSAYRKLLGETLLRLPGVNDTRTYVVMEEVKQSNRLVIKTR
120 130 140 150 160

Figure 2. Correlation between the total charge of the *E. cloacae* UW4 LRP-like protein and optimal ACC deaminase activity as a function of pH. Data for the dependence of ACC deaminase activity on pH are adapted from Honma and Shimomura (1978). Unfilled square, protein charge as deduced with PepTool for Windows; filled square, α-ketobutyrate.

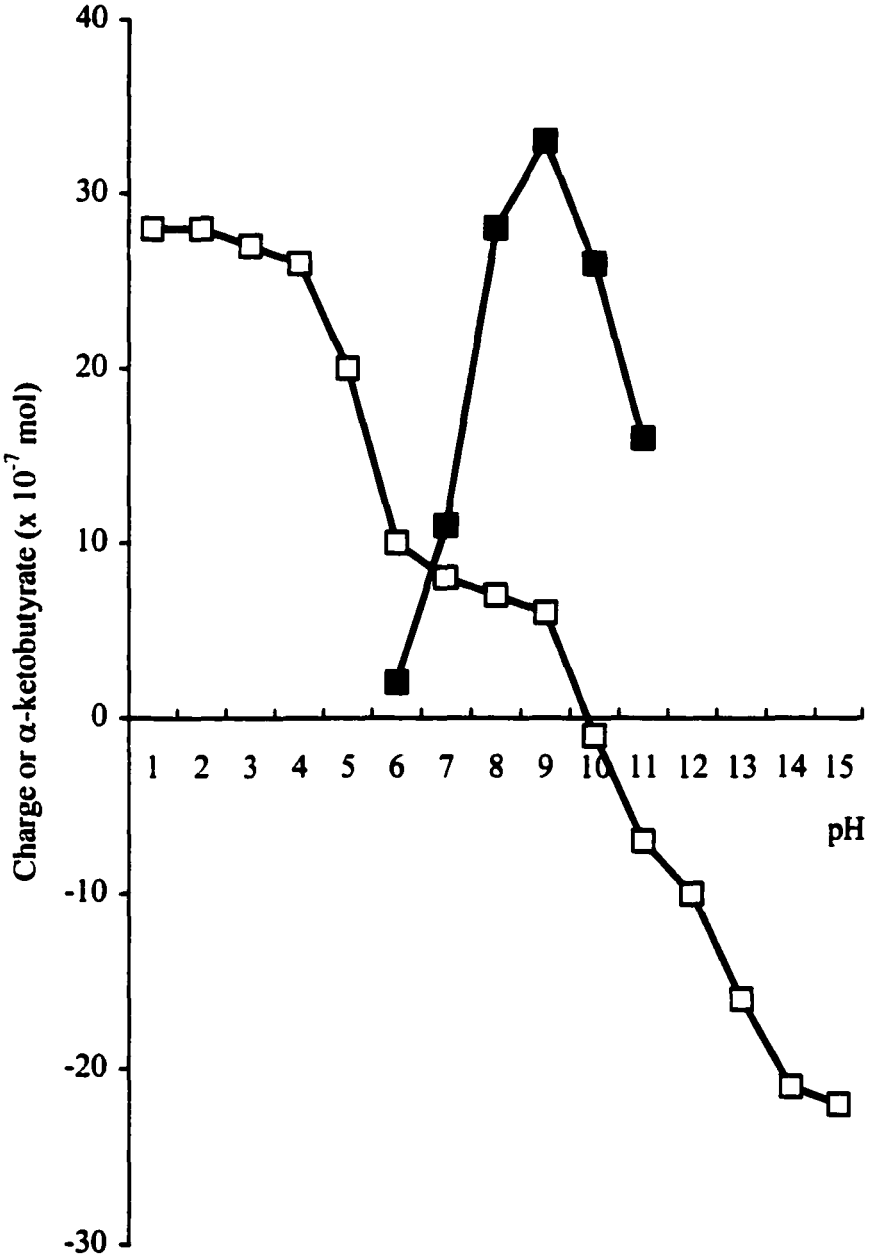
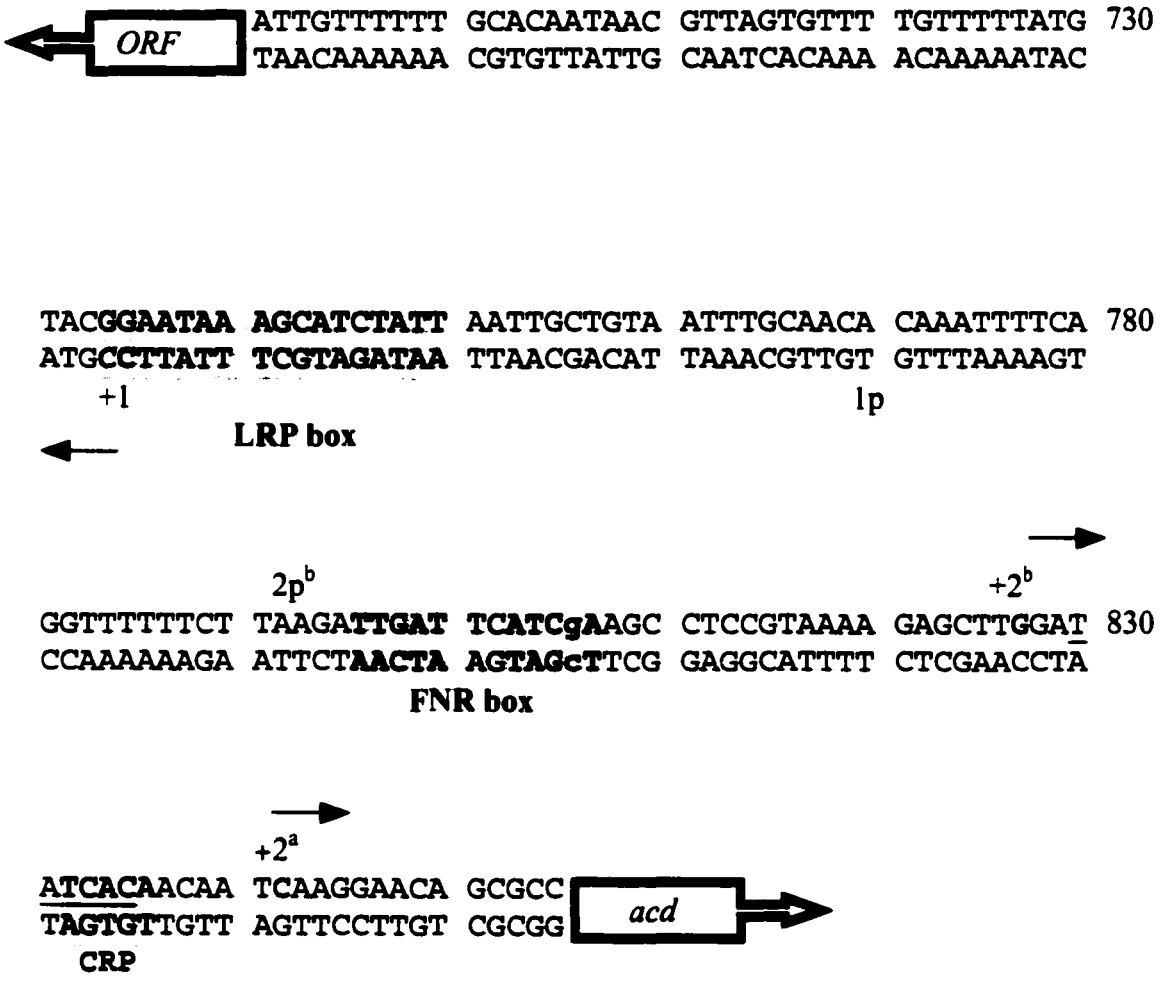

40
30
20
10
0
-10
-20
-30
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
pH
Charge or α-ketobutyrate (x 10⁻⁷ mol)

Figure 3. Nucleotide sequence between the *acd* gene and gene for the LRP-like protein from *E. cloacae* UW4. 1p, ORF promoter with score 0.94; $2p^a$, *acd* promoter with score 0.42; $2p^b$, *acd* promoter with score 0.46. FNR-binding box, LRP-binding site and CRP-binding half-site are highlighted. The -10 sequence of the deduced FNR-dependent promoter is underlined. The 5' end of the promoter regions are shown as 1p and $2p^b$. The transcription start sites are designated as +1, $+2^a$ and $+2^b$.


ORF
ATTGTTTTTT GCACAATAAC GTTAGTGTTT TGTTTTTATG 730
TAACAAAAAA CGTGTTATTG CAATCACAAA ACAAAAATAC
TACGGAATAA AGCATCTATT AATTGCTGTA ATTTGCAACA CAAATTTTCA 780
ATGCCTTATT TCGTAGATAA TTAACGACAT TAAACGTTGT GTTTAAAAGT
+1
lp
LRP box
2p^b
+2^b
GGTTTTTTCT TAAGATTGAT TCATCgAAGC CTCCGTAAAA GAGCTTGGAT 830
CCAAAAAAGA ATTCTAACTA AGTAGcTTCG GAGGCATTTT CTCGAACCTA
FNR box
+2^a
ATCACAACAA TCAAGGAACA GCGCC
TAGTGTTGTT AGTTCCTTGT CGCGG
acd
CRP

In *E. cloacae* UW4, the palindromic FNR-binding site lacks two central spacers and hence, is moved 2 bp toward the start of transcription initiation that could lower the strength of FNR-dependent transcription from the putative promoter $2p^a$. It is also possible that a single TTGAT half-site is functional. The GC base pair in the TTGAT half-site is vital for recognition by the FNR protein (Sawers, 1993) and a single functional DNA half-site is sufficient to direct binding and transcription by an FNR protein (Melville and Gunsalus, 1996; Sawers et al., 1997). The second potential promoter of the *acd* gene is located between the *E. cloacae* UW4 ORF and the *acd* gene and is designated as the promoter $2p^b$ in Fig. 3. The promoter $2p^b$ overlaps promoter $2p^a$ similar to the overlapping promoters controlling FNR-dependent transcription of the *focA* gene of the *E. coli focApfl* operon (Kaiser and Sawers, 1997). It is also possible that the ORF may activate transcription of the *acd* gene from the $2p^b$ promoter under aerobic condition when the FNR-dependent promoter is inactive. The presence of a consensus binding half-site of CRP which overlaps the Pribnow box of the putative FNR-dependent promoter of the *acd* gene (Fig. 3) suggests the possible inhibitory effect of low glucose levels and high concentrations of cAMP on the expression of ACC deaminase in *E. cloacae* UW4.

3.1.3. ACC deaminase activity under different physiological conditions

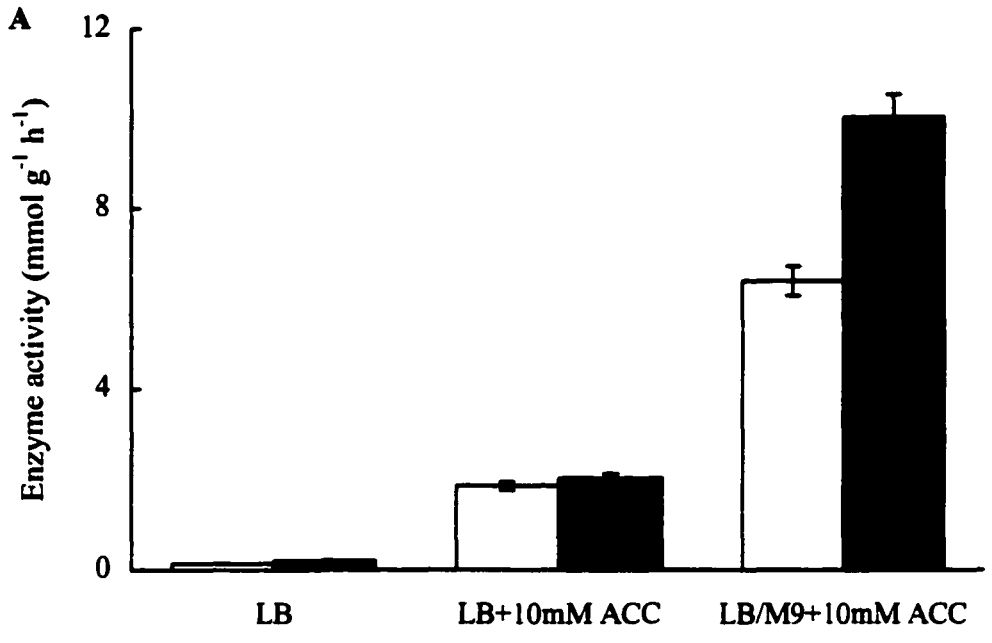
The presence of ORF and various putative transcriptional regulatory sequences upstream of the *E. cloacae* UW4 *acd* gene suggests a complex mechanism of regulation for the expression of this gene. However, despite the intriguing regulatory possibilities that are suggested by the DNA sequence analysis, the involvement of any particular

element in transcriptional regulation of the *acd* gene remains to be demonstrated. In this regards, a plasmid complementation study based on the DNA sequence analysis reported here revealed, when the *acd* gene was replaced by *luxAB*, that both the entire ORF and the DNA sequence between the ORF and the *luxAB* were required for maximal *luxAB* activity (Li, Shah, Moffatt and Glick, manuscript in preparation).

To prove that the deduced mechanisms are operative, the ACC deaminase activity of a number of different constructs, transformed into various host cells and grown under different conditions was examined. Growth of *E. coli* DH5α/p4U2 cells in LB medium in the absence of ACC under both aerobic and anaerobic conditions yielded only a very low level of ACC deaminase activity (Fig. 4A). The addition of ACC to the LB growth medium resulted in a significant increase in enzyme activity in both aerobically and anaerobically induced *E. coli* DH5α/p4U2. In *E. coli*, ACC deaminase activity was increased considerably when the cells were first grown in LB medium then washed and resuspended in M9 medium supplemented with ACC. Moreover, this "second growth" resulted in an additional increase in enzyme specific activity when it was performed under anaerobic conditions (Fig. 4A). In *Enterobacter cloacae* UW4, the highest level of enzyme activity was observed in cells grown anaerobically in rich medium and induced in M9 medium lacking ammonium and supplemented with ACC under aerobic conditions (Table 2).

If the LRP and the novel LRP-like protein encoded by the ORF upstream of the *acd* gene in *E. cloacae* UW4 are important in regulating the expression of ACC deaminase then the presence of high levels of leucine in the induction medium should have a significant effect on ACC deaminase expression. In this experiment, induction in

Figure 4. Induction of ACC deaminase in different strains of *E. coli*. (A) Induction of ACC deaminase in *E. coli* DH5α/p4U2 in different media. White bars, aerobic conditions; grey bars, aerobic growth conditions followed by 12 h anaerobic incubation. (B). ACC deaminase specific activity in *E. coli* JC1552/p4U2 (lrp^+) and *E. coli* AE84064/p4U2 (lrp^-). Black bars, aerobic growth in LB medium followed by induction in M9 medium plus 5 mM ACC as a nitrogen source for 4 h under limited oxygen conditions; gray bars, aerobic growth in LB medium followed by induction in M9 plus 100 mM ACC for 4 h under anaerobic conditions. LB medium was supplemented with 0.4% glucose. Values are means ± SE, n = 5. $P < 0.05$.


A
12
8
4
0
Enzyme activity (mmol g-1 h-1)
LB
LB+10mM ACC
LB/M9+10mM ACC

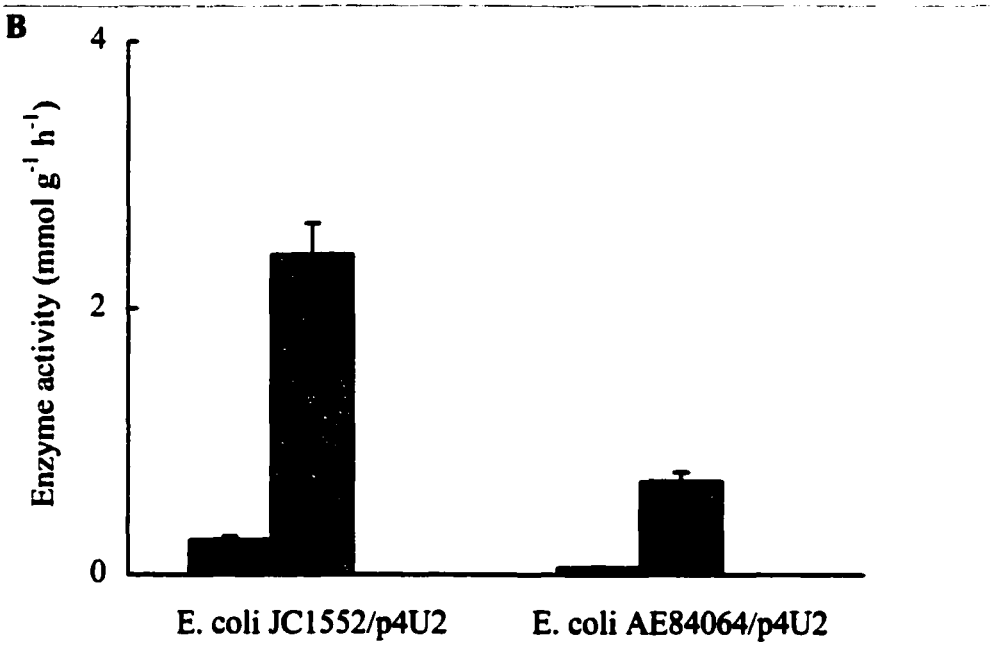

B
4
2
0
Enzyme activity (mmol g-1 h-1)
E. coli JC1552/p4U2
E. coli AE84064/p4U2

Table 2. ACC deaminase activity (mmol g^{-1} h^{-1}) *in E. cloacae* UW4.

Induction medium	Growth/induction condition		
	$TSB+O_2/+O_2$	$TSB+O_2/-O_2$	$TSB-O_2/+O_2$
M9 salts, 10 mM ACC, 0.4% glucose for 12 h	4.7 ± 0.4	0.9 ± 0.1	16.3 ± 1.6

Values are means ± SE, n = 5. $P < 0.05$. $TSB+O_2$, aerobic growth in TSB; $TSB-O_2$, anaerobic growth in TSB. $+O_2$, aerobic induction; $-O_2$, anaerobic induction.

M9 medium plus 1 mM leucine and 10 mM ACC but no ammonium salts decreased the ACC specific activity by 20% with *E. coli* DH5α/p4U2 and by 50% with *E. cloacae* UW4, both grown under aerobic conditions (data not shown). In another experiment, plasmid p4U2 was used to transform *E. coli* strains JC1552 (which contains LRP) and AE84064 (which is devoid of LRP). Following aerobic growth in LB medium and then prolonged anaerobic induction in minimal medium with two different levels of ACC, strain JC1552 showed a much higher level of ACC deaminase activity (Fig. 4B). The higher ACC concentration resulted in a higher specific ACC deaminase activity in both of these host strains (Fig. 4B). Introduction of 100 mM NH_4Cl into the growth medium did not affect the ACC deaminase specific activity in the lrp^- strain while the specific activity in the lrp^+ strain was substantially increased (data not shown). These data are consistent with the involvement of the LRP-like protein encoded by the ORF upstream of the *acd* gene together with ACC (which presumably binds to the LRP-like protein) as a positive regulator of *acd* transcription. Based on this assumption, the protein encoded by the ORF is hereafter referred to as Bacca (binding 1-aminocyclopropane-1-carboxylic acid).

In the absence of glucose, in Czapex Dox medium supplemented with 25 mM ACC the enzyme activity was low in *E. coli* strains. In *E. cloacae* UW4, ACC deaminase specific activity in the absence of glucose was always lower than the activity in the presence of glucose. In addition, the presence of 2 mM cAMP in the growth medium decreased the enzyme activity in all experiments. These data, combined with the presence of a potential CRP binding site just upstream of the *acd* gene, are consistent with cAMP being a negative regulator of ACC deaminase expression.

In *E. cloacae* UW4, the strongest putative promoter with a score of 0.99 (by the program Promoter Prediction) is located 158 bp upstream of the *acd* gene. To examine both the possible independent functioning of this promoter and to confirm that Bacca activates the expression of *acd*, plasmid p53997 was constructed by inserting a fragment from plasmid p4U2 containing the *acd* gene along with the 580 bp upstream sequence into the shuttle vector pME6030 (Fig. 5 and 6). *E. coli* DH5α/p53997 cells did not express ACC deaminase activity (either under aerobic or anaerobic conditions) despite the fact that *E. coli* DH5α is an lrp^+ strain. On the other hand, transformants of *E. coli* DH5α/p53997 that contain either pEag or pEco (Fig. 6) carrying the *bacca* gene were able to grow both on solid and liquid medium containing ACC as a sole nitrogen source. Under aerobic conditions the ACC deaminase activity of *E. coli* DH5α/p53997/pEag reached approximately 20% of the activity found in *E. coli* DH5α/p4U2 (data not shown). The lower activity of *trans* complemented strain may be a result of both the low plasmid copy number and methabolic load. With SDS-PAGE of protein extracts of *E. coli* DH5α/p53997/pEag there was accumulation of a protein with a molecular mass of 35,000 daltons, the size of the ACC deaminase monomer, while no corresponding band was observed for *E. coli* DH5α/p53997 (data not shown).

Under anaerobic conditions, oxygen can be replaced by alternative electron acceptor such as nitrate which is a constituent of Czapex Dox medium and the corresponding anaerobic respiratory chain is induced. When *E. coli* DH5α/p4U2 was transformed with pRK2501*fnr*, anaerobic growth followed by oxygen limited induction in Czapex Dox medium supplemented with ACC resulted in 18-fold increase in enzyme activity in *E. coli* DH5α/p4U2/pRK2250l*fnr* compared to *E. coli* DH5α/p4U2 probably

Table 3. Effect of the composition of induction medium on ACC deaminase enzyme specific activity (mmol g^{-1} h^{-1}).

Induction medium	Growth/induction	*E. coli* DH5α/ p4U2	*E. coli* DH5α/ p4U2/pRK2501*fnr*
Czapex Dox, 25 mM ACC	Czapex Dox-O_2/-O_2	0.03	0.53
M9 salts (no NH_4^+), 100 mM ACC, 4 h	LB+O_2/+O_2	7.20	3.90
M9 salts (no NH_4^+), 5 mM ACC, 0.4% glucose, 4 h	LB+O_2/-O_2	10.22	5.64
M9 salts, 100 mM NH_4^+, 5 mM ACC, 0.4% glucose, 4 h	LB+O_2/-O_2	9.84	10.68

Values are means of at least three assays where the standard errors did not exceed 15%. LB+O_2, aerobic growth in LB medium; Czapex Dox-O_2, anaerobic growth in Czapex Dox medium; +O_2, aerobic induction; -O_2, anaerobic induction.

Figure 5. Construction of plasmid p53997. (A), lane 2, a 1% agarose gel containing undigested plasmid p4U2 (B), lanes 2 and 3, *Sph*I complete digest of the p4U2 plasmid DNA. The 1.7-kb fragment was gel-purified and used to construct plasmid p53997; (C), lanes 1 and 2, purified plasmid p53997 (1,7-kb *Sph*I fragment with *acd* gene from p4U2 inserted in the *Sph*I site of pME6030) digested with *Sph*I revealing the fragment of approximately 1.7 kb as expected; lanes 3, 4, 5, and 6, linearized plasmid p53997. The DNA was visualized by ethidium bromide staining. Lanes 1 and 4 of panels A and B and lane 7 of panel C, GeneRuler™ DNA 1 kb Ladder (MBI Fermentas Inc., Flamborough, ON, Canada).

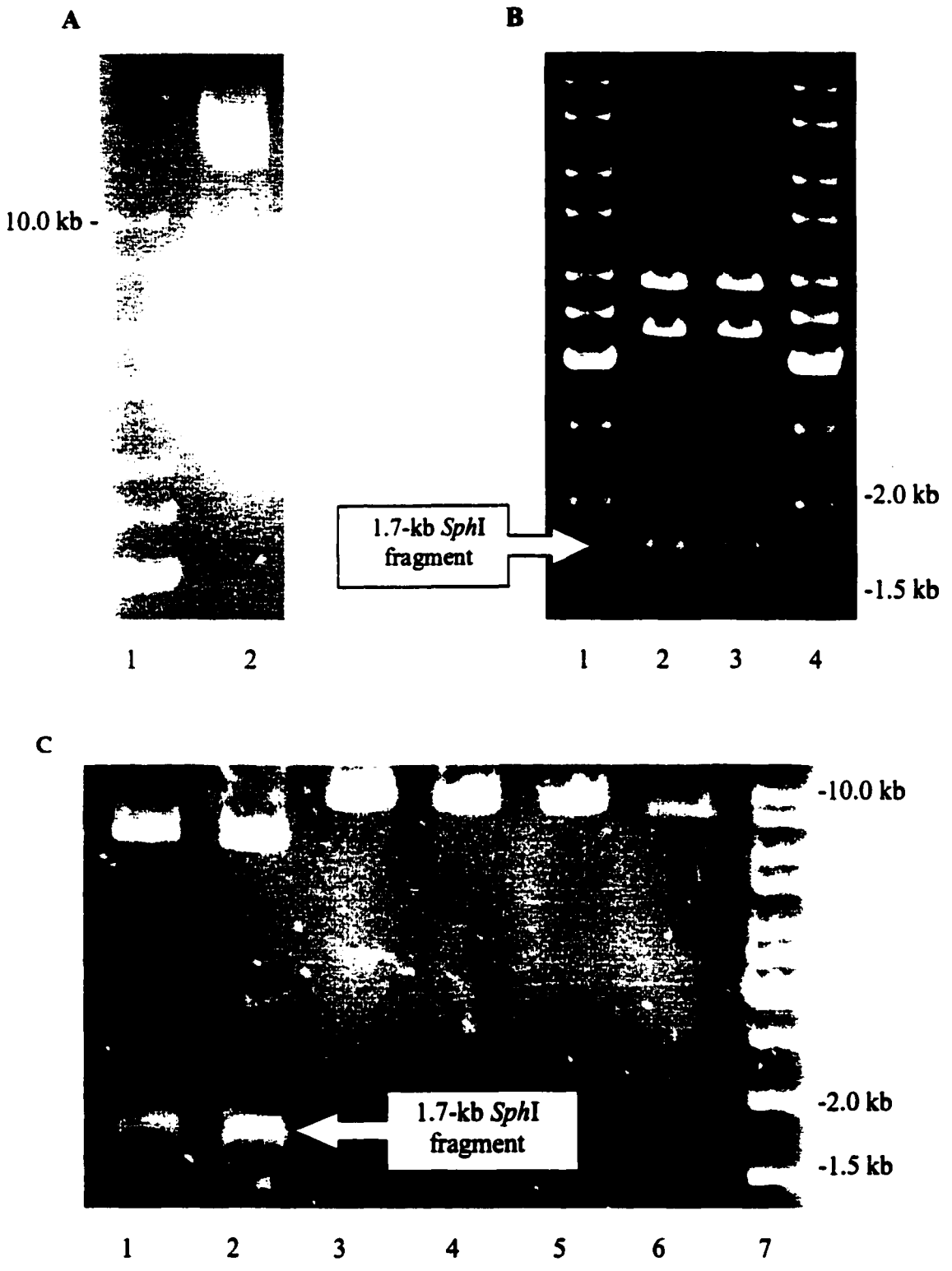
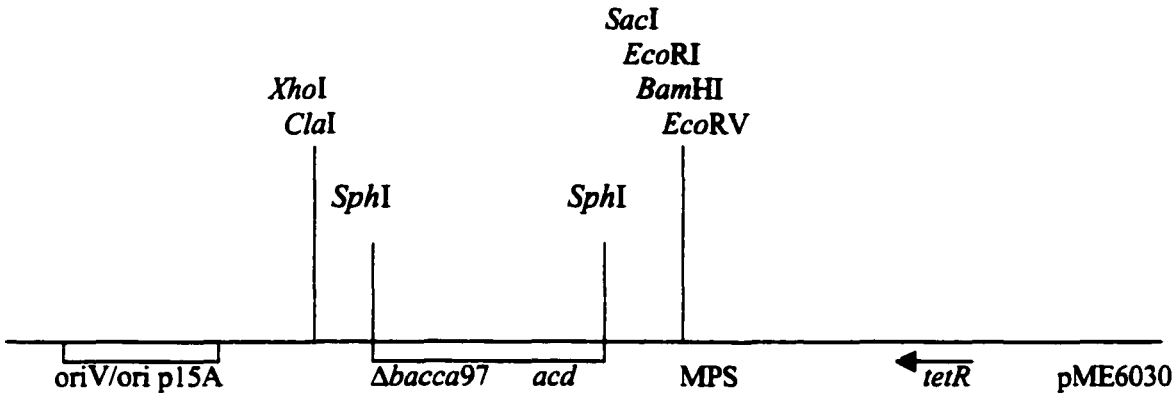

A
10.0 kb -
1
2
B
1.7-kb SphI fragment
-2.0 kb
-1.5 kb
1
2
3
4
C
-10.0 kb
1.7-kb SphI fragment
-2.0 kb
-1.5 kb
1
2
3
4
5
6
7

Figure 6. Restriction map of (A) plasmid p53997; (B) plasmid pEag; (C) plasmid pEco.

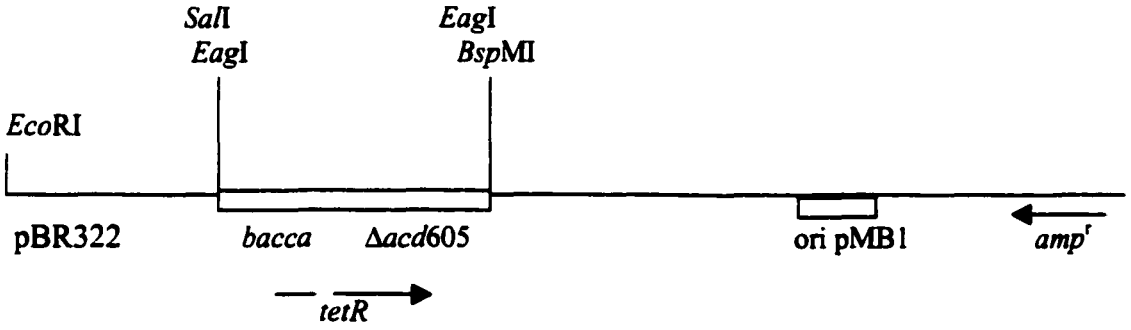
A

p53997 (9.7 kb)



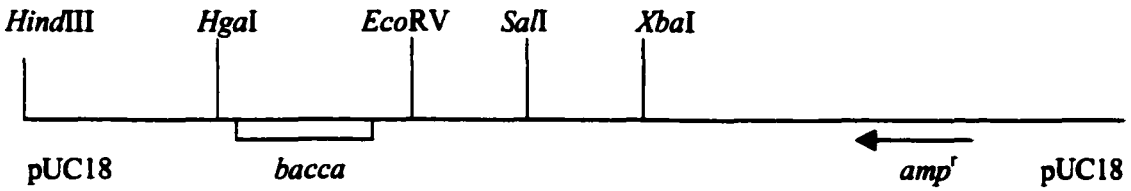
B

pEag (5.5 kb)



C

pEco (8.4 kb)



because the intracellular concentration of FNR was increased (Table 3). The highest ACC deaminase activity was observed in cells grown in LB under oxygen-sufficient conditions and induced in M9 medium supplemented with ACC under oxygen limiting conditions which were designed to favor genes involved in fermentative growth rather than anaerobic respiration (Table 3). In this case the release of ammonium may make a substantial contribution to deacidification (Winkelman and Clark, 1986). Similar results were observed for *P. aeruginosa* PAO1 and *P. fluorescens* CHA0 (Winteler et al., 1996; Laville et al., 1998). Decrease in ACC deaminase activity upon introducing pRK2501*fnr* into *Escherichia coli coli* DH5α/p4U2 under both aerobic and anaerobic induction in M9 minimal medium lacking ammonium salts and supplemented with ACC may reflect the metabolic load that is a consequence of synthesizing the plasmid pRK2501*fnr* encoded proteins.

ACC deaminase activity was higher in *Escherichia coli* JRG465 (fnr^+ strain) by approximately 5-fold compared to the enzyme activity in *Escherichia coli* SD7 (fnr^- strain) in cells induced under oxygen limiting conditions in Czapex Dox growth medium supplemented with 0.4% glucose and 25 mM ACC for 16 hours (Fig. 5). In this case, the highest amount of activity was observed in *E. coli* strain AE84064/p4U2 (lrp^-). In addition, ACC deaminase specific activity reached 2 mmol g^{-1} h^{-1} in *E. coli* DH5α/p4U2 cells which were grown and induced in M9 salt medium lacking ammonium and supplied with 10 mM ACC, 50 mM glycerol, 15 mM maltose and 20 mM KNO_3 under anaerobic conditions.

In the absence of leucine, the ACC deaminase specific activity in the *E. coli* lrp^- strain was substantially higher compared to the activity in the lrp^+ strain under anaerobic

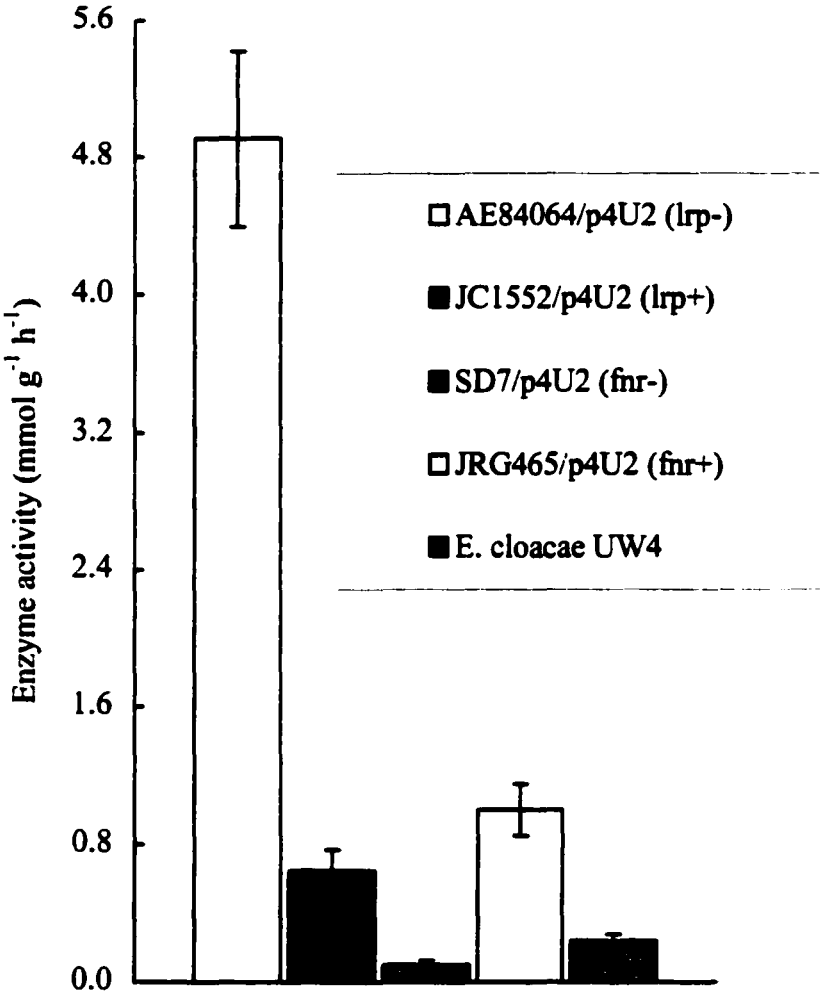
induction in Czapex Dox medium (Fig. 7) suggesting that LRP may repress the transcription of the *bacca* gene in the absence of leucine. The excessive amount of available leucine may result in the binding of Bacca protein to leucine followed by a decrease in ACC activity.

Enterobacter cloacae UW4 behaved somewhat differently compared to *Escherichia coli*. Cells grown aerobically in rich medium and induced under oxygen limiting conditions for 12 h had a 5-fold lower amount of ACC deaminase activity than did *Enterobacter cloacae* UW4 which was grown and induced under aerobic conditions (Table 2). The amount of ACC deaminase activity in *E. cloacae* UW4 which was grown and induced under anaerobic conditions in Czapex Dox medium supplied with 10 mM ACC and 0.4% glucose was similar to the ACC deaminase activity in the *E. coli* fnr$^-$ strain (Fig. 7) suggesting a low concentration of the FNR-like protein in this bacterium. Although the data are consistent with the involvement of FNR in the transcriptional regulation of ACC deaminase, precisely how this occurs is unclear at the present time. However, it should be kept in mind that ACC is both a substrate of ACC deaminase and an inducer of the *acd* gene transcription (Honma and Shimomura, 1978) and that during prolonged incubations with ACC the concentration of this molecule continues to change.

3.2. Effect of constitutive and root-specific ACC deaminase gene expression on tomato plant growth and development

3.2.1. Enzyme activity

Figure 7. ACC deaminase activity in different bacterial strains. Cells were induced anaerobically in Czapex Dox media supplemented with 0.4% glucose and 25 mM ACC for 16 h. Values are means ± SE, n = 5. $P < 0.05$.


5.6
4.8
4.0
3.2
2.4
1.6
0.8
0.0
Enzyme activity (mmol g-1 h-1)
AE84064/p4U2 (lrp-)
JC1552/p4U2 (lrp+)
SD7/p4U2 (fnr-)
JRG465/p4U2 (fnr+)
E. cloacae UW4

As expected, there was a significant amount of ACC deaminase activity in the leaf, root and pericarp tissues of transgenic plants but none or negligible amounts in the non-transformed plants (Table 4). In plants with constitutive and root-specific ACC deaminase gene expression, enzyme activities were similar in roots (Table 4). The apparent activity seen in non-transformed plants may be the consequence of the non-specific cleavage of ACC.

To date, there is no evidence suggesting the presence of ACC deaminase in tomato plants despite the fact that this enzyme has been found in yeast and fungi (Minami et al., 1998; Jia et al., 1999) as well as bacteria. Treatment of transgenic plants with Co^{2+}, which is an inhibitor of ACC oxidase, resulted in a substantial increase in ACC deaminase activity in parallel to the uptake of Co^{2+} into tomato plants leaves (Fig. 8).

3.2.2. Germination

To examine the effect of ACC deaminase on the germination of tomato seeds, surface sterilized seeds were germinated in sterile growth pouches containing either 1 mM nitric acid pH 5.7 as a control or 1 mM silver nitrate pH 5.7. In non-transformed plants, silver delayed germination while with both types of transgenic seeds germination was hastened (Fig. 9). Since PRB-1*b* seeds germinated much more slowly than any of the other plants, they were not included in these experiments. In tomatoes and many other plants, ethylene promotes seed germination and its effect is antagonized by the ethylene

Table 4. ACC deaminase activity (mmole g^{-1} total protein h^{-1}) in mature non-transformed and transgenic tomato plants cv. Heinz 902.

Plant organ	Non-transformed plants	Transgenic plants: promoter fused to ACC deaminase gene		
		35*S*	*rolD*	PRB-1*b*
Leaf	0.022 ± 0.009 (6)	0.805 ± 0.243 [a] (13)	0.029 ± 0.007 (13)	0.061 ± 0.030 (11)
Root	0.067 ± 0.016 (2)	0.641 ± 0.130 [a] (5)	0.830 ± 0.144 [a] (4)	0.151 ± 0.045 (6)
Green fruit	ND	0.781 ± 0.122 (28)	0.126 ± 0.036 (10)	ND
Red fruit	ND	0.170 ± 0.050 (7)	0.142 ± 0.026 (17)	0.084 ± 0.018 (3)

The values are means ± SE, $P < 0.05$. a, significantly different from non-transformed plants. ND, not determined. The values in brackets indicate the number of plants examined.

Figure 8. ACC deaminase activity and Co^{2+} content in leaves of non-transformed plants and transgenic plants treated with Co^{2+}. White bars, ACC deaminase activity; black bars, cobalt content in leaves.

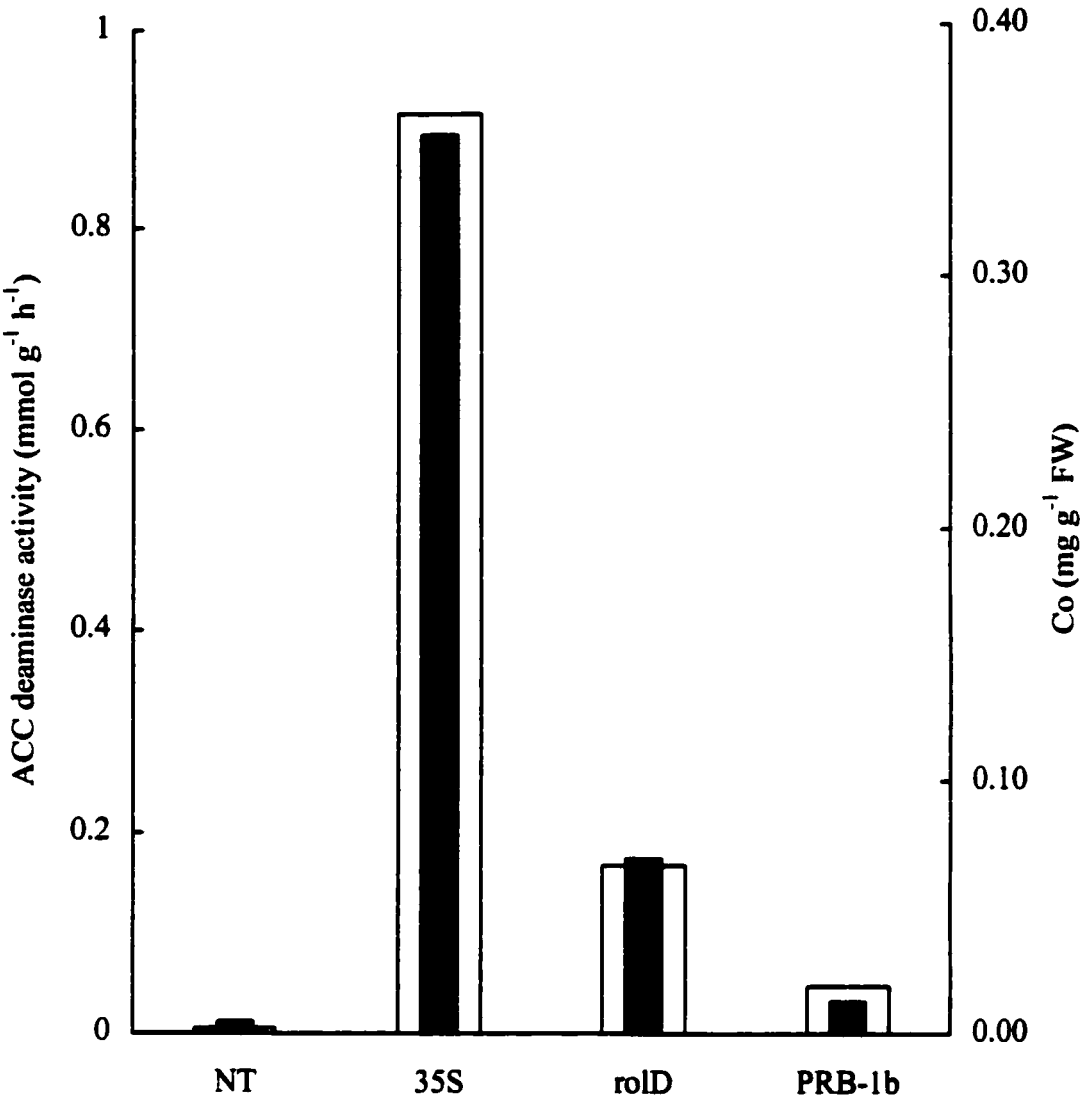
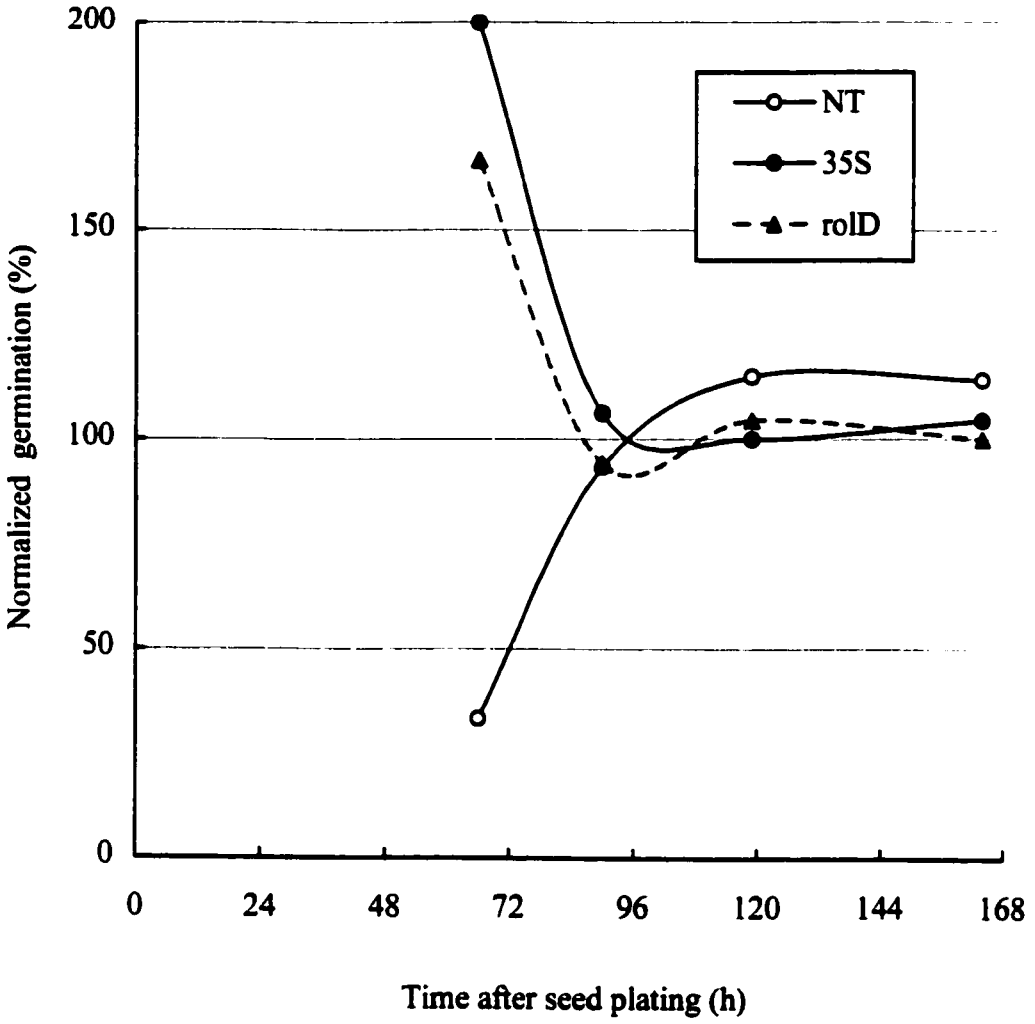

ACC deaminase activity (mmol g-1 h-1)
Co (mg g-1 FW)
1
0.8
0.6
0.4
0.2
0
0.40
0.30
0.20
0.10
0.00
NT
35S
rolD
PRB-1b

Figure 9. Effect of Ag^+ on the germination of tomato seeds (n = 25). In each case the data for the number of seeds germinated in the presence of Ag^+ are normalized to the control (i.e., the number of seeds germinated in plants in the absence of Ag^+). NT, non-transformed. Promoter constructs present in the transgenic plants are found in insert.


200
150
100
50
0
Normalized germination (%)
0
24
48
72
96
120
144
168
Time after seed plating (h)
NT
35S
rolD

action inhibitor Ag^+ (Abeles et al., 1992). Non-transformed seeds treated with silver probably did not produce enough ethylene for normal germination. The binding of ethylene to the receptor removes repression in the signaling pathway leading to the characteristic triple response. ACC deaminase gene expression could affect the pH shift and rate of the cleavage of ethylene-metal bond in receptor complex followed by increase in tissue sensitivity to ethylene. In this case, treatment with Ag^+ would stimulate both transgenic seed germination and early plant growth.

3.2.3. Early root and shoot development

When non-transformed and the 35*S* and *rolD* promoter transgenic tomato plants were grown in sterile growth pouches filled either with distilled water or 1 mM nitric acid pH 5.7, after one week the roots of both types of transgenic seedlings were significantly longer than the roots of non-transformed seedlings (Table 5). In addition, both 1-week-old transgenic plants with the *rolD* promoter and plants with the 35*S* promoter had similar root weights of 7.870 mg (n = 172) and 8.035 mg (n = 172), respectively, while non-transformed plants exhibited a significantly lower root weight of 6.543 mg (n = 131). Noticeably, root specific ACC deaminase gene expression resulted in a significant increase in the fresh shoot weight of one-week-old plants up to 18.99 mg (n =172) compared to 17.17 mg (n = 131) in non-transformed plants. By contrast, the shoot weights of 35*S* and PRB-1*b* plants were not significantly increased over the non-transformed plants. The treatment of tomato plants with Ag^+ resulted in a significant increase in both root length (Fig. 10) and weight (data not shown) in 35*S* and PRB-1*b* plants and similar levels of silver accumulated in the roots of non-transformed, 35*S* and *rolD* plants (Fig. 11).

Table 5. Root length (mm) of one-week-old tomato plants cv. Heinz 902 grown in sterile growth pouches.

	Genotype		
	NT	35*S*	*rolD*
Distilled water	75 ± 2	80 ± 2 [a]	82 ± 2 [a]
	(56)	(67)	(58)
1 mM HNO_3, pH 5.7	58 ± 2	72 ± 2 [a]	73 ± 2 [a]
	(131)	(172)	(172)

The values are means ± SE, $P < 0.05$. a, significantly different from non-transformed plants. The numbers in brackets indicate the number of plants examined. NT, non-transformed.

Figure 10. Effect of silver on root elongation. a, significantly different than in the absence of silver. b, significantly different from non-transformed plants. NT, non-transformed. Promoters in the ACC constructs indicated in insert.

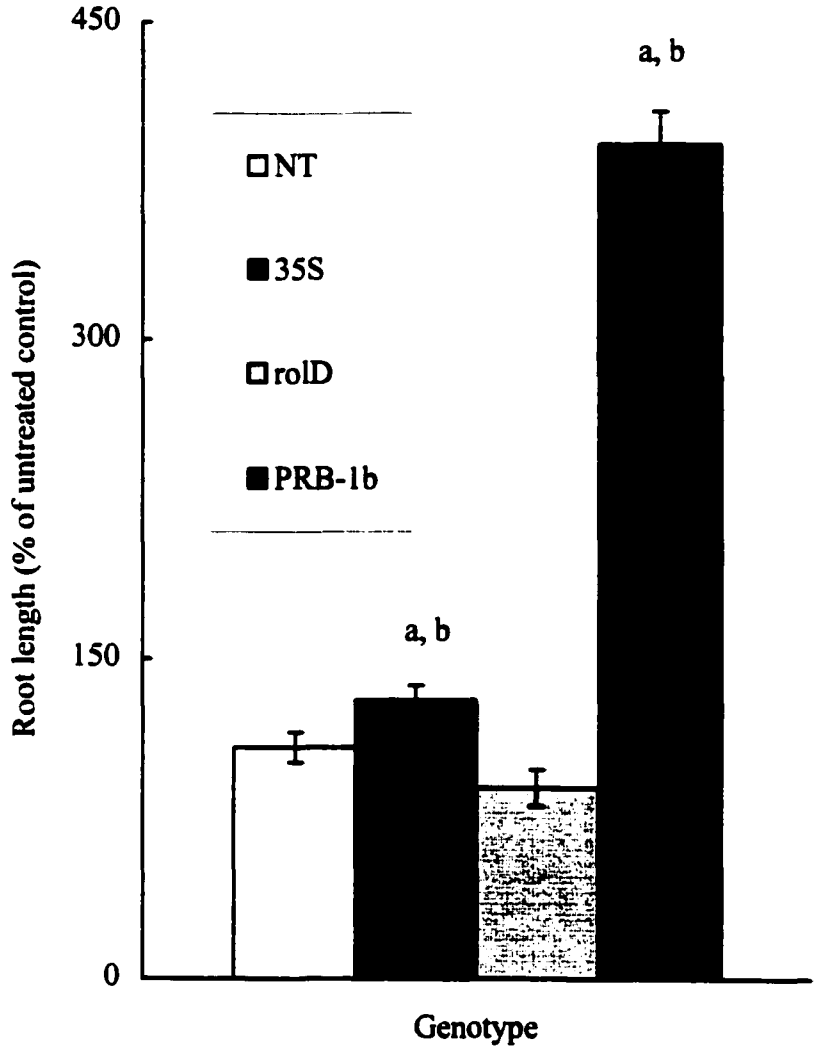
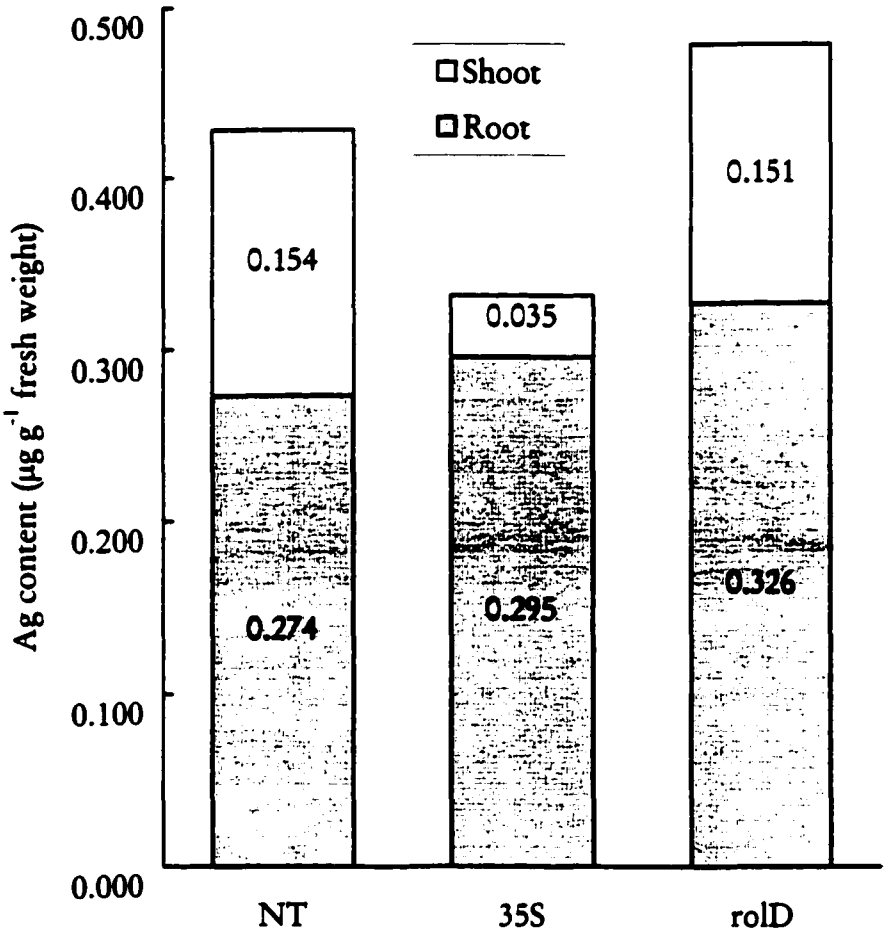

450
300
150
0
Root length (% of untreated control)
NT
35S
rolD
PRB-1b
a, b
a, b
Genotype

Figure 11. Accumulation of silver by 1-week-old tomato plants grown in sterile growth pouches. NT, non-transformed.


0.500
0.400
0.300
0.200
0.100
0.000
Ag content (µg g-1 fresh weight)
Shoot
Root
0.154
0.274
0.035
0.295
0.151
0.326
NT
35S
rolD

The fresh shoot weights of silver treated plants: non-transformed, 8.9 ± 0.5 mg (n = 18); 35*S*, 11.5 ± 0.8 mg (n = 16); *rolD*, 10.4 ± 0.9 mg (n = 18) were inversely correlated with the accumulated amounts of silver. The ratios of fresh shoot weight to fresh root weight, which were similar before the treatment with silver in all plants, did not change in *rolD* plants while it dropped by 17% in both non-transformed plants and 35*S* plants (Fig. 12).

3.2.4. Development of plants grown in soil

Following seventeen days of growth in Pro-Mix growth medium in a greenhouse, transgenic plants had significantly greater fresh shoot weight compared to non-transformed plants (Table 6). Transgenic plants also showed a decrease in their root hair production, as was observed by SEM (data not shown). The ability of transgenic plants to develop adventitious roots after prolonged flooding was also diminished compared to non-transformed plants (data not shown). All transgenic plants grown for fifty-one days in the greenhouse had significantly higher shoot fresh and dry weights compared to non-transformed (Fig. 13A and 13B). Fifty-one day plants with either the 35*S* or *rolD* promoter also had a higher ratio of dry/fresh shoot weight (Fig. 13C). Treatment with silver resulted in significant increases in both shoot fresh and dry weight compared to non-transformed plants in *rolD* plants (Fig. 13A and 13B) and in the ratio of dry/fresh shoot weight in plants with the PRB-1*b* promoter (Fig. 13C). Transgenic 35*S* plants absorbed a significantly greater amount of silver in their roots than did non-transformed plants while *rolD* plants were much less efficient than non-transformed at absorbing silver into their roots (Fig. 14).

Table 6. Effect of ACC deaminase expression on the fresh shoot weight of seventeen-day-old tomato plants.

Plant genotype	n	Mean ± SE (mg)
Non-transformed	63	110 ± 6
35*S*	35	153 ± 7 [a, b]
rolD	55	171 ± 6 [a]
PRB-1*b*	34	140 ± 10 [a, b]

The values are means ± SE, $P < 0.05$. a, significantly different from non-transformed plants; b, significantly different from *rolD* plants.

Figure 12. Effect of treatment with silver on the ratio of fresh shoot weight to fresh root weight of one-week-old plants. Legend: blank, non-transformed; gray, *rolD*; black, 35*S* plants (n = 20).

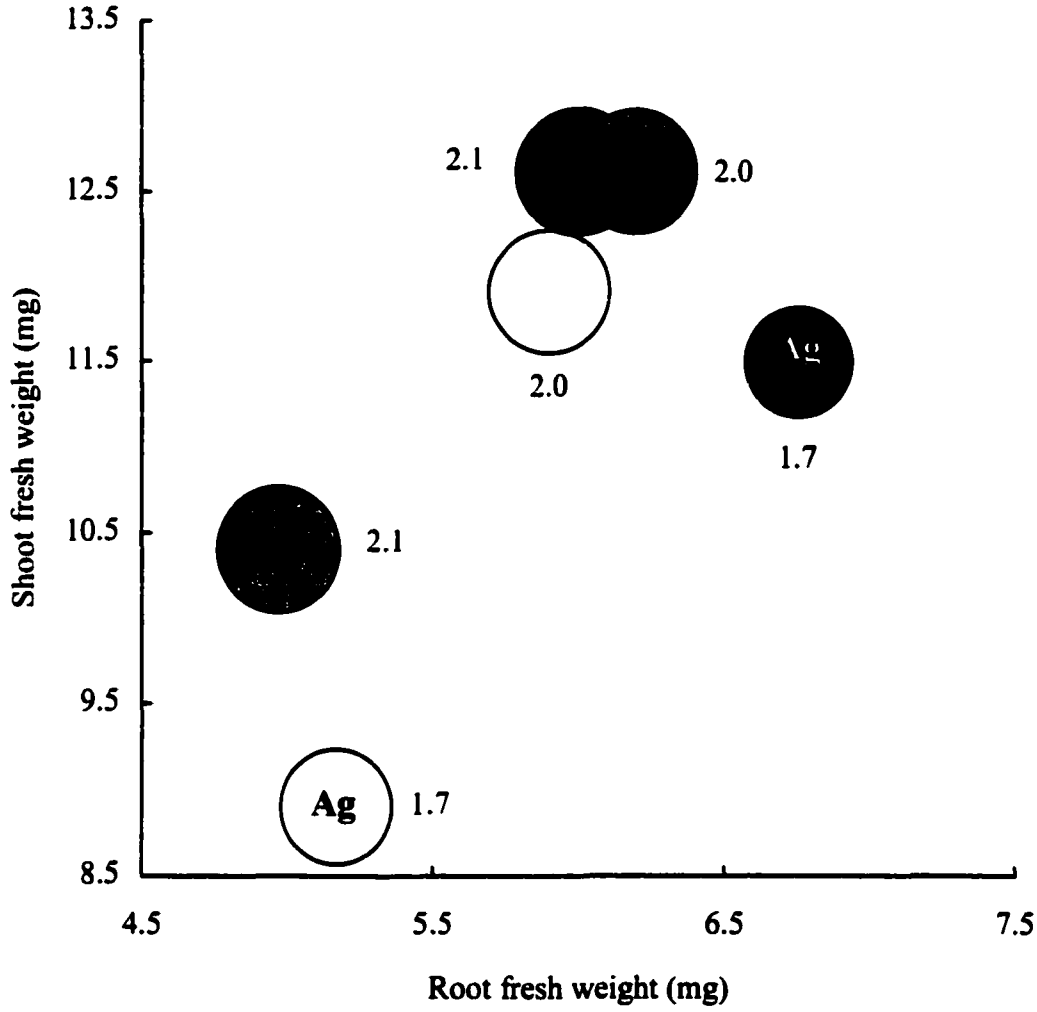
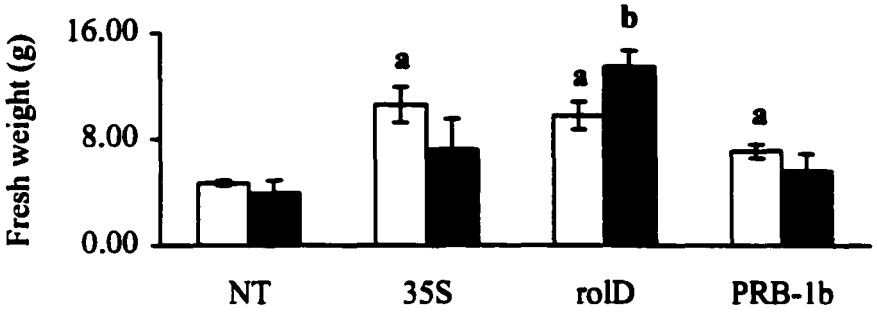
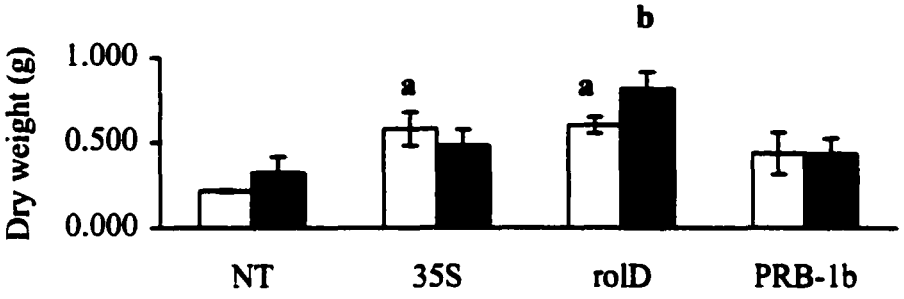

13.5
12.5
11.5
10.5
9.5
8.5
Shoot fresh weight (mg)
2.1
2.0
2.0
1.7
2.1
Ag
1.7
4.5
5.5
6.5
7.5
Root fresh weight (mg)

Figure 13. Shoot weight of untreated plants (open bars) and plants treated with Ag^{+} (solid bars). (A) Fresh shoot weight of 51-day-old tomato plants. (B) Dry shoot weight. (C) Dry shoot weight/fresh shoot weight ratio. a, significantly different from non-transformed plants. b, significantly different from untreated plants. NT, non-transformed.

A


Fresh weight (g)
16.00
8.00
0.00
a
a
b
a
NT
35S
rolD
PRB-1b

B


Dry weight (g)
1.000
0.500
0.000
a
a
b
NT
35S
rolD
PRB-1b

C

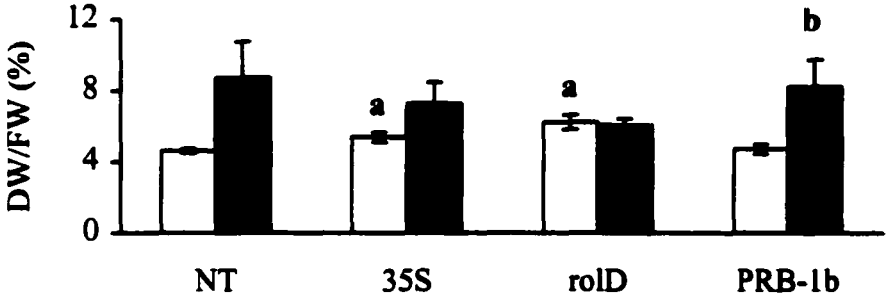
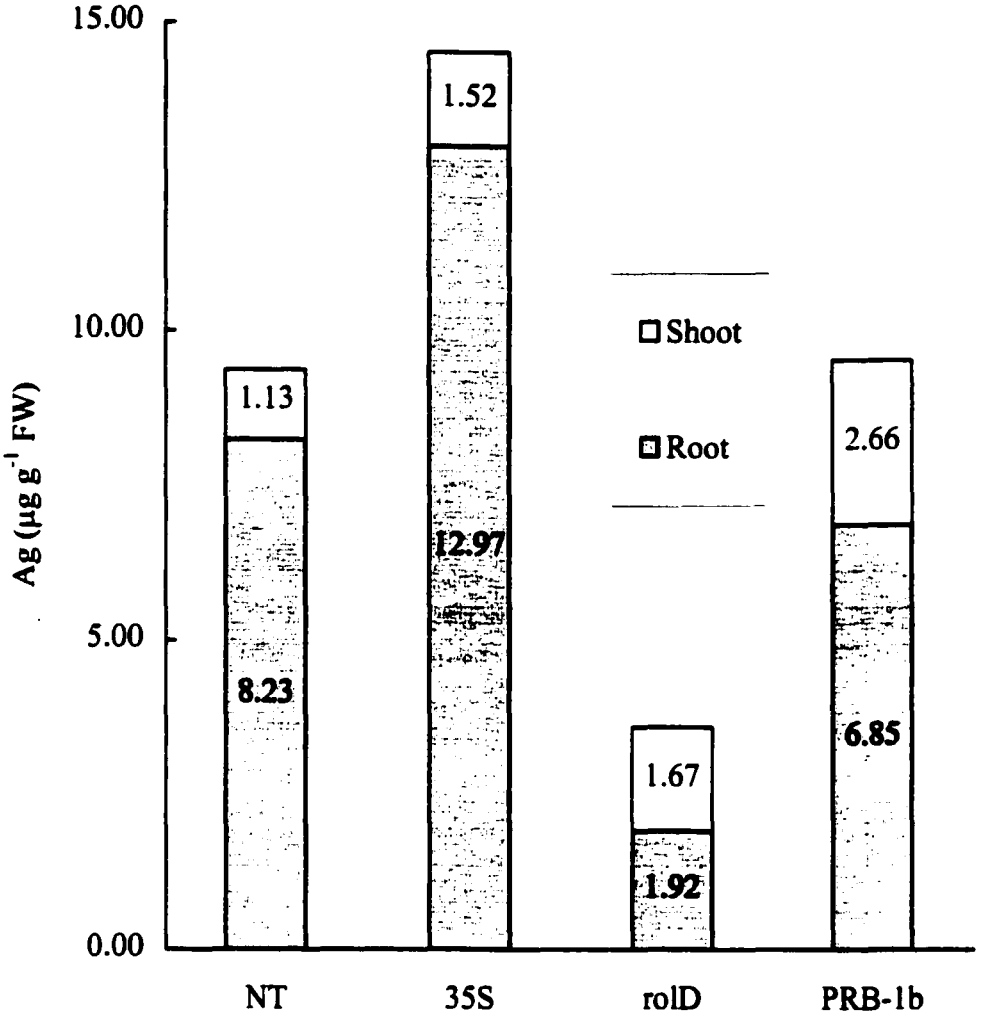

DW/FW (%)
12
8
4
0
a
a
b
NT
35S
rolD
PRB-1b

Figure 14. Accumulation of silver by 51-day-old tomato plants. Plants were treated with 10 mL of a 10 mM solution of $AgNO_3$, pH 5.7 on a daily basis for ten days (n = 5). NT, non-transformed.


15.00
10.00
5.00
0.00
Ag (μg g-1 FW)
1.13
8.23
1.52
12.97
1.67
1.92
2.66
6.85
Shoot
Root
NT
35S
rolD
PRB-1b

3.2.5. Chlorophyll, total protein and photosystem II activity

Similar to the observation by Knoester et al. (1998) that ethylene insensitive tobacco mutants appeared to be greener than control plants, all of the transgenic plants that were studied tended to be greener and more vigorous than the non-transformed plants. In transgenic tomato plants, the leaf chlorophyll content was significantly higher in plants in which ACC deaminase was expressed from the *rolD* promoter (Table 7). The leaf chlorophyll *a* fluorescence ratio F_v/F_m, a measure of the quantum yield of PSII *in vivo*, falls into the relatively narrow range of 0.70-0.82 for both untreated plants and plants treated with silver (Fig. 15). In the presence of Ag^+, the F_v/F_m ratio decreased slower in both 35*S* and PRB-1*b* plants compared to non-transformed plants while *rolD* plants did not exhibit any significant decline in F_v/F_m ratio during the course of the experiment (Fig. 15).

As well as having the highest photosynthetic quantum yield of all of the transgenic tomato plants examined in the presence of Ag^+, the *rolD* plants also had the highest level of leaf chlorophyll (Table 7) and the highest total leaf protein (Table 8). Interestingly, these plants did not accumulate as much silver in their roots as did the other plants (Fig. 14), and following the treatment with Ag^+ they showed a significant increase both in fresh shoot weight and in dry shoot weight and, as a consequence, they had a constant ratio of dry shoot weight and fresh shoot weight (Fig. 12, 13).

3.2.6. Fruits and lycopene

Attached transgenic plants did not exhibit a noticeable delay in the onset of flowering or fruit ripening.

Table 7. Leaf chlorophyll content in mature tomato plants.

		Transgenic plants: Promoter fused to ACC deaminase gene		
	NT	35*S*	*rolD*	PRB-1*b*
Chlorophyll	1.55 ± 0.07	1.65 ± 0.10	1.94 ± 0.07 [a]	1.71 ± 0.08
($mg \cdot g^{-1}$ FW)	(12)	(56)	(56)	(55)

The values are means ± SE, $P < 0.05$. a, significantly different from others. The values in brackets represent the number of plants examined. NT, non-transformed plants.

Figure 15. Effect of silver treatment on non-transformed and transgenic tomatoes: Temporal changes in the quantum efficiency of the the primary photochemical reaction of photosynthesis. Inserted legend: days of silver nitrate treatment; NT, non-transformed.

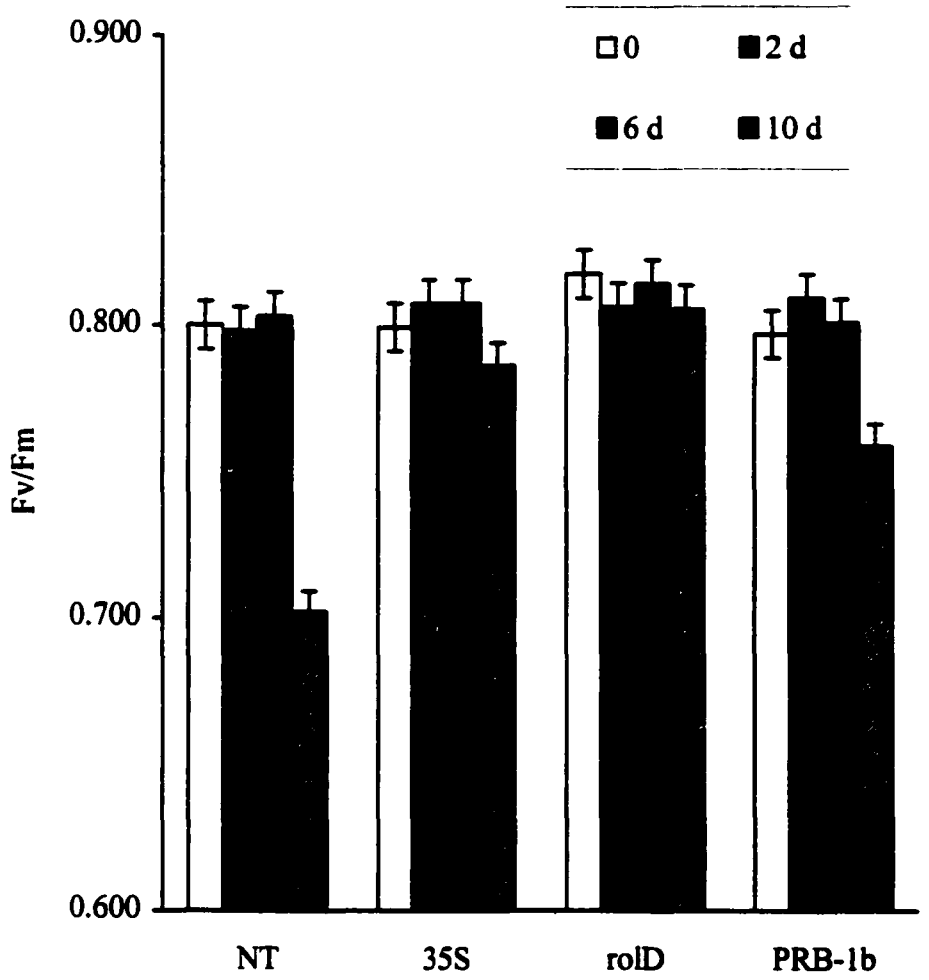

0.900
0.800
0.700
0.600
Fv/Fm
0
2 d
6 d
10 d
NT
35S
rolD
PRB-1b

Table 8. Protein concentration (mg · g^{-1} FW) in five-month-old tomato plants.

Organ		Transgenic plants: Promoter fused to ACC deaminase gene		
	NT	35*S*	*rolD*	PRB-1*b*
Leaf	39.57 ± 1.98 (5)	43.19 ± 0.67 [a] (9)	53.95 ± 0.36 [a, b] (9)	28.81 ± 0.26 [b] (9)
Root	11.38 ± 1.16 (6)	8.14 ± 0.28 (9)	10.79 ± 0.54 [b] (9)	10.47 ± 0.99 [b] (9)

The data are means ± SE, $P < 0.05$. a, significantly different from PRB-1*b* plants. b, significantly different from the 35*S* plants. The values in brackets represent the number of plants examined. NT, non-transformed.

In transgenic plants expressing ACC deaminase under the control of the *35S* promoter in green fruits which displayed a high level of ACC deaminase activity (Table 4), the ethylene production was quite low (Fig. 16). The lower level of ACC deaminase activity in attached red fruits (Table 4) resulted in a near normal level of ethylene (Fig. 16). The increases in respiration were identical in non-transformed and *35S* plants (Fig. 16), suggesting the similar amounts of energy used in catabolic processes during fruit ripening. The average fruit weight of transgenic *rolD* plants turned to be significantly larger compared to non-transformed, *35S* and PRB-1*b* plants when the entire harvest period was considered (Table 9).

The average number of carpels in non-transformed and transgenic tomato plants cv. Heinz 902 was three although two carpels were frequently found. The typical number of carpels in tomato fruit is two, and cultivated tomatoes other than the cherry variety, show a variable number of carpels. In this study, the phenomenon of parthenocarpicy was often observed in transgenic plants.

Heinz 902 is an indeterminate cultivar, which grows in the field until frost stops it. Both non-transformed plants and transgenic plants cv. Heinz 902 have a prolonged ripening period except for *rolD* plants which behave much more like a determinate cultivar (Esau, 1977) (Fig. 17).

Tomato fruits were also assayed for lycopene and β-carotene (Table 10). Lycopene is an antioxidant and is one of the major human serum carotenoids (Bendich and Olson, 1989). Both *35S* and *rolD* plants had a significantly higher content of lycopene and β-carotene in ripe fruit pericarp tissue compared to non-transformed plants.

Figure 16. Ethylene and CO_2 emission by tomato fruits: Effects of ACC deaminase gene expression and fruit maturity. At 0 h, a tomato fruit was placed in a measuring cuvette. Each data point was obtained by subtracting either ethylene or CO_2 concentration for the control empty cuvette from that for the cuvette with tomato fruit. Solid line, ethylene; dashed line, CO_2; NT, non-transformed.

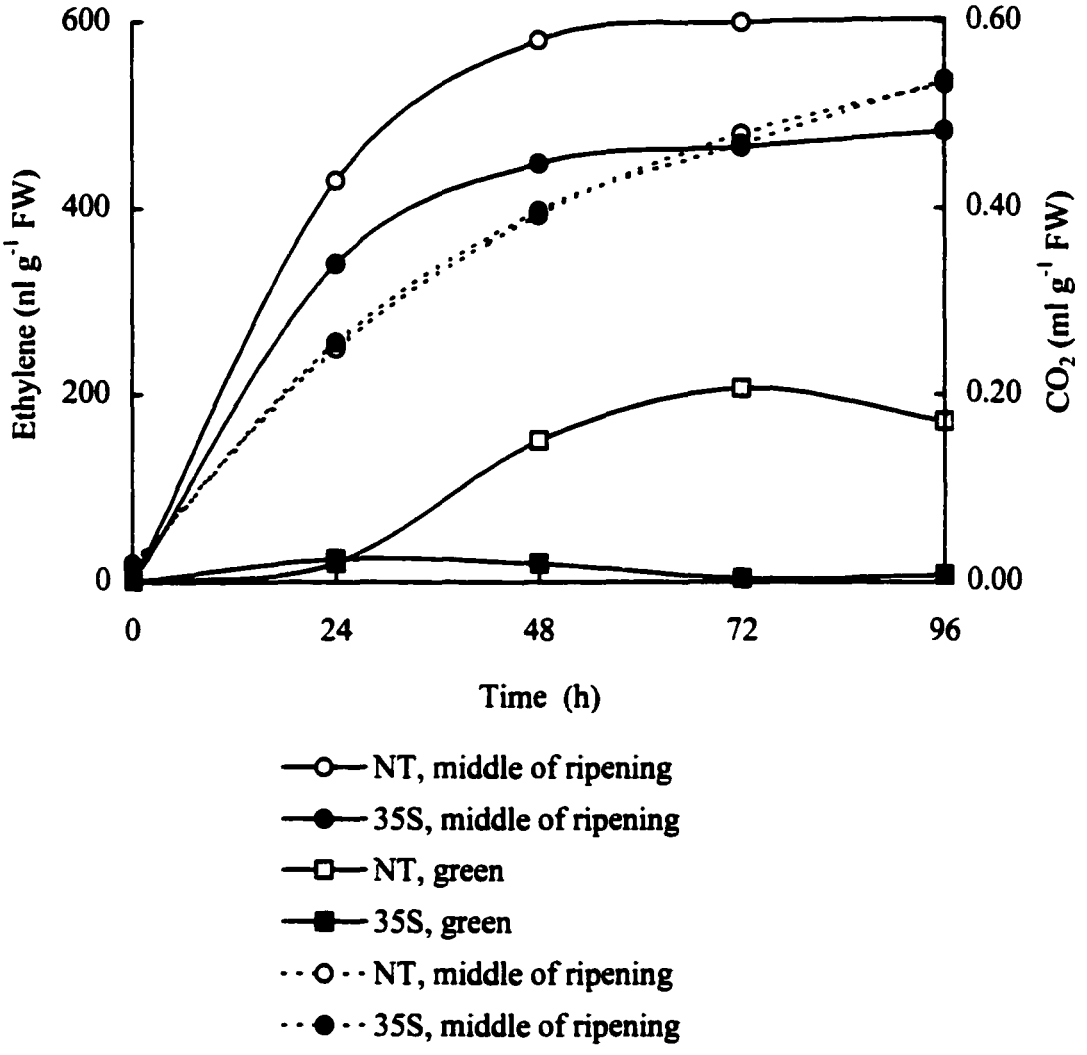

600
400
200
0
0.60
0.40
0.20
0.00
Ethylene (nl g-1 FW)
CO2 (ml g-1 FW)
0
24
48
72
96
Time (h)
NT, middle of ripening
35S, middle of ripening
NT, green
35S, green
NT, middle of ripening
35S, middle of ripening

Table 9. Average fresh ripe fruit weight (g) for a harvesting period of 60 days.

Non-transformed plants	Transgenic plants: Promoter fused to ACC deaminase gene		
	35S	*rolD*	PRB-1*b*
$26.7 \pm 0.7^{a,b}$ (94)	29.5 ± 0.5^{b} (421)	30.9 ± 0.3^{a} (1275)	22.7 ± 2.6^{b} (4)

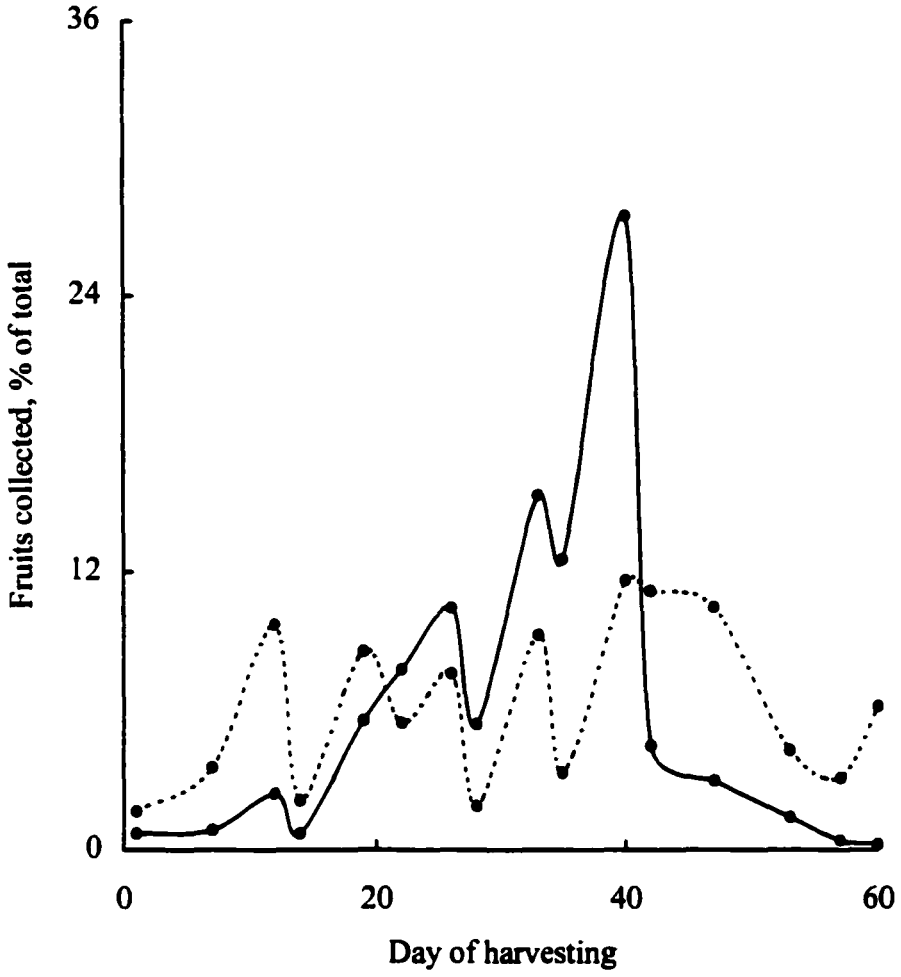
The values are means ± SE, $P < 0.05$. a, significantly different from *35S* plants. b, significantly different from *rolD* plants. The values in brackets represent the number of plants examined. ND, not determined. Tomatoes were picked when they reached the fully red stage.

Table 10. Lycopene and β-carotene content of mature tomato fruits.

Concentration ($\mu g\ g^{-1}$ FW)	Non-transformed	Transgenic plants: Promoter fused to ACC deaminase gene	
		35S	*rolD*
Lycopene	8.7 ± 0.9 (25)	13.8 ± 1.0 [a] (25)	13.7 ± 1.1 [a] (25)
β-carotene	7.5 ± 0.7 (25)	11.7 ± 0.8 [a] (25)	11.2 ± 0.8 [a] (25)

The values are means ± SE, $P < 0.05$. a, significantly different from non-transformed plants. The values in brackets represent the number of plants examined.

Figure 17. Temporal productivity of transgenic plants. Solid line, *rolD*; dashed line, 35*S* plants.


36
24
12
0
Fruits collected, % of total
0
20
40
60
Day of harvesting

3.3. Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn

3.3.1. Cd

Both roots and shoots of one-week-old plants accumulated Cd (Fig. 18A). Transgenic seeds (35*S* and *rolD*) germinated faster in the presence of Cd than non-transformed seeds (Fig. 19A); initial root growth was suppressed by cadmium in the order of WT >> *rolD* > 35*S* (Table 11); PRB-1*b* seeds were not included in these short term experiments since they germinate much more slowly than non-transformed seeds. The 35*S* plants treated with Cd developed roots that were significantly longer but weighed the same as roots of non-transformed plants treated with Cd and at the same time the 35*S* roots took up more Cd than non-transformed plants. Roots of 51-day-old plants accumulated Cd in the order of PRB-1*b* >> *rolD* > 35*S* > WT (Fig. 18A); *rolD* plants did not accumulate Cd in their shoots and did not exhibit a decrease in shoot growth (Table 12). These plants had the highest leaf chlorophyll content after treatment with Cd (Table 13). The 35*S* and *rolD* plants took up a similar amount of Cd; PRB-1*b* plants acquired an almost 4-fold higher amount of Cd, mostly in the roots (Fig. 18A).

3.3.2. Co

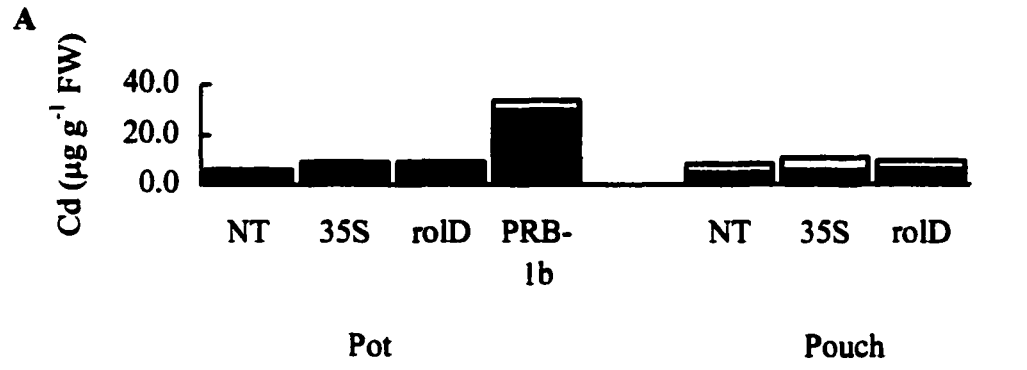
Cobalt, which resembles iron in its chemical behavior, inhibits ethylene synthesis by ACC oxidase which relies on iron as a cofactor. The 35*S* transgenic plants germinated faster than the non-transformed seeds in the presence of Co while the behavior of the *rolD* seeds was more complex (Fig. 19B). In these plants, the major target organ for cobalt accumulation is roots for young plants and shoots for older plants (Fig. 18B).

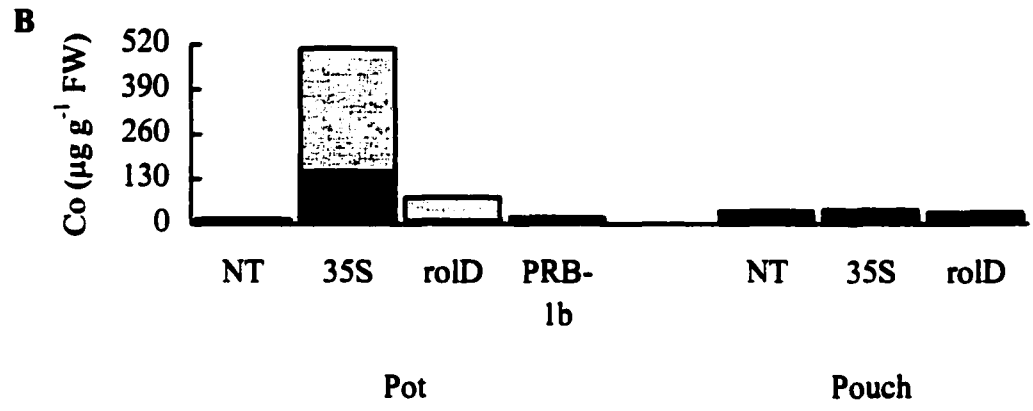
Table 11. Effect of Cd^{2+} and Zn^{2+} on growth of 7-day-old non-transformed and transgenic tomato plants.

	Metal 1 mM	Non-transformed plants	ACC deaminase transgenic plants	
			35S	*rolD*
Root length (mm)	- Cd	42 ± 3 (n = 24)	46 ± 4 (n = 24)	45 ± 4 (n = 24)
	+ Cd	27 ± 2 [a] (n = 23)	37 ± 2 [a,b] (n = 24)	32 ± 3 [a] (n = 21)
Root FW (mg)	- Cd	5.3 ± 0.3 (n = 24)	5.6 ± 0.4 (n = 24)	5.9 ± 0.5 (n = 24)
	+ Cd	4.8 ± 0.3 (n = 23)	5.1 ± 0.2 (n = 24)	5.6 ± 0.6 (n = 21)
Shoot FW (mg)	- Cd	12.0 ± 0.5 (n = 24)	12.1 ± 0.9 (n = 24)	13.0 ± 0.6 (n = 24)
	+ Cd	11.3 ± 0.5 (n = 23)	11.1 ± 0.7 (n = 24)	13.2 ± 0.8 (n = 21)
Root length (mm)	- Zn	50 ± 4 (n = 24)	57 ± 4 (n = 20)	53 ± 4 (n = 24)
	+ Zn	46 ± 4 (n = 20)	50 ± 4 (n = 21)	45 ± 3 (n =23)
Root FW (mg)	- Zn	6.3 ± 0.4 (n = 24)	6.5 ± 0.3 (n = 20)	7.1 ± 0.4 (n = 24)
	+ Zn	7.9 ± 0.5[a] (n = 20)	6.3 ± 0.3 (n = 21)	6.3 ± 0.3 (n = 23)
Shoot FW (mg)	- Zn	12.4 ± 0.5 (n = 24)	14.3 ± 0.7 (n = 20)	14.4 ± 0.7 (n = 24)
	+ Zn	13.9 ± 0.9 (n = 20)	13.6 ± 0.6 (n = 21)	13.6 ± 0.7 (n = 23)

Values are means ± SE, $P < 0.05$. a, significantly different from – metal control; b, significantly different from non-transformed plants.

Figure 18. Metal accumulation in roots and shoots of ACC deaminase transgenic tomato plants. (A) Cadmium uptake by 7-pouch-grown seedlings and 51-day-old potted tomato plants. (B) Cobalt uptake by 9-day pouch-grown seedlings and 40-day-old potted tomato plants. (C) Copper uptake as in B. Solid bar, root; gray bar, shoot. NT, non-transformed.


A
Cd (µg g-1 FW)
40.0
20.0
0.0
NT
35S
rolD
PRB-1b
NT
35S
rolD
Pot
Pouch


B
Co (µg g-1 FW)
520
390
260
130
0
NT
35S
rolD
PRB-1b
NT
35S
rolD
Pot
Pouch

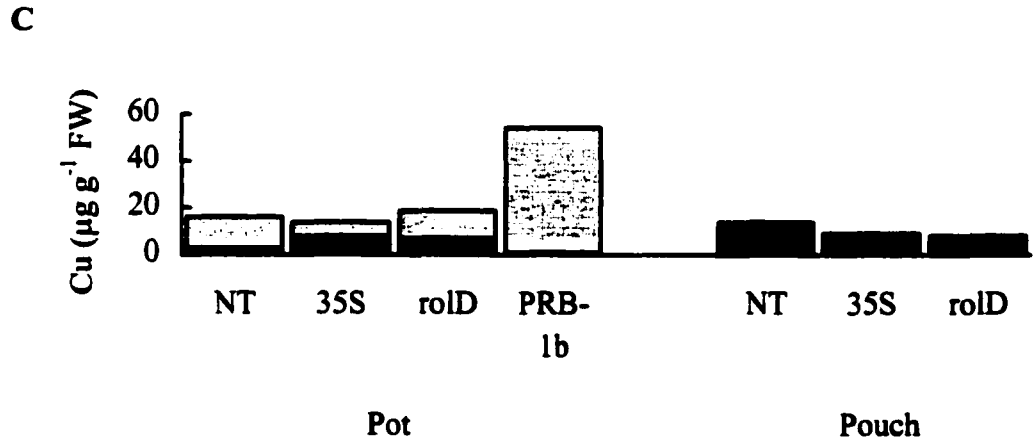
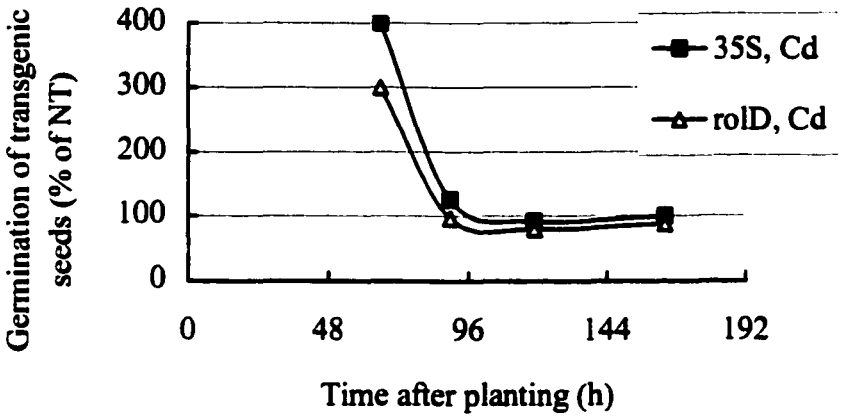
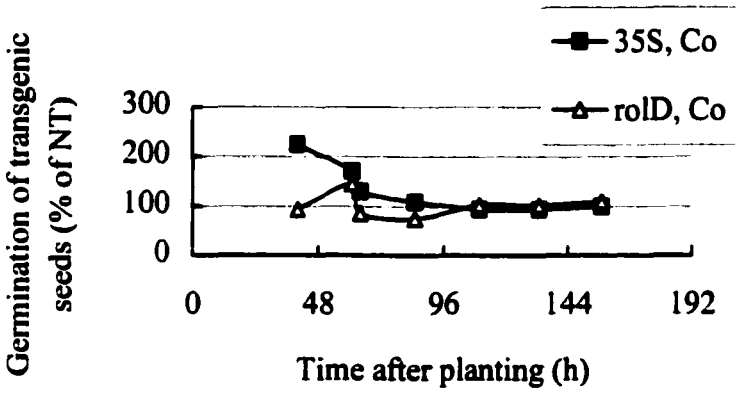

C
Cu (µg g-1 FW)
60
40
20
0
NT
35S
rolD
PRB-1b
NT
35S
rolD
Pot
Pouch

Figure 19. Effect of metals on germination of transgenic seeds. (A) Cd; (B) Co; (C) Cu and Pb. NT, non-transformed.

A


Germination of transgenic seeds (% of NT)
400
300
200
100
0
0
48
96
144
192
Time after planting (h)
35S, Cd
rolD, Cd

B


Germination of transgenic seeds (% of NT)
300
200
100
0
0
48
96
144
192
Time after planting (h)
35S, Co
rolD, Co

C

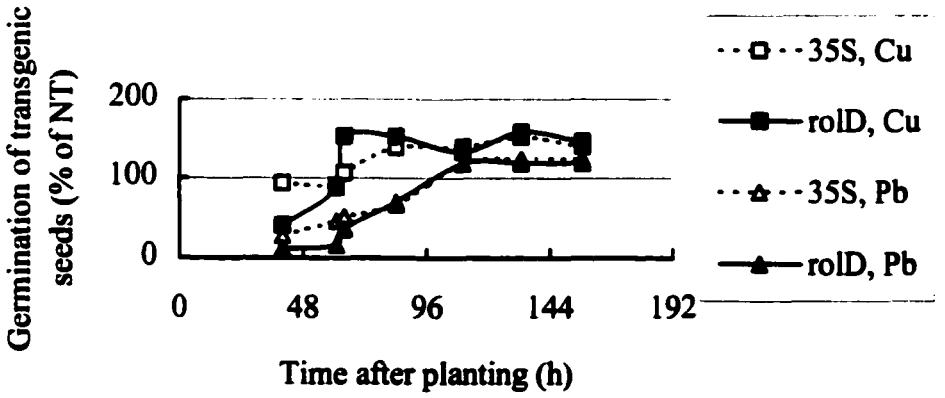

Germination of transgenic seeds (% of NT)
200
100
0
0
48
96
144
192
Time after planting (h)
35S, Cu
rolD, Cu
35S, Pb
rolD, Pb

Table 12. Effect of metals on tomato plant fresh and dry shoot weight (g).

Metal	Age (d)	Weight	Non-transformed	ACC deaminase Transgenic Plants		
				35S	*rolD*	PRB-1*b*
-	40	Fresh	2.89 ± 0.32	3.36 ± 0.18	4.31 ± 0.18[b]	3.24 ± 0.21
		Dry	0.1380 ± 0.0181	0.1634 ± 0.0144	0.2203 ± 0.0078[a]	0.1679 ± 0.0063
-	51	Fresh	4.71 ± 0.19	10.56 ± 1.34[b]	9.71 ± 1.04[b]	7.01 ± 0.52
		Dry	0.2172 ± 0.0057	0.5778 ± 0.0978[a]	0.5975 ± 0.0474[a]	0.4351 ± 0.1209
Cd	51	Fresh	5.22 ± 0.91	6.43 ± 1.20	12.09 ± 2.04[b]	4.35 ± 1.96
		Dry	0.1615 ± 0.0351	0.4256 ± 0.0724[a]	0.6308 ± 0.0939[a]	0.2788 ± 0.0624
Co	40	Fresh	1.98 ± 0.03[a]	2.21 ± 0.12[a]	2.75 ± 0.25[a,b]	2.31 ± 0.44
		Dry	0.0988 ± 0.0023	0.1167 ± 0.0121	0.1477 ± 0.0156[a, b]	0.1248 ± 0.0256[b]
Cu	40	Fresh	2.53 ± 0.1	2.37 ± 0.21	3.06 ± 0.05[a,b]	3.34 ± 0.12[b]
		Dry	0.1232 ± 0.0048	0.1119 ± 0.0131	0.1524 ± 0.0036[a,b]	0.1608 ± 0.0039[a]
Ni	40	Fresh	1.73 ± 0.02[a]	2.15 ± 0.19[a]	3.04 ± 0.02[a,b]	2.81 ± 0.40[b]
		Dry	0.0894 ± 0.0097	0.1076 ± 0.0133[b]	0.1512 ± 0.0024[a,b]	0.1412 ± 0.0219
Pb	40	Fresh	2.03 ± 0.22	2.35 ± 0.31[a]	2.85 ± 0.33[a]	2.62 ± 0.45
		Dry	0.0981 ± 0.0073	0.1202 ± 0.0151	0.1586 ±0.0192[a,b]	0.1342 ± 0.0236
Zn	51	Fresh	5.29 ± 1.84	10.28 ± 1.43	13.08 ± 1.30	9.79 ± 0.05[a]
		Dry	0.3286 ± 0.0505[a]	0.4867 ± 0.0728	0.6029 ± 0.0483[a]	0.4916 ± 0.0142[a]

The values are means ± SE, n = 3, $P < 0.05$. a, significantly different from control; b, significantly different from non-transformed plants.

Table 13. Effect of Cd and Zn on tomato plant leaf chlorophyll content (mg g^{-1} FW).

Metal	Non-transformed	35*S*	*rolD*	PRB-1*b*
Control	1.550 ± 0.067 [a] (12)	1.652 ± 0.097 [a] (56)	1.936 ± 0.072 (56)	1.707 ± 0.079 [a] (55)
Cd	0.835 ± 0.206	0.811 ± 0.069	1.374 ± 0.139	0.786 ± 0.065
Zn	1.330 ± 0.586	1.000 ± 0.032	1.794 ± 0.029	1.669 ± 0.113

The data are means ± SE. n = 2 if not shown. The values in brackets represent the number of plant examined. a, significantly different from *rolD* plants for $P < 0.05$.

This results in a significant decrease in both root length and fresh weight in young plants, and an increase in the fresh shoot/root weight ratio (Table 14). Cobalt accumulated in the roots and shoots of 35*S* plants to a much greater extent than in roots of the other plants. Interestingly, ACC deaminase activity was considerably higher in shoots of 35*S* plants treated with Co compared with control plants (data not shown).

3.3.3. Cu

Initially, the germination of transgenic seeds relative to the non-transformed plants is inhibited by Cu(II), although it is stimulated at later times (Fig. 19C). Young plants (especially non-transformed) accumulate Cu in their roots (Fig. 18C) concomitant with a decrease in root length (Table 14). On the other hand, 40-day-old tomato plants were able both to transport copper to shoots (Fig. 18C) and grow normally (Table 12). PRB-1*b* plants accumulated 53 $\mu g\ g^{-1}$ FW of Cu without a noticeable change in shoot weight (Table 12); the ratio of shoot/root Cu content was the highest in these plants. The highest level of ACC deaminase activity in this study was observed in leaves of 35*S* plants treated with Cu (Fig. 20); the roots of 35*S* plants did not show such a dramatic elevation in enzyme activity despite a content of acquired copper similar to leaves (Fig. 18C). Copper may affect ACC oxidation which uses a radical-based mechanism (Pirrung et al., 1998).

3.3.4. Ni

In the presence of Ni, the germination of 35*S* and *rolD* transgenic seeds occurred at a rate that was not very different from the non-transformed plants (Fig. 21A).

Table 14. Effect of Co^{2+} and Cu^{2+} on 9-day-old non-transformed and transgenic tomato plants.

	Metal 1 mM	Non-transformed plants	ACC deaminase transgenic plants	
			35S	*rolD*
Root length (mm)	- Co	77 ± 8 (n = 13)	97 ± 4 [b] (n = 23)	91 ± 6 (n = 21)
	+ Co	64 ± 6 (n = 15)	64 ± 7 [a] (n = 21)	88 ± 5 [b] (n =23)
Root FW (mg)	- Co	9.4 ± 0.9 (n = 13)	10.7 ± 0.6 (n = 23)	10.2 ± 0.6 (n = 21)
	+ Co	7.2 ± 0.6 [a] (n = 15)	7.3 ± 0.7 [a] (n = 21)	8.5 ± 0.4 [a] (n = 23)
Shoot FW (mg)	- Co	25.0 ± 1.0 (n = 13)	22.7 ± 0.8 (n = 23)	26.0 ± 0.8 (n = 21)
	+ Co	23.9 ± 2.5 (n = 15)	23.3 ± 1.2 (n = 21)	25.4 ± 0.9 (n = 23)
Root length (mm)	- Cu	83 ± 5 (n = 14)	94 ± 4 (n = 22)	95 ± 5 (n = 23)
	+ Cu	64 ± 8 [a] (n = 11)	83 ± 5 [b] (n = 24)	90 ± 4 [b] (n = 25)
Root FW (mg)	- Cu	6.7 ± 0.4 (n = 14)	9.2 ± 0.6 [b] (n = 22)	9.4 ± 0.5 [b] (n = 23)
	+ Cu	6.7 ± 0.7 (n = 11)	6.1 ± 0.4 [a] (n = 24)	8.2 ± 0.5 (n = 25)
Shoot FW (mg)	- Cu	24.2 ± 1.7 (n = 14)	23.3 ± 0.8 (n = 22)	24.9 ± 1.0 (n = 23)
	+ Cu	23.9 ± 2.5 (n = 11)	23.4 ± 1.3 (n = 24)	26.9 ± 1.4 (n = 25)

Values are means ± SE, $P < 0.05$. a, significantly different from – metal control group; b, significantly different from non-transformed plants.

Figure 20. ACC deaminase activity in 40-day-old tomato plants treated with Cu, Ni or Pb. NT, non-transformed.

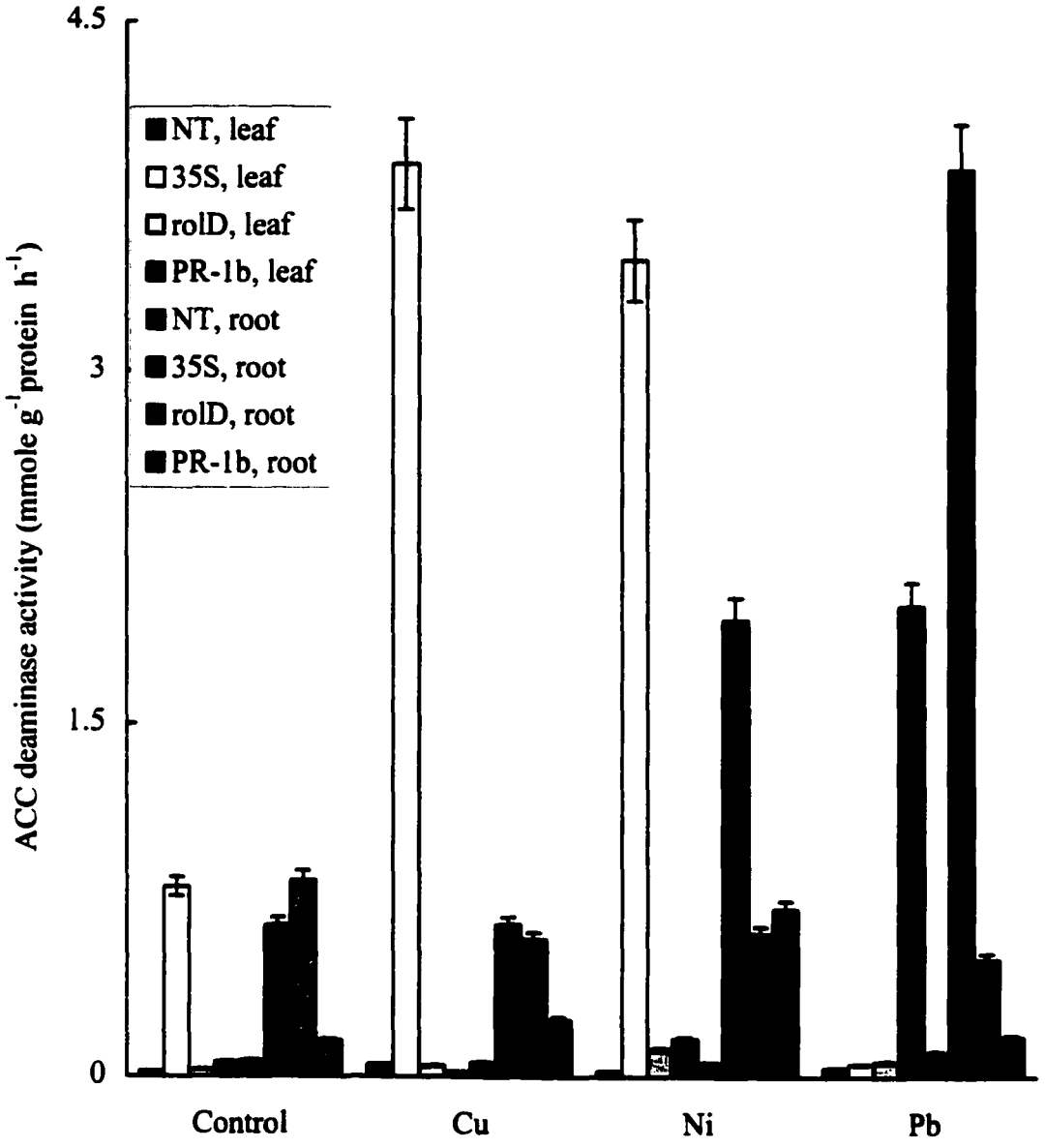
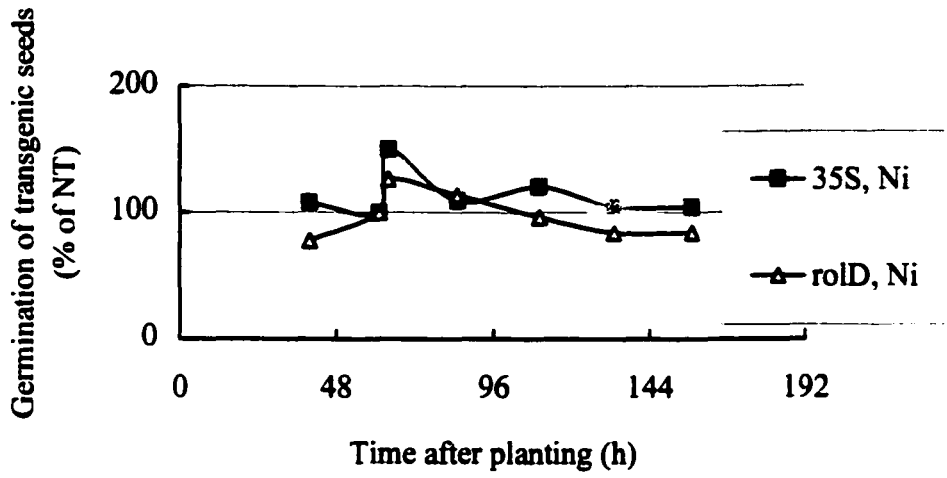
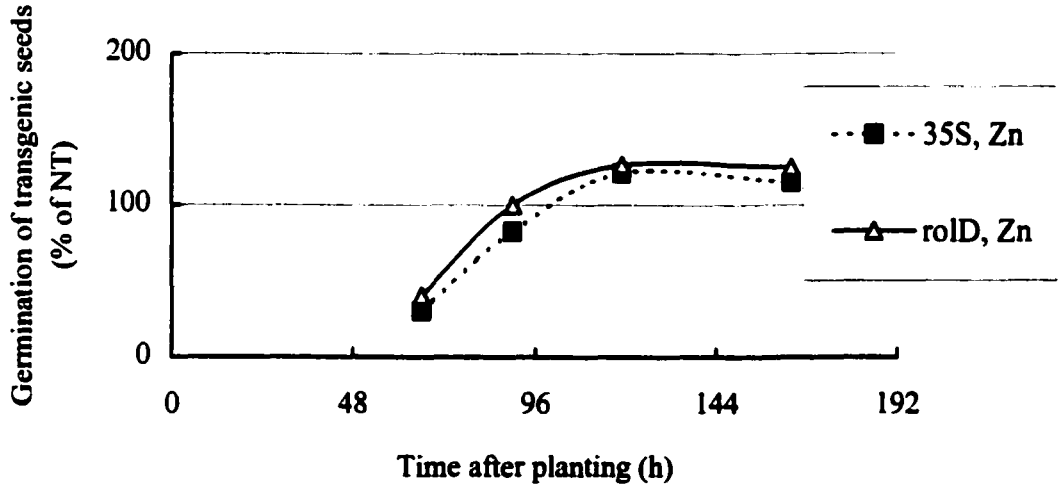

4.5
3
1.5
0
ACC deaminase activity (mmole g-1protein h-1)
NT, leaf
35S, leaf
rolD, leaf
PR-1b, leaf
NT, root
35S, root
rolD, root
PR-1b, root
Control
Cu
Ni
Pb

Figure 21. Effect of metals on germination of transgenic tomato seeds. (A) Ni; (B) Zn. NT, non-transformed.

A


Germination of transgenic seeds (% of NT)
200
100
0
0
48
96
144
192
Time after planting (h)
35S, Ni
rolD, Ni

B


Germination of transgenic seeds (% of NT)
200
100
0
0
48
96
144
192
Time after planting (h)
35S, Zn
rolD, Zn

With 9-day-old *rolD* plants Ni was accumulated largely in roots (Fig. 22A). In 40-day-old plants, Ni accumulated mostly in shoots in the order of PRB-1*b* > 35*S* > WT >> *rolD* (Fig. 23A). Both root length and fresh root weight were larger in 35*S* and *rolD* 9-day-old plants compared to that of non-transformed plants both in the presence and absence of nickel (Table 15). Except for the PRB-1*b* plants, Ni had a severe effect on both fresh and dry shoot weight in 40-day-old tomato plants (Table 12) and on PSII as well (Fig. 23). This notwithstanding, all of the transgenic plants grew better in both the presence and absence of Ni. Nickel substantially increased ACC deaminase activity in 35*S* plants (Fig. 20); the activity in leaves was almost twice the enzyme activity found in roots, perhaps reflecting ACC oxidase inhibition by Ni.

3.3.5. Pb

Of all of the metals tested, lead had the most inhibitory effect on transgenic seed germination compared to non-transformed seeds (Fig. 19C). While forty-day-old PRB-1*b* plants accumulated Pb in both roots and shoots, young plants did not transport lead from roots to shoots (Fig. 22B). In general, transgenic plants grew better than non-transformed plants in presence of lead (Tables 12 & 15). Moreover, no change in either the F_v/F_m ratio or q_p in both non-transformed and transgenic plants treated with lead was observed (data not shown). The leaves in PRB-1*b* plants and the roots in 35*S* plants responded to lead treatment by significantly increasing ACC deaminase activity (Fig. 20).

3.3.6. Zn

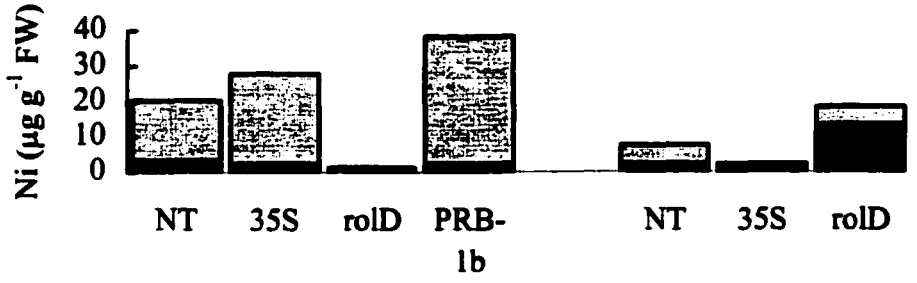
Table 15. Effect of Ni^{2+} and Pb^{2+} on growth of 9-day-old non-transformed and transgenic tomato plants.

	Metal 1 mM	Non-transformed	ACC deaminase transgenic plants	
			35S	*rolD*
Root length (mm)	- Ni	76 ± 8 (n = 15)	103 ± 4 [b] (n = 21)	100 ± 5 [b] (n = 22)
	+ Ni	62 ± 9 (n = 13)	97 ± 4 [b] (n = 23)	89 ± 3 [b] (n =19)
Root FW (mg)	- Ni	7.1 ± 0.8 (n = 15)	10.0 ± 0.5 [b] (n = 21)	9.3 ± 0.5 [b] (n = 22)
	+ Ni	6.5 ± 0.9 (n = 13)	8.7 ± 0.4 [b] (n = 23)	9.1 ± 0.4 [b] (n = 19)
Shoot FW (mg)	- Ni	23.8 ± 1.4 (n = 15)	25.0 ± 0.7 (n = 21)	23.6 ± 1.1 (n = 22)
	+ Ni	21.7 ± 1.4 (n = 13)	22.1 ± 1.2 [a] (n = 23)	25.0 ± 1.0 (n = 19)
Root length (mm)	- Pb	50 ± 7 (n = 15)	88 ± 5 [b] (n = 22)	94 ± 5 [b] (n = 22)
	+ Pb	72 ± 8 (n = 15)	88 ± 5 (n = 19)	98 ± 4 [b] (n =23)
Root FW (mg)	- Pb	6.7 ± 0.7 (n = 15)	9.1 ± 0.5 [b] (n = 22)	10.3 ± 0.7 [b] (n = 22)
	+ Pb	7.9 ± 0.8 (n = 15)	8.2 ± 0.5 (n = 19)	9.0 ± 0.4 (n = 23)
Shoot FW (mg)	- Pb	23.4 ± 1.3 (n = 15)	24.0 ± 0.5 (n = 22)	26.1 ± 1.0 (n = 22)
	+ Pb	27.9 ± 1.8 (n = 15)	27.0 ± 1.5 (n = 19)	26.6 ± 0.8 (n = 23)

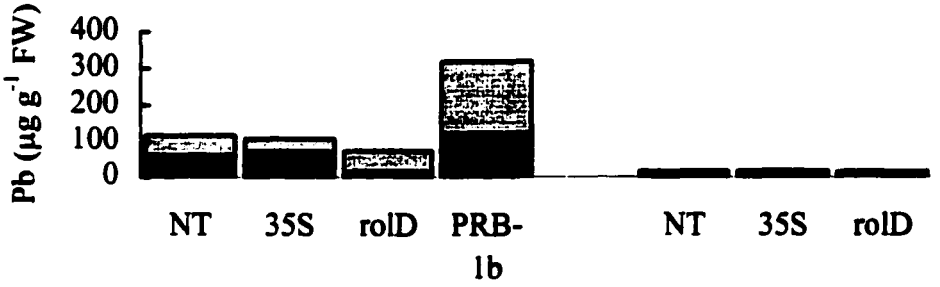
The valuaes are means ± SE, $P < 0.05$. a, significantly different from – metal control; b, significantly different from non-transformed plants.

Figure 22. (A) Nickel uptake by 9- (pouch) and 40-day-old (pot) tomato plants. (B) Lead uptake by 9- (pouch) and 40-day-old (pot) tomato plants. (C) Zinc uptake by 7- (pouch) and 51-day-old (pot) tomato plants. Solid bar, root; gray bar, shoot. NT, non-transformed.

A


Ni (μg g-1 FW)
40
30
20
10
0
NT
35S
rolD
PRB-1b
NT
35S
rolD
Pot
Pouch

B


Pb (μg g-1 FW)
400
300
200
100
0
NT
35S
rolD
PRB-1b
NT
35S
rolD
Pot
Pouch

C

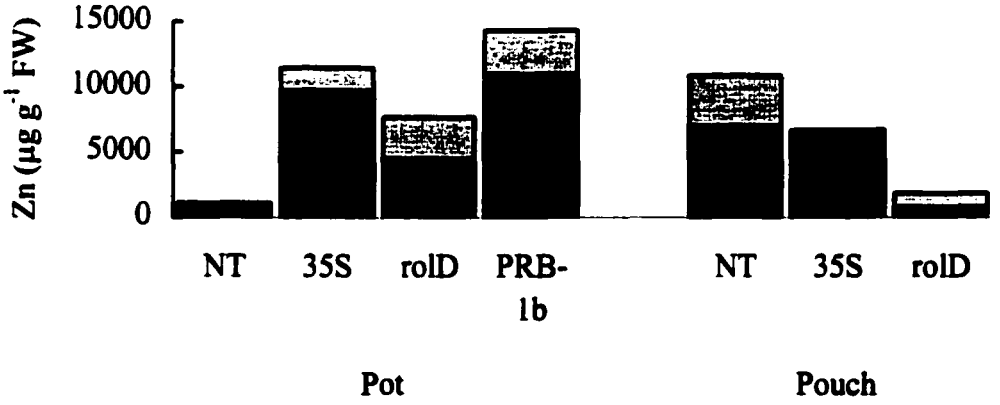
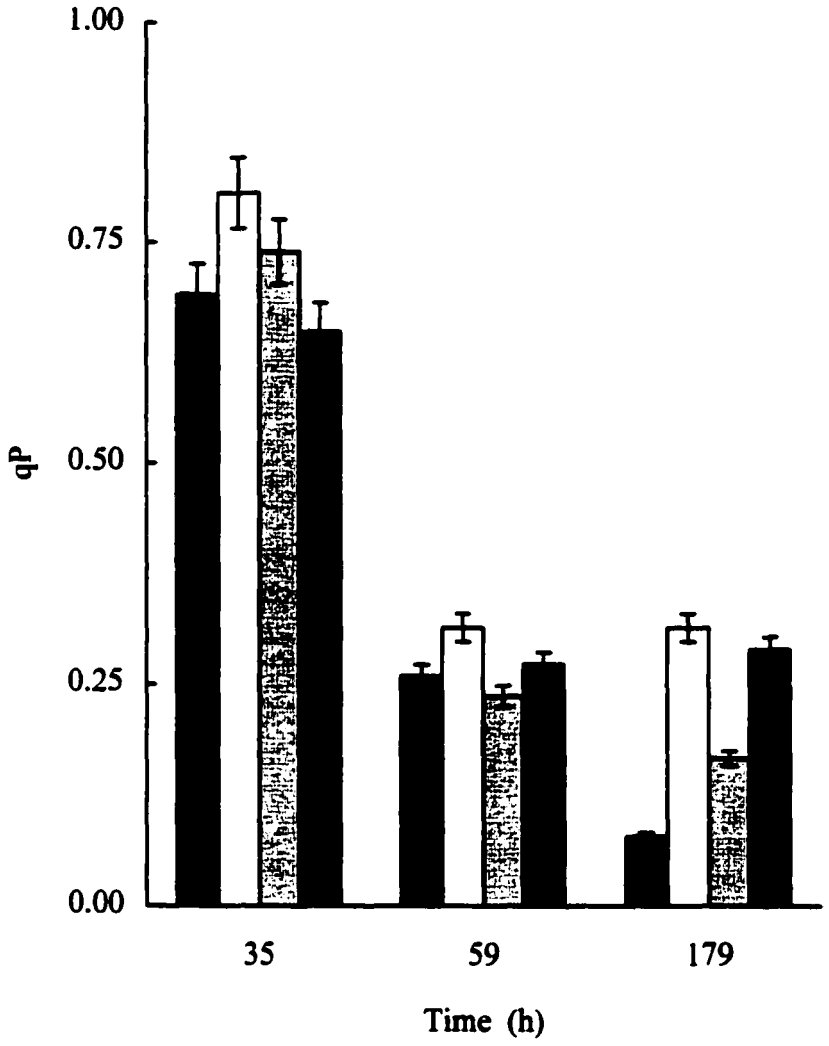

Zn (μg g-1 FW)
15000
10000
5000
0
NT
35S
rolD
PRB-1b
NT
35S
rolD
Pot
Pouch

Figure 23. Effect of Ni on the coefficient of photochemical quenching (qp) in tomato plants. Solid bar, non-transformed plants; clear bar, 35*S* plants; light gray bar, *rolD* plants; dark gray bar, PRB-1*b* plants.


1.00
0.75
0.50
0.25
0.00
qP
35
59
179
Time (h)

Zinc is an important trace metal that can be toxic at elevated concentrations (Raskin et al., 1997). Both 51-day-old transgenic plants and 7-day-old non-transformed plants hyperaccumulated Zn (Fig. 22C). The shoot/root ratio of Zn content was lower in older non-transformed plants. Previously, this ratio was shown to fall in *Thlaspi caerulescens* with an increase in available Zn (Brown et al., 1994). Leaf chlorophyll tended to decline in treated plants compared to the untreated control in the absence of Zn, except for PRB-1*b* plants (Table 13). Zn had little effect on the growth of 7- and 51-day-old plants (Tables 11, 12).

3.4. Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35*S*, *rolD* or PRB-1*b* promoter

Flooded ACC deaminase transgenic tomato plants appeared healthier and greener compared to non-transformed plants. Flooding resulted in a decrease in shoot height, fresh and dry shoot weight, and the ratios derived from these values in all of the plants that were flooded for nine days (Table 16; Fig. 24). However, in a number of cases the differences between non-transformed and transgenic plants were statistically significant; flooded tomato plants showed a much higher degree of stress tolerance as: NT < 35*S* < PRB-1*b* < *rolD*. Interestingly, 35*S* and PRB-1*b* transgenic plants exhibited faster development of epinastic curvature at the beginning of the flooding experiment (Fig. 25 & 26) compared to either the non-transformed or the *rolD* plants. Based on the final values after 144 h, the severity of flooding stress on the epinasty in petioles was as: PRB-1*b* > NT > 35*S* > *rolD*. No significant changes in epinastic curvature were observed in control groups of well-drained plants (data not shown).

Table 16. Growth characteristics of 55-day-old non-transformed and transgenic tomato plants flooded for nine days.

Geno-type	Flood-ing	Shoot height (cm)	Fresh shoot weight (g)	Fresh shoot weight/height (g/cm)	Shoot dry weight (g)	Dry shoot weight/fresh shoot weight
NT	-	16.8 ± 0.5 (n = 27)	26.03 ± 1.83 (n = 27)	1.51 ± 0.08 (n = 27)	1.3624 ± 0.1314 (n = 27)	0.051 ± 0.003 (n = 27)
NT	+	11.3 ± 0.5 (n = 21)	11.71 ± 0.86 (n = 21)	1.01 ± 0.05 (n = 21)	0.3151 ± 0.0478 (n = 21)	0.025 ± 0.002 (n = 21)
35*S*	-	16.6 ± 0.5 (n = 13)	30.50 ± 1.76 (n = 13)	1.84 ± 0.08 [a] (n = 13)	1.5753 ± 0.1413 (n = 13)	0.051 ± 0.002 (n = 13)
35*S*	+	11.7 ± 0.4 (n = 14)	14.4 ± 1.16 (n = 14)	1.21 ± 0.06 [b] (n = 14)	0.3731 ± 0.0438 (n = 14)	0.026 ± 0.002 (n = 14)
rolD	-	16.9 ± 0.5 (n = 14)	29.20 ± 1.81 (n = 14)	1.71 ± 0.07 (n = 14)	1.5354 ± 0.1169 (n = 14)	0.052 ± 0.002 (n = 14)
rolD	+	11.9 ± 0.4 (n = 12)	15.45 ± 0.88 [b] (n = 12)	1.29 ± 0.04 [b] (n = 12)	0.8709 ± 0.0528 [b] (n = 12)	0.056 ± 0.002 [b] (n = 12)
PRB-1*b*	-	17.1 ± 0.8 (n = 10)	29.09 ± 2.47 (n = 10)	1.68 ± 0.09 (n = 10)	1.3743 ± 0.1633 (n = 10)	0.046 ± 0.002 (n = 10)
PRB-1*b*	+	11.8 ± 0.6 (n = 17)	15.64 ± 1.67 [b] (n = 17)	1.28 ± 0.08 [b] (n = 17)	0.4475 ± 0.0633 (n = 17)	0.0291 ± 0.0027 (n = 17)

Values are means ± SE. a, significantly different vs. control non-transformed plants; b, significantly different vs. flooded non-transformed plants, $P < 0.05$.

Figure 24. Photograph of non-transformed tomato plant (left) and a *rolD* transgenic tomato plant (right) flooded for nine days.



Figure 25. Epinastic response of a non-transformed 55-day-old tomato plant flooded for five days.

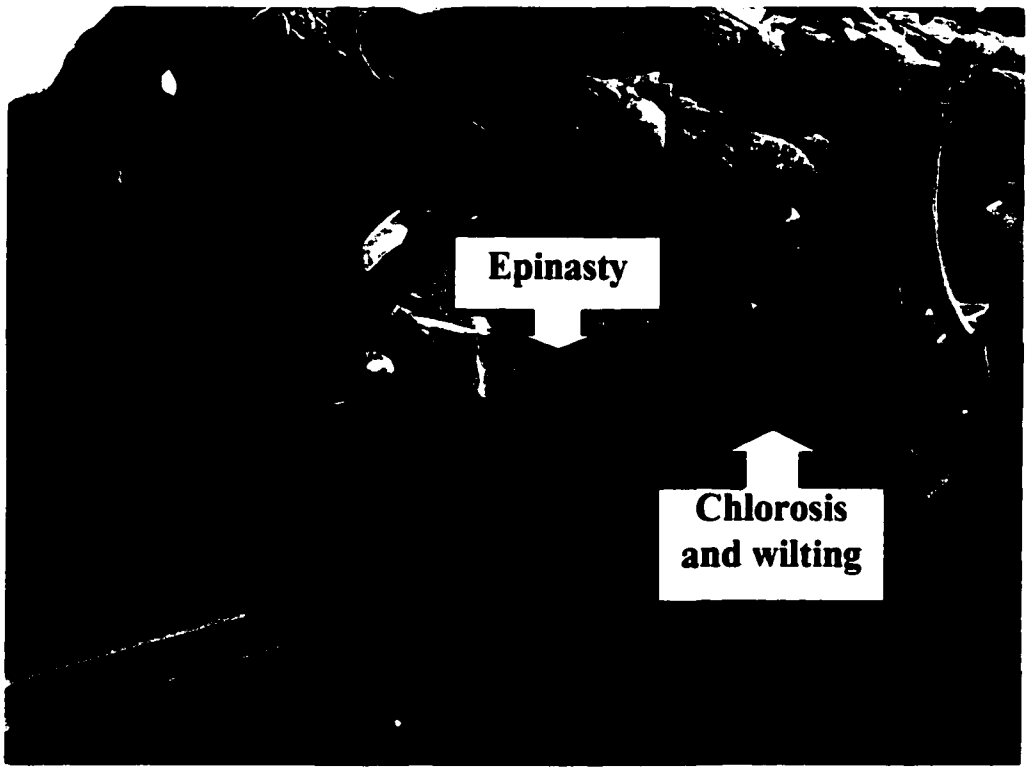
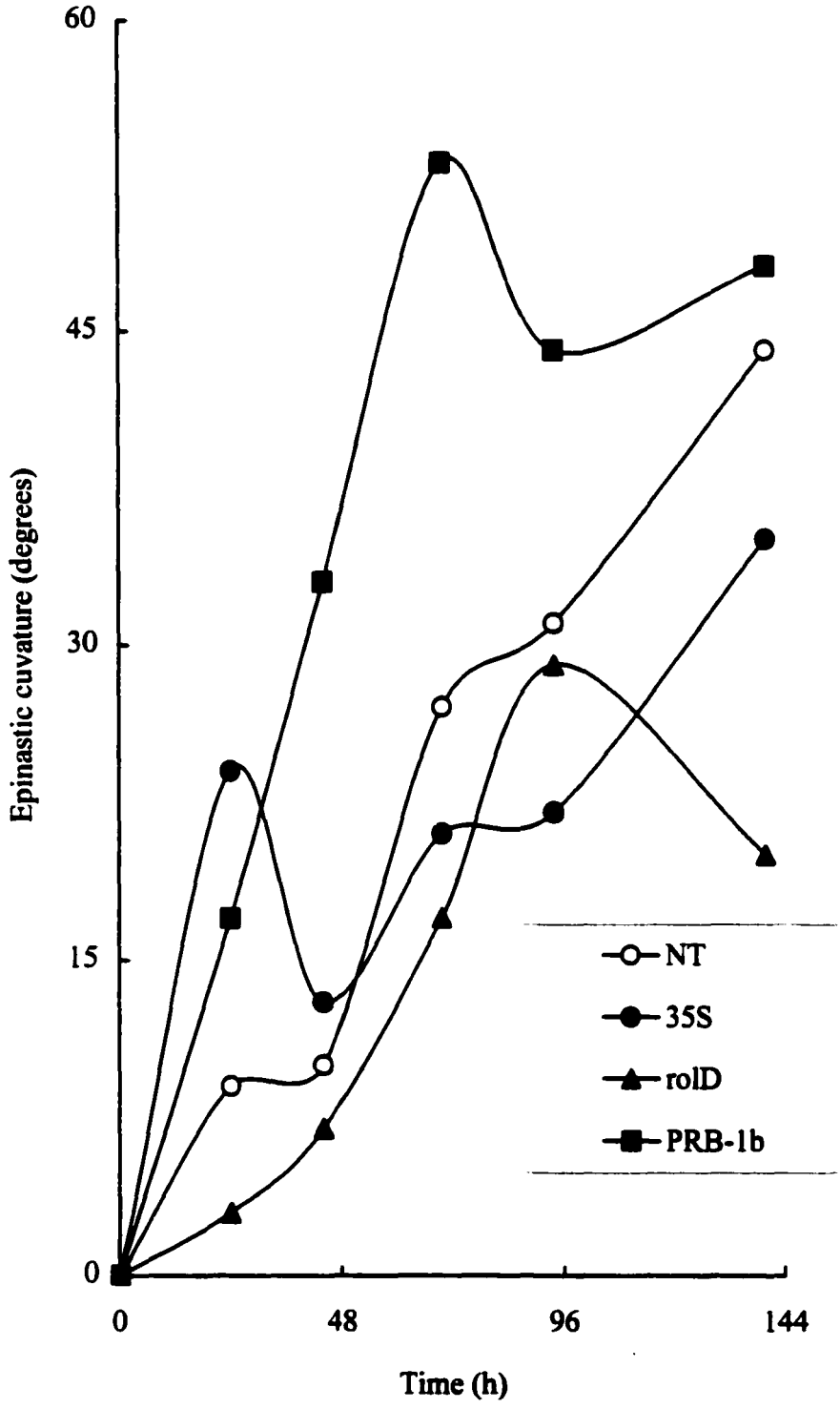

Epinasty
Chlorosis
and wilting

Figure 26. Epinastic curvature in the petiole of the fifth top leaf of non-transformed and ACC deaminase transgenic 55-day-old plants that were flooded for up to 144 h. The values shown are the changes in the angle from the 0-h data (n = 3). NT, non-transformed. 35*S*, *rolD* and PRB-1*b* are the promoters used for the construction of ACC deaminase transgenic plants.


60
45
30
15
0
Epinastic cuvature (degrees)
0
48
96
144
Time (h)
NT
35S
rolD
PRB-1b

As expected, the decreased endogenous ACC concentration, as a consequence of a functional ACC deaminase, in all of the transgenic tomato plants resulted in slower development of both adventitious roots and stem aerenchyma in these plants compared to non-transformed plants (data not shown). Development of epinasty in petioles is normally fast in relatively young leaves and it is reversible in tomato plants if the flooding stress is not too severe. The recovery of a plant from flooding stress is generally associated with the formation of adventitious roots. As a rule these two events are almost coincident in time.

Reflooding above the adventitious root level results in the rapid appearance of a second peak of epinastic curvature (Fig. 27). AVG (L-α-(aminoethoxyvinyl)-glycine), which is a competitive inhibitor of pyridoxal phosphate (PLP) dependent enzymes such as ACC synthase, can reduce the development of petiolar epinasty in flooding-stressed tomato plants in a dose-dependent manner (Fig. 27). Epinasty depends on many factors and falls in a wide range for different cultivars depending on both leaf and plant age. In addition, all transgenic plants developed petiolar epinasty more slowly after adventitious root submergence than did non-transformed plants (data not shown).

Flooding resulted in increased production of ethylene in both non-transformed plants and transgenic plants. The ethylene concentrations in flooded plants was NT ≈ PRB-1*b* > 35*S* > *rolD* after 24, 48 or 72 hours of flooding (Fig. 28). None of the transgenic plants completely inhibited the synthesis of ethylene in response to flooding. The greatest decrease in ethylene concentration was observed after 48 or 72 h of flooding of *rolD* plants; the ethylene concentration in this case was approximately 30% of the level found with non-transformed plants. Leaf chlorophyll content decreased in all of the

flooded plants that were tested (Table 17). However, the concentration of total chlorophyll, and both chlorophyll A and B was significantly higher in flooded ACC deaminase transgenic plants than in flooded non-transformed plants. Based on the observed leaf chlorophyll content, the tolerance of different genotypes to flooding can be represented as: NT < PRB-1*b* ≤ 35*S* < *rolD*.

Flooding changes the range of organic and inorganic compounds available to be taken up by a plant (Nilsen and Orcutt, 1997). Because the ACC deaminase transgenic plants that were used in this study were previously found to have an increased ability to accumulate a number of metals from the environment into both roots and shoots (Grichko, V.P., Filby, B. and Glick, B.R. submitted for publication), the effect of exogenous magnesium on seed germination and plant growth was estimated. Mg suppressed germination of transgenic seeds compared to non-transformed seeds during the initial four days and after that the transgenic seeds germinated at a similar rate to non-transformed seeds (Fig. 29A). In 51-day-old plants, treated with Mg for ten days, fresh shoot weight increased slightly in non-transformed and PRB-1*b* plants, and to a greater extent in *rolD* plants, while the fresh shoot weight decreased in 35*S* plants (Fig. 29B). After the treatment with Mg, the dry shoot weight was changed in all the plants tested (Fig. 29B) and became 2.5-fold higher in *rolD* plants compared to non-transformed plants (data not shown). Magnesium had a stimulatory effect on leaf chlorophyll in *rolD* and 35*S* plants and was inhibitory in non-transformed and PRB-1*b* plants (Fig. 29C). The total chlorophyll content was about 2-fold higher in *rolD* plants compared to non-transformed plants, following prolonged treatment with magnesium.

Figure 27. Epinasty in the petiole of the third oldest leaf of 41-day-old non-transformed tomato plants cv Heinz 1439 treated with AVG. Plants were flooded above the level of the adventitious roots after eight days from the beginning of the experiment. Data are changes in the angle from 0-h values, n = 5.

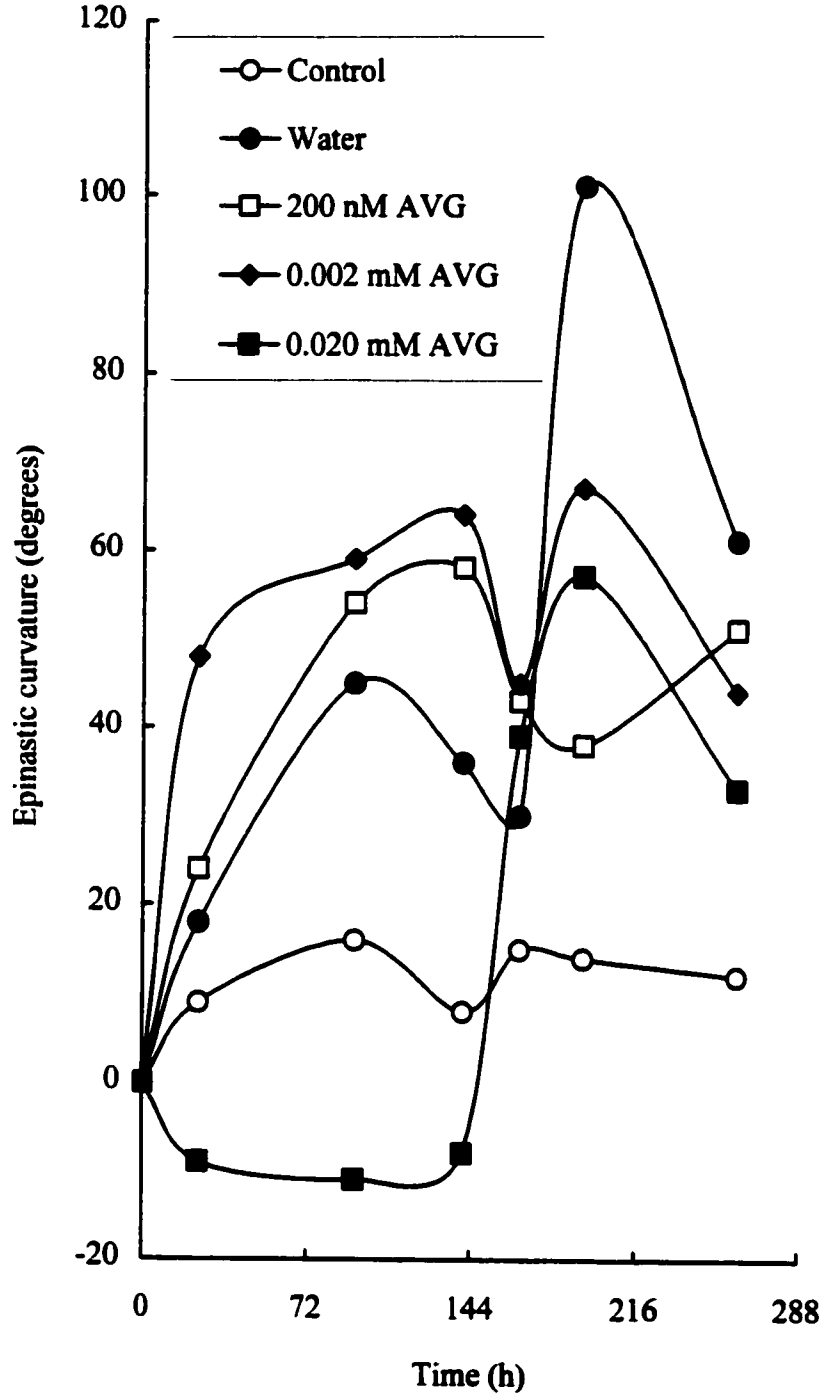

120
100
80
60
40
20
0
-20
Epinastic curvature (degrees)
Control
Water
200 nM AVG
0.002 mM AVG
0.020 mM AVG
0
72
144
216
288
Time (h)

Figure 28. Ethylene production in petioles sampled from the fifth top leaves of non-transformed and ACC deaminase transgenic plants ($P < 0.05$, $n = 4$).

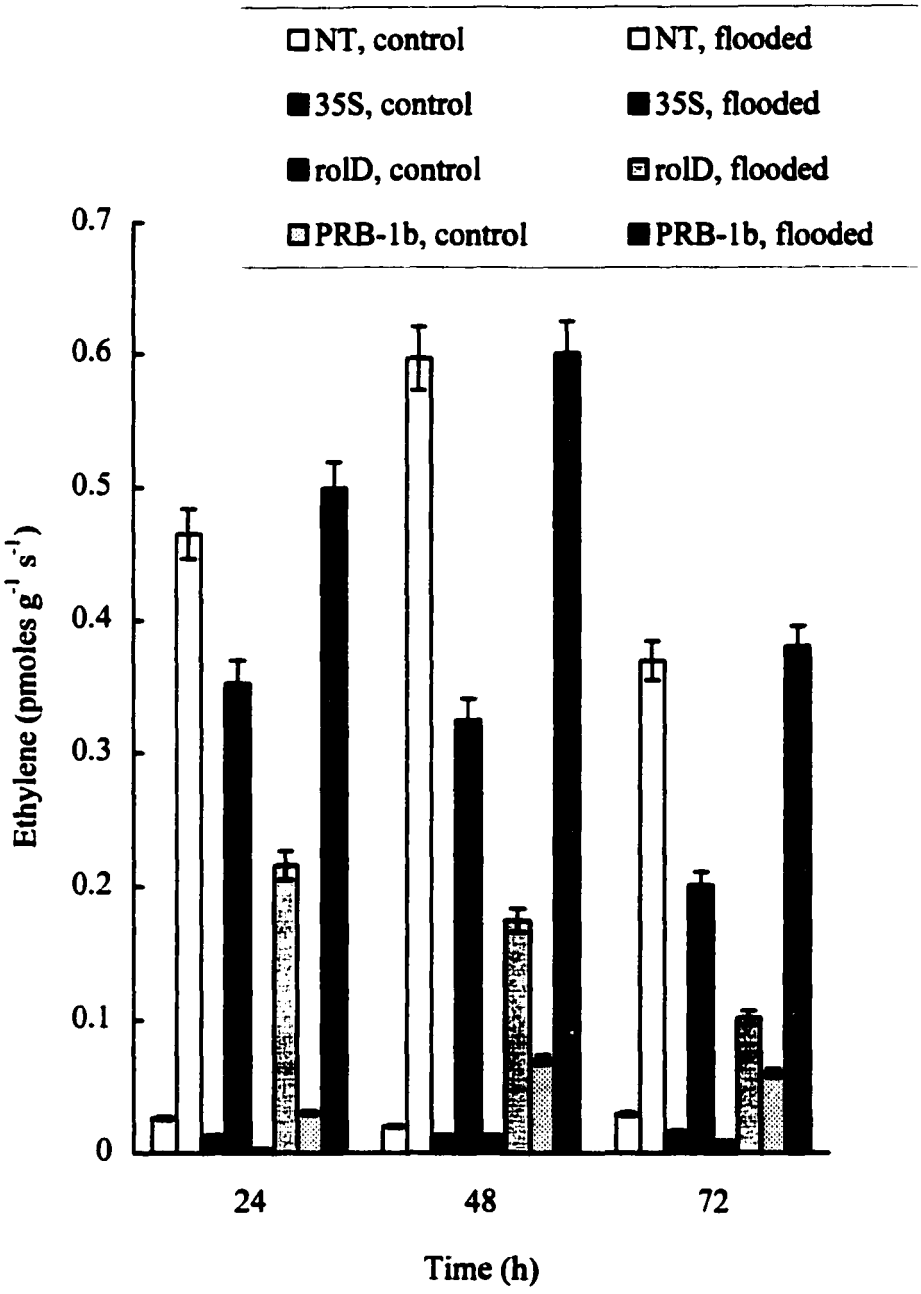
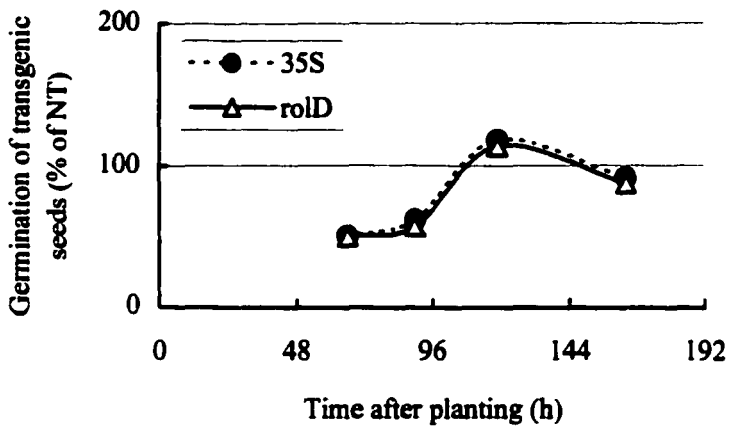
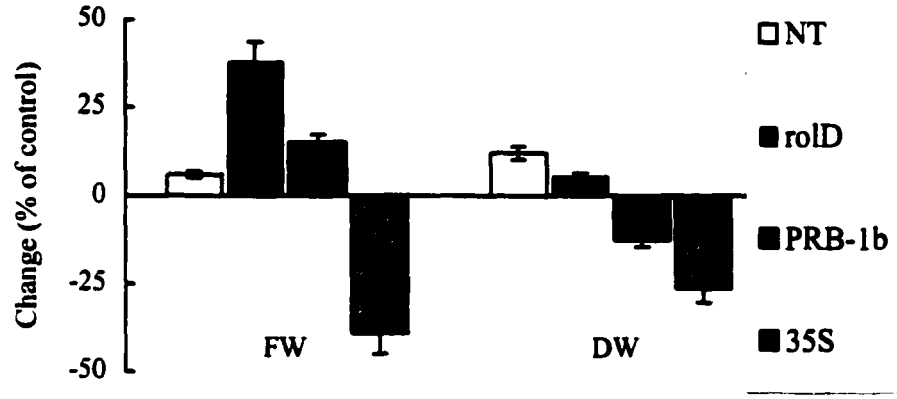

NT, control
NT, flooded
35S, control
35S, flooded
rolD, control
rolD, flooded
PRB-1b, control
PRB-1b, flooded
Ethylene (pmoles g-1 s-1)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
24
48
72
Time (h)

Figure 29. Effect of Mg on transgenic plants. (A) Germination. NT, non-transformed. (B) Change in fresh (FW) and dry (DW) shoot weight in 51-day-old non-transformed and transgenic tomato plants treated with magnesium (n = 3). NT, non-transformed. (C) Change in leaf chlorophyll in 51-day-old non-transformed and transgenic tomato plants treated with magnesium (n = 12 - 56). NT, non-transformed.

A


Germination of transgenic seeds (% of NT)
200
100
0
35S
rolD
0
48
96
144
192
Time after planting (h)

B


Change (% of control)
50
25
0
-25
-50
FW
DW
NT
rolD
PRB-1b
35S

C

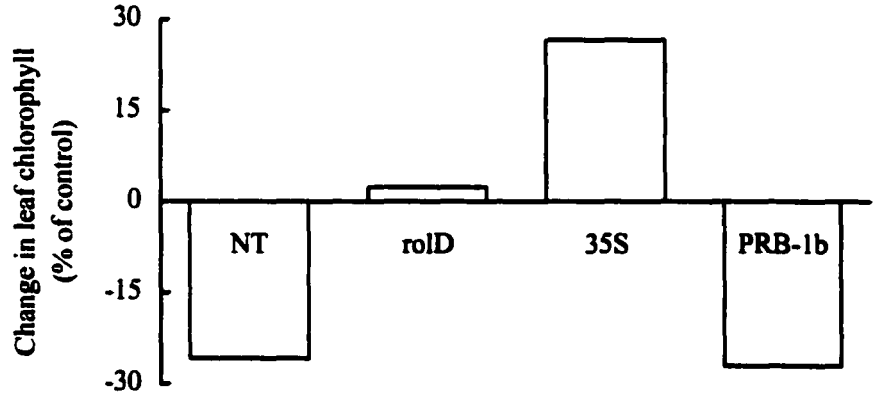

Change in leaf chlorophyll (% of control)
30
15
0
-15
-30
NT
rolD
35S
PRB-1b

Table 17. Leaf chlorophyll concentration (mg g^{-1} FW) in 55-day-old non-transformed and transgenic tomato plants flooded for nine days.

Geno-type	Floo-ding	Total chlorophyll	Chlorophyll A	Chlorophyll B	Chloropyll A/ B
NT	-	1.169 ± 0.043 (n = 27)	0.900 ± 0.034 (n = 27)	0.270 ± 0.010 (n = 27)	3.35 ± 0.08 (n = 27)
NT	+	0.537 ± 0.042 (n = 21)	0.416 ± 0.032 (n = 21)	0.122 ± 0.010 (n = 21)	3.45 ± 0.05 (n = 21)
35*S*	-	1.322 ± 0.114 (n = 13)	1.016 ± 0.089 (n = 13)	0.306 ± 0.025 (n = 13)	3.30 ± 0.08 (n = 13)
35*S*	+	0.770 ± 0.074 [a] (n = 14)	0.581 ± 0.051 [a] (n = 14)	0.189 ± 0.023 [a] (n = 14)	3.23 ± 0.12 (n = 14)
rolD	-	1.194 ± 0.126 (n = 14)	0.920 ± 0.100 (n = 14)	0.275 ± 0.027 (n = 14)	3.36 ± 0.09 (n = 14)
rolD	+	1.077 ± 0.084 [a] (n = 12)	0.820 ± 0.063 [a] (n = 12)	0.257 ± 0.021 [a] (n = 12)	3.21 ± 0.06 [a] (n = 12)
PRB-1*b*	-	1.270 ± 0.124 (n = 10)	0.997 ± 0.104 (n = 10)	0.273 ± 0.023 (n = 10)	3.63 ± 0.20 (n = 10)
PRB-1*b*	+	0.789 ± 0.038 [a] (n = 17)	0.605 ± 0.029 [a] (n = 17)	0.184 ± 0.010 [a] (n = 17)	3.33 ± 0.07 (n = 17)

Values are means ± SE. a, significantly different compared with flooded non-transformed plants, $P < 0.05$.

3.5. Amelioration of flooding stress in mature plants by FNR transcriptionally regulated ACC deaminase-containing plant growth-promoting bacteria

It was previously observed that ACC deaminase-containing plant growth-promoting bacteria can promote root, and to a lesser extent shoot growth in ethylene sensitive dicots such as canola, tomato and lettuce (Hall et al., 1996; Glick et al., 1997). However, most of these experiments reported the effect of plant growth-promoting bacteria on relatively young plants. It was therefore of interest to determine whether these organisms could also affect the growth of older and larger plants. As can be seen in Table 18, preliminary experiments with 17-day-old tomato plants indicated that inoculation of tomato seeds with bacteria expressing an ACC deaminase gene is beneficial for the growth of tomato plants. Of the bacteria tested *P. putida* ATCC 17399/pRKACC (Shah et al., 1998) had the strongest growth-stimulating effect.

Following the demonstration that bacteria expressing ACC deaminase can stimulate normal plant growth, the effect of these bacteria on flooded plants was assessed. Compared to untreated control plants, flooded tomato plants grown from seeds that were inoculated with plant growth-promoting bacteria appeared to be taller and greener (data not shown). Moreover, bacteria with ACC deaminase activity noticeably stimulated the development of both adventitious roots and stem aerenchyma in flooded tomato plants compared to non-bacterized flooded tomato plants or flooded plants bacterized with organisms that do not contain ACC deaminase (data not shown). Nine consecutive days of flooding resulted in a decrease in shoot height, both fresh and dry shoot weight, and the ratios derived from these values in all groups of plants that were tested (Table 19).

Table 18. Effect of plant growth-promoting bacteria on seventeen-day-old tomato plants cv Heinz 902

Strain used for seed inoculation	ACC deaminase activity (μmole mg^{-1} protein h^{-1})	Fresh shoot weight (mg)
Non-bacterized	-	110.0 ± 5.5 (63)
P. putida ATCC 17399/pRKACC	3.1 ± 0.5 (3)	121.8 ± 6.5 (39)
P. putida ATCC 17399/pRK415	0.1 ± 0.05 (3)	95.9 ± 6.6 [a] (28)
CAL2	5.2 ± 0.6 (3)	111.7 ± 5.8 (29)
UW4	3.6 ± 0.4 (3)	114.0 ± 7.5 (30)

The values are means ± SE, $P < 0.05$. The values in brackets indicate the number of plants examined. a, significantly different from *P. putida* ATCC 17399/pRKACC.

Table 19. Effect of flooding and bacteria on shoots of 55-day-old tomato plants

Strain	Flo-od	Shoot height (cm)	Fresh shoot weight (g)	FW/height (g/cm)	Shoot dry weight (g)	DW/FW
Control	-	16.8 ± 0.5 (n = 27)	26.03 ± 1.83 (n = 27)	1.51 ± 0.08 (n = 27)	1.362 ± 0.131 (n = 27)	0.051 ± 0.003 (n = 27)
	+	11.3 ± 0.5 (n = 21)	11.71 ± 0.86 (n = 21)	1.01 ± 0.05 (n = 21)	0.315 ± 0.048 (n = 21)	0.025 ± 0.002 (n = 21)
CAL2	-	21.6 ± 0.7 [a] (n = 20)	33.64 ± 2.05 [a] (n = 20)	1.56 ± 0.08 (n = 20)	1.504 ± 0.105 (n = 20)	0.045 ± 0.003 (n = 20)
	+	16.3 ± 0.5 [b] (n = 20)	22.82 ± 1.20 [b] (n = 20)	1.40 ± 0.06 [b] (n = 20)	0.855 ± 0.118 [b] (n = 20)	0.037 ± 0.005 (n = 20)
UW4	-	21.0 ± 0.5 [a] (n = 18)	32.95 ± 2.40 [a] (n = 18)	1.57 ± 0.11 (n = 18)	1.642 ± 0.182 (n = 18)	0.048 ± 0.002 (n = 18)
	+	18.0 ± 0.6 [b] (n = 18)	25.77 ± 0.92 [b] (n = 18)	1.44 ± 0.05 [b] (n = 18)	1.054 ± 0.050 [b] (n = 18)	0.041 ± 0.001 (n = 18)
P. putida/ pRKACC	-	17.1 ± 0.8 (n = 10)	29.09 ± 2.47 (n = 10)	1.68 ± 0.09 (n = 10)	1.374 ± 0.163 (n = 10)	0.046 ± 0.002 (n = 10)
	+	16.1 ± 0.4 [b] (n = 19)	22.26 ± 1.17 [b] (n = 19)	1.38 ± 0.06 [b] (n = 19)	0.857 ± 0.062 [b] (n = 19)	0.038 ± 0.001 (n = 19)
P. putida/ pRK415	–	19.1 ± 0.6 [a] (n = 18)	27.37 ± 2.06 (n = 18)	1.41 ± 0.08 (n = 18)	1.345 ± 0.121 (n = 18)	0.048 ± 0.002 (n = 18)
	+	13.6 ± 0.2 [b] (n = 21)	13.91 ± 0.73 (n = 21)	1.02 ± 0.05 (n = 21)	0.463 ± 0.072 (n = 21)	0.033 ± 0.005 (n = 21)

Values are means ± SE, $P < 0.05$. a, significantly different vs. non-flooded non-bacterized plants; b, significantly different vs. flooded non-bacterized plants.

However, the differences between the obtained parameters for control tomato plants and plants treated either with UW4, CAL2 or *P. putida*/pRKACC deaminase were unexpectedly high and statistically significant. Leaf chlorophyll content decreased in all types of flooded plants compared to control plants (Table 20). However, the concentrations of total chlorophyll, and of both chlorophyll A and B were significantly higher in flooded plants that were treated with ACC deaminase-containing bacteria compared to flooded plants. In addition, plants treated with *P. putida*/pRKACC had a significantly increased chlorophyll content - plants treated with CAL2 also had an increased chlorophyll content although it was not statistically significant - in the absence of flooding. Moreover, *P. putida*/pRK415 which does not contain ACC deaminase had the smallest effect of any of the added bacteria on chlorophyll levels following flooding. The onset of flooding caused a rapid increase in the concentration of ACC in the soil water (i.e., exuded by the plant roots) that declined below the limit of detection of HPLC analysis after six days of the root submergance (Fig. 30).

Examination of the roots of plants, that were grown from bacterized and non-bacterized seeds both before and after the flooding by scanning electron microscopy, revealed the presence of dense bacterial biofilms (Fig. 31 & 32). The highest density of bacteria was observed at the root tips. The root tips were excised, washed with distilled water, and placed aseptically on solid minimal medium containing ACC as the nitrogen source. Following three days of growth, no colonies appeared on the plates with the root samples from control plants. However, vigorous growth was observed around the roots on the plates which contained samples from the roots of the plants whose seeds were treated with bacteria harboring an ACC deaminase gene (data not shown).

Table 20. Leaf chlorophyll concentration (mg g^{-1} FW) in 55-day-old tomato plants

Strain	Flooding	Total chlorophyll	Chlorophyll A	Chlorophyll B	Chlorophyll A/ B
Non-bacterized	-	1.169 ± 0.043 (n = 27)	0.900 ± 0.034 (n = 27)	0.270 ± 0.010 (n = 27)	3.35 ± 0.08 (n = 27)
	+	0.537 ± 0.042 (n = 21)	0.416 ± 0.032 (n = 21)	0.122 ± 0.010 (n = 21)	3.45 ± 0.05 (n = 21)
CAL2	-	1.398 ± 0.190 (n = 20)	1.065 ± 0.144 (n = 20)	0.334 ± 0.046 (n = 20)	3.22 ± 0.06 (n = 20)
	+	0.995 ± 0.059 [b] (n = 20)	0.766 ± 0.046 [b] (n = 20)	0.229 ± 0.013 [b] (n = 20)	3.35 ± 0.06 (n = 20)
UW4	-	1.227 ± 0.073 (n = 18)	0.922 ± 0.056 (n = 18)	0.306 ± 0.017 (n = 18)	3.01 ± 0.04 [a] (n = 18)
	+	1.194 ± 0.062 [b] (n = 18)	0.913 ± 0.048 [b] (n = 18)	0.281 ± 0.015 [b] (n = 18)	3.26 ± 0.07 [b] (n = 18)
P. putida/ pRKACC	-	1.384 ± 0.111 [a] (n = 19)	1.051 ± 0.084 (n = 19)	0.333 ± 0.028 [a] (n = 19)	3.17 ± 0.06 (n = 19)
	+	1.131 ± 0.062 [b] (n = 19)	0.857 ± 0.047 [b] (n = 19)	0.274 ± 0.015 [b] (n = 19)	3.13 ± 0.04 [b] (n = 19)
P. putida/ pRK415	-	1.095 ± 0.079 (n = 18)	0.841 ± 0.063 (n = 18)	0.254 ± 0.017 (n = 18)	3.30 ± 0.07 (n = 18)
	+	0.862 ± 0.046 [b] (n = 21)	0.660 ± 0.034 [b] (n = 21)	0.202 ± 0.012 [b] (n = 21)	3.31 ± 0.05 (n = 21)

Values are means ± SE, $P < 0.05$. a, significantly different vs. non-flooded non-bacterized plants; b, significantly different vs. flooded non-bacterized plants.

Figure 30. Release of ACC by non-transfoormed tomato plant roots into the surrounding water after the onset of flooding stress.

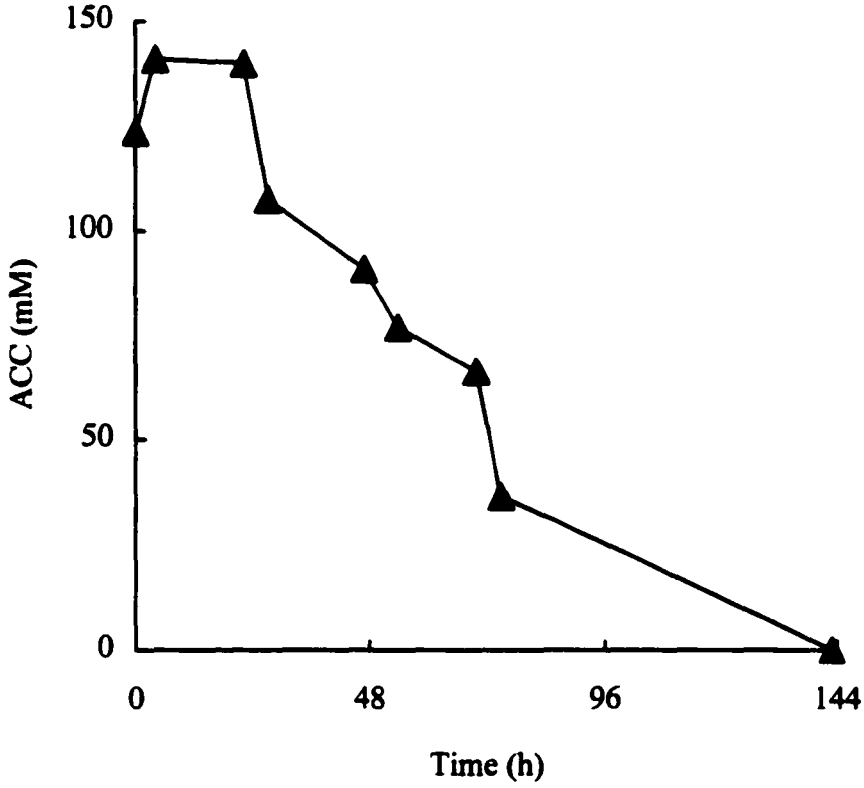

150
100
50
0
ACC (mM)
0
48
96
144
Time (h)

Figure 31. Scanning electron micrograph of tomato plant root colonized by *P. putida*/pRKACC taken at the end of the flooding experiment.



Figure 32. Scanning electron micrograph of tomato plant root colonized by *E. cloacae* UW4 taken at the end of the flooding experiment.



Figure 33. Epinastic curvature in the petiole of the fifth top leaf of control tomato plants and plants treated with bacteria following flooding. Plants were fifty-five days old. Values are changes in angle from the 0-h data (n = 3).

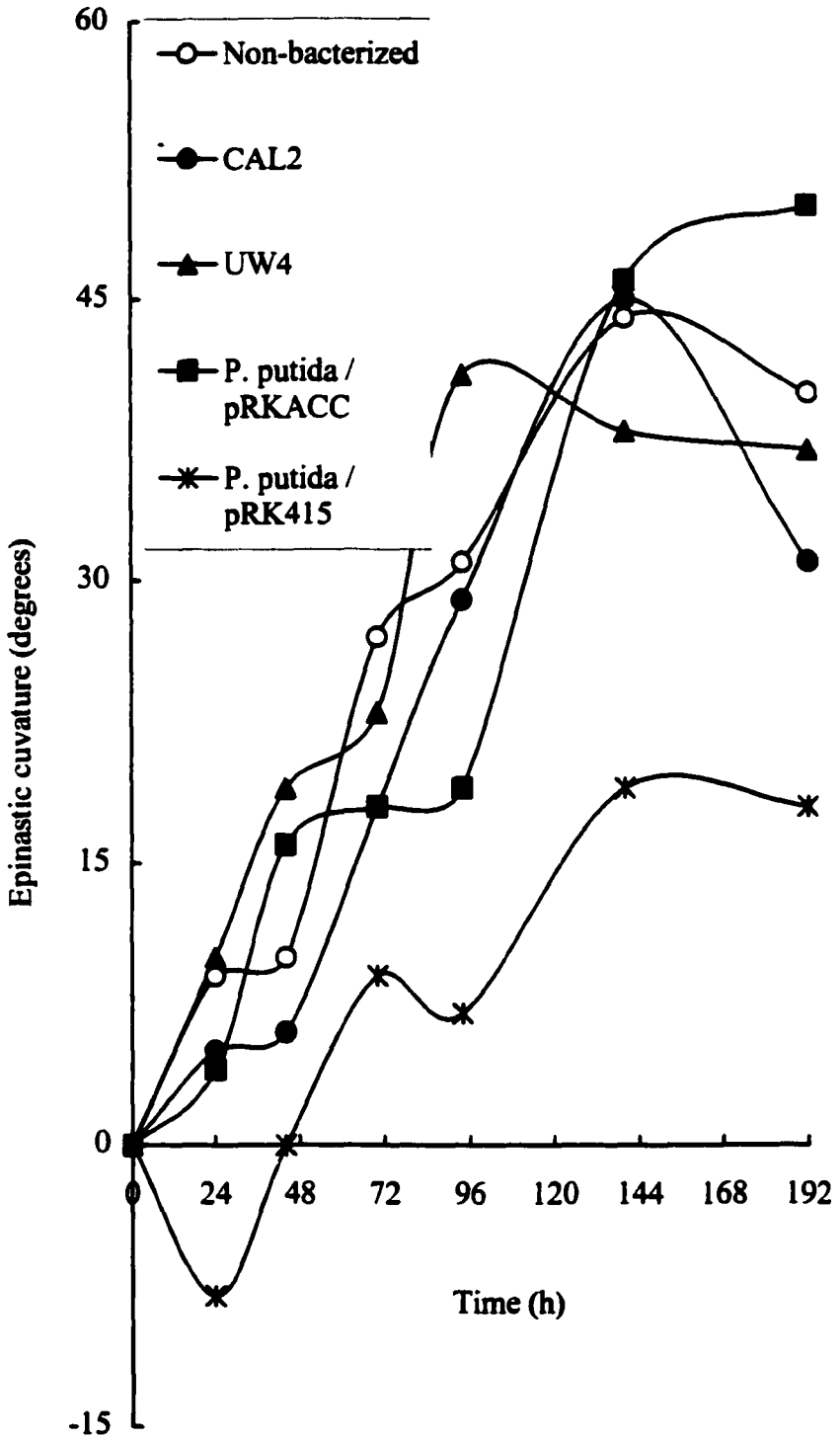
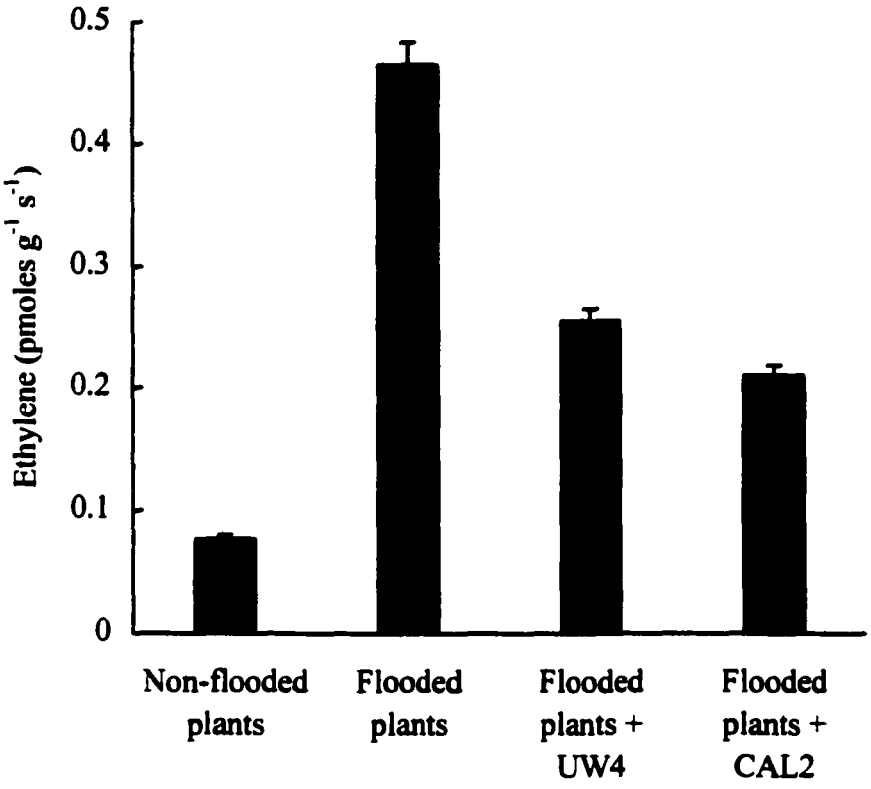

60
45
30
15
0
-15
Non-bacterized
CAL2
UW4
P. putida / pRKACC
P. putida / pRK415
Epinastic cuvature (degrees)
0
24
48
72
96
120
144
168
192
Time (h)

Figure 34. Ethylene production by petioles of tomato plants treated with *Enterobacter cloacae* and flooded for nine days. Values are means ± SE, $P < 0.05$, ($n = 3 - 7$).


0.5
0.4
0.3
0.2
0.1
0
Ethylene (pmoles g-1 s-1)
Non-flooded plants
Flooded plants
Flooded plants + UW4
Flooded plants + CAL2

Unexpectedly, with the notable exception of strain *P. putida* ATCC 17399/pRK415, all of the tomato plants that developed from bacterized seeds followed essentially the same kinetics of epinastic curvature following flooding as did tomato plants that developed from non-bacterized seeds (Fig. 33). This surprising result is despite the fact that flooding resulted in a significant increase in the production of ethylene by petiolar tissue in non-bacterized plants and had a much less pronounced effect on the production of ethylene by plants treated with *E. cloacae* UW4 or CAL2 (Fig. 34). In addition, ethylene production in petiolar tissue of tomato plants bacterized with *P. putida*/pRKACC was almost two-fold lower compared to the rate of ethylene production by leaf petioles of tomato plants bacterized with *P. putida*/pRK415 (data not shown).

4. DISCUSSION

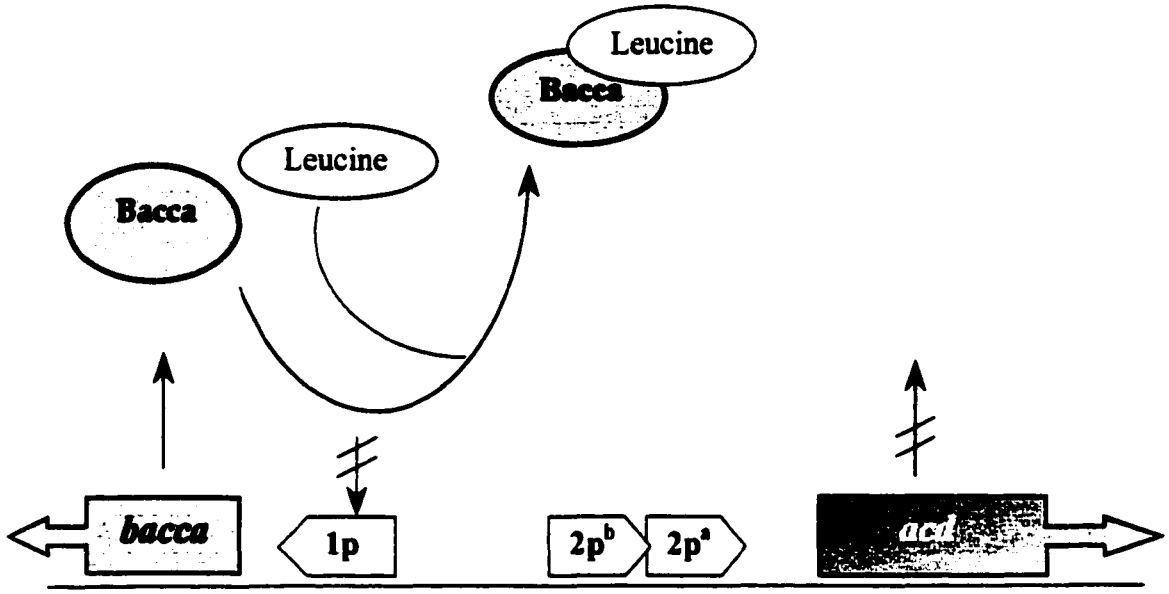
4.1. *acd* gene regulation in *E. cloacae* UW4

The regulation of *acd* gene transcription in *E. cloacae* UW4 is complex and seems to involve novel protein Bacca, LRP, CRP and FNR proteins. Indeed, most of the FNR-dependent promoters in *E. coli* are regulated by FNR in co-operation with other regulators such as NarL, nARp (nitrate regulation) or CRP, in either a positive or negative mode (Unden et al., 1995). The complex regulatory machinery that controls the *E. cloacae* UW4 *acd* gene is apparently well suited for normal as well for stress conditions. The FNR protein directly senses oxygen concentrations so that FNR-dependent promoters are activated by 5 μM O_2 (Laville et al., 1998), a trait that is clearly beneficial for the proliferation of root-associated bacteria.

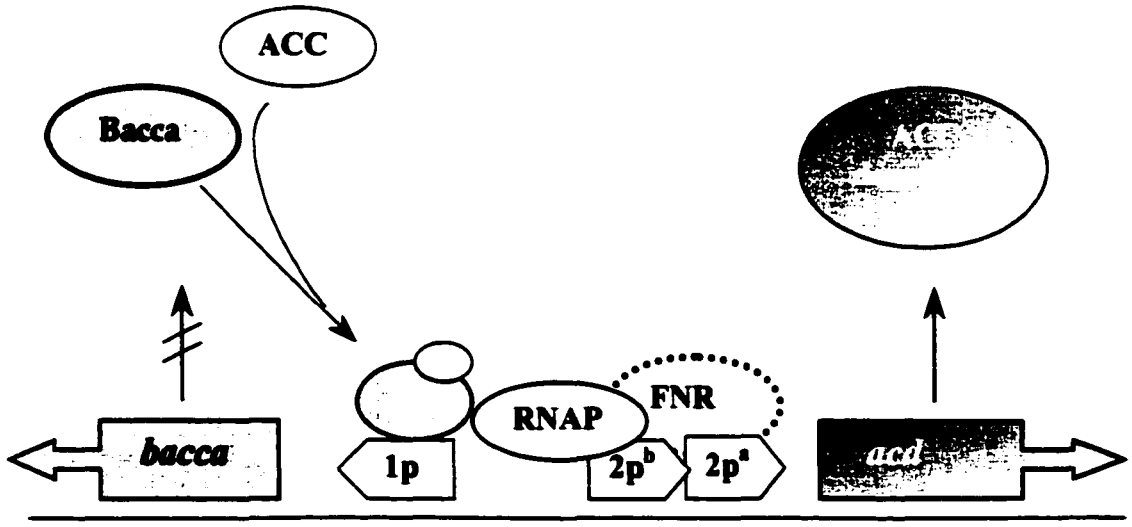
The product of ACC degradation, α-ketobutyrate is utilized to form isoleucine, which belongs to pyruvate-derived amino acids similar to leucine. LRP stimulates transcription of the *ilvIH* operon of *E. coli* encoding acetohydroxy-acid synthase III, which catalyzes the common step in the biosynthesis of isoleucine and leucine from pyruvate (Willins et al., 1991). LRP binds to the promoter/regulatory region of *ilvIH* operon in the absence of leucine, which represses expression of the operon by preventing the binding of LRP (Willins et al., 1991). A similar type of regulation may exist for the ACC deaminase gene from *E. cloacae* UW4 with ACC-bound Bacca protein acting as an activator and leucine acting as a repressor of transcription (Fig. 4A & 4B).

Figure 35. A model of the regulation of the *acd* gene transcription in *E. cloacae* UW4. (A) Bacca regulatory protein is transcribed from promoter 1p in the absence of ACC. In this case, there is no transcription from promoters $2p^a$ and $2p^b$ and therefore no ACC deaminase produced. It is likely that Bacca binds to leucine. (B) Bacca gene expression is autoregulated and the bound Bacca-ACC complex is required to activate transcription of the *acd* gene. In presense of ACC, the ACC-bound Bacca binds to its own promoter, ceases transcription of the *bacca* gene and initiates transcription of *acd* gene from promoter $2p^b$. Under anaerobic conditions the FNR protein binds to the FNR box and initiates transcription from promoter $2p^a$. In this case the bound FNR protein acts as an adjunct to the bound Bacca-ACC complex. RNAP, RNA polymerase.

A


Leucine
Bacca
Leucine
Bacca
bacca
1p
2p^b
2p^a
acl

B


ACC
Bacca
bacca
1p
RNAP
FNR
2p^b
2p^a
acl

According to the model elaborated here (Fig. 35) the *bacca* gene appears to be transcribed from a strong promoter 1p which contains an LRP-like binding site in the absence of ACC in a medium (Fig. 35A). Indeed, in *E. cloacae* UW4, the 5' upstream region of the *bacca* gene is exceptionally A/T rich and might serve as a binding site for an LRP-like protein (Napoli et al., 1999). This LRP-binding cite overlaps a Pribnow box, consistent with autoregulation of *bacca* gene expression *in E. cloacae* UW4 (Fig. 3). A number of LRP proteins including that of *E. coli* are autoregulated (Napoli et al., 1999).

ACC-bound Bacca is thought to be able to bind to DNA (Fig. 35B); once bound the protein both ceases its own transcription and activates the transcription of *acd* gene from the weak promoter $2p^b$ under aerobic conditions or from the FNR-dependent promoter $2p^a$ under limited oxygen conditions in the presence of glucose. Interestingly, a high but still non-significant score was observed for the sequence starting from amino acid 37, i.e., ISNVALAEKVKLSPPACLRRVD, when the program HTH (NPSA server, France) was used to predict the presence of a helix-turn-helix motif in the Bacca protein. Binding of ACC may result in the conformational change in Bacca protein making possible its binding to the DNA. Under anaerobic conditions both ACC-bond Bacca protein and FNR are thought to be needed for maximal activity of ACC deaminase. At the present time the molecular mechanism of transcriptional regulation by the LRP-AsnC protein family is unknown and no interactions with the basal transcriptional machinery have been elucidated (Napoli et al., 1999). Roesch and Blomfield showed (1998) that in *E. coli* a single LRP binding site in a three-binding site complex is sensitive to leucine-bound LRP. It is possible that a similar relationship of the Bacca protein to the LRP-binding sites exist in *E. cloacae* UW4.

The Bacca protein contains seven copies of 6-amino acid leucine periodic patterns resembling the structure of heptad leucine zipper proteins found in eukaryotes including yeast and has an unusually elevated content of leucine suggesting the presence of a leucine dependent regulatory feedback. The role of leucine in *acd* gene transcription is complex and dependent on the balance of available leucine, ACC, LRP and Bacca protein. The LRP protein has a high affinity for leucine (Napoli et al., 1999), and Bacca protein seems to bind both leucine and ACC (Fig. 6A). If leucine binds to the Bacca protein making it unavailable for binding ACC, the effect may be overcome by increasing the level of ACC in the growth medium. Indeed, an increase in ACC deaminase activity was observed in the lrp^+ strain following addition of ACC to the leucine-containing LB growth medium. In some cases, multiple copies of the LRP protein could stimulate ACC deaminase gene expression by binding excess leucine (Fig. 4A & 4B). The lrp^- *E. coli* strain may produce sufficient LRP to bind leucine while leaving the Bacca protein to bind ACC. LRP is toxic to the cell at a high level (Chen and Newman, 1998).

The data indicate that the *acd* gene is induced to a greater extent in the presence of glucose and is able to function under both aerobic and oxygen limiting conditions due to the presence of two different promoters. Under anaerobic conditions or in the presence of excess glucose (even under aerobic conditions) the citric acid cycle is transformed into a non-cyclic form (Guest 1992) generating fumarate as an electron acceptor. As a result of glucose fermentation acetate, ethanol, formate, and lactate are produced from pyruvate. Anaerobiosis activates FNR, which might result in transcription initiation from promoter $2p^a$ and reduce initiation from promoter $2p^b$. The FNR half-site (TTGAT) has been shown to be functional in the *E. coli ndh* promoter (Page and Guerinot, 1995).

Switching mechanisms in overlapping promoters is common and is observed in both the *gal* and *focApfl* operons in *E. coli*, and in the *fixRnifA* operon in *Bradyrhizobium japonicum* (Kaiser and Sawers, 1997). Limiting oxygen levels and a relatively high level of glucose are characteristics of the natural habitats of fruit-associated yeast and root-associated bacteria. In this regard, the induction of ACC deaminase is routinely carried out in the presence of 0.4-2% of glucose (Honma and Shimomura, 1978; Minami et al., 1998).

It is noteworthy that in bacterium *Klebsiella aerogenes*, the D-alanine catabolic operon (*dad*) is controlled by CRP, LRP and a nitrogen assimilation control protein (Janes and Bender, 1999; Roesch and Blomfield, 1998). Regulation of ACC deaminase expression in *E. cloacae* UW4 appears to be similar to regulation of the D-alanine catabolic operon in *K. aerogenes* and in fact ACC deaminase is active toward a number of amino acids, including D-alanine (Penrose and Glick, 1997).

4.2. ACC deaminase transgenic plants

The bacterial enzyme ACC deaminase is considered to be the only non-plant enzyme that can metabolize ACC; the enzyme converts ACC to α-ketobutyrate and ammonia (Honma and Shimomura, 1978; Honma, 1983). The α-ketobutyrate can be used as a precursor in the sythesis of isoleucine; isoleucine, leucine, and α-aminobutyrate accumulate in bacteria expressing ACC deaminase (Honma and Shimomura, 1978). Both ACC synthase and ACC deaminase are pyridoxal phosphate-dependent enzymes; the optimum pH of ACC deaminase is 8.5 and ACC synthase has matching pH optimum of 8.5-9.5. (Abeles et al., 1992; Honma and Shimomura, 1978; Jacobson et al., 1994).

Simultaneous expression of ACC synthase and ACC deaminase in *Esherichia coli* strain JHM-544, which cannot normally synthesize isoleucine, results in vigorous growth on minimal medium without the addition of isoleucine (Tarun et al., 1998).

Expression of ACC deaminase in plants is directed toward decreasing ACC levels and, hence, reducing ethylene production (Reed et al., 1995). The advantage of the approach is that it is not species and gene specific and may be applied to a number of plant species. Because the functioning of ACC deaminase does not interfere with the main biochemical pathways in a plant, some potential side effects that could occur as a consequence of modulating plant ethylene levels by other types of genetic manipulation may be avoided. For example, in contrast to the control of plant ethylene levels based on ACC deaminase expression, the expression of antisense ACC oxidase reduces ethylene levels while allowing a potentially dangerous buildup of ACC to occur within the transgenic plant.

The K_m values for ACC deaminases from different bacterial strains are 4.1-17.4 mM (Klee, 1992) compared to K_m values of 1-120 μM for ACC oxidases (Abeles et al., 1992). Enzyme competition for ACC can be affected by a number of factors. While optimum temperatures for ACC deaminase and ACC oxidase are close, 25-30°C and 26°C, respectively, the optimum pH of ACC oxidase is 7.5, the pH optimum of ACC deaminase is 8.5 (Abeles et al., 1992; Honma and Shimomura, 1978; Jacobson et al., 1994). Discriminatory conditions are often the case; for example, ACC oxidation is arrested under anaerobiosis while the reaction catalyzed by ACC deaminase proceeds normally under these conditions. In tomato roots, *LE-ACS7* (the earliest gene, which is expressed in 5 h), *LE-ACS3* and *LE-ACS2* genes are induced under flooding followed by

accumulation of ACC (Shiu et al., 1998). ACC is transported in xylem and is responsible for the stress seen in shoots (Jackson, 1997). The presence of ACC in xylem sap in both healthy and stressed plants raises the question about the role of ACC synthesized by roots in the physiology of non-stressed plants (Else and Jackson, 1998). During one 12 h light period, approximately 20 nmoles of ACC, equivalent to about 40 nl h^{-1} of ethylene, is transported in the xylem sap from the roots of well-drained tomato plants (Jackson, 1997). In addition, long-distance translocation of ACC in phloem may take place as a component of interorgan signaling (Morris and Larcombe, 1995). In tomato, the EIN3 binding site has been identified in the promoter sequence of the early ethylene responsive gene *LE-ACO1*, which is rapidly induced in shoots following root anaerobiosis (Shiu et al., 1998; Theologis, 1998).

In this study, tomato plants were transformed with an ACC deaminase gene under the control of three different plant promoters, i.e. 2x 35*S* (Christopher et al., 1987), *rolD* (Elmayan and Terfer, 1995) and PRB-1*b* (Eyal et al., 1992). All three types of transgenic plants were previously shown to be homozygous for a single copy of the ACC deaminase gene (Robison et al., in preparation). Moreover, immunological analysis indicates that, as expected, with the 35*S* promoter ACC deaminase is found in both leaves and roots, with the *rolD* promoter ACC deaminase is detected only in roots and with the PRB-1*b* promoter ACC deaminase is detectable following abiotic stress (Robison et al., in preparation). Here, the effect of the transgene on a number of physiological properties of tomato plants was examined. While numerous studies have examined the impact of lower plant ethylene levels using ACC deaminase or other ethylene transgenes and their effect on fruit ripening, flower and leaf senescence, and resistance to pathogens (Klee et

al., 1991; Oeller et al., 1991; Picton et al., 1993; Good et al., 1994; John et al., 1995; Bolitho et al., 1997), this appear to be the first report that genetically reducing plant ethylene levels can improve normal plant growth and development.

To confirm that endogenous ethylene was the main cause of the observed phenotypes in a number of instances we compared control tomato plants to plants treated with Ag^+. Since Ag^+ is a noncompetitive inhibitor of ethylene action (Abeles et al., 1992) which may interact with ethylene receptors, relying presumably on Cu^+ (Rodriguez et al., 1999), treatment of non-transformed plants with silver should make them comparable to the transgenic plants in terms of the ability to ameliorate some of the effects of stress ethylene production. Change in the stability of the ethylene-metal bond in the occupied ethylene receptor complex could result in the development of a specific response in transgenic seeds resembling the triple response. In tobacco, a several-fold increase in ethylene production was detected in plants expressing a mutant *Arabidopsis etr-1* (ethylene triple response) gene (Knoester et al., 1998). In sterile growth pouches, Ag^+ stimulated root growth in 35*S* plants and both shoot and root growth in PRB-1*b* plants. These plants produced a high level of anthocyanin, which is regulated by ethylene (data not shown). PRB-1*b* promoter responds to a number of stimuli, i.e., virus infection, ethephon treatment, UV light, ethylene and wounding (Brederode et al., 1991) that could be a reason for the high anthocyanin production observed in young PRB-1*b* plants. In order to completely inhibit these receptors a 30,000-fold molar excess of silver is required (Abeles et al., 1992). Young plants absorbed about 3 μM silver in roots and 0.3-1.4 μM in shoots. Based on the estimated concentration of ethylene binding sites of 3.5 nM (Schaller and Bleecker, 1995), it is possible that the inhibitory effect of ethylene on

plant growth was overcome in both 35*S* and PRB-1*b* plants due to the synergetic effect of ACC deaminase and Ag^+. In the light, silver is less toxic for plants than in the dark (Abeles et al., 1992). The formation of metallic spots at a near-rib region at the adaxical side of tomato plant leaves treated with silver nitrate under high light was detected. Silver salts, like a number of other ethylene action inhibitors are photosensitive, and colloids produced by photoreduction of silver (Zhang and Long, 1993) may be the cause of the metallic spots. Ag^0 produces stronger complexes with ethylene than Ag^+ (Kiselev and Krylov, 1989).

For the most part in this study, plants with root specific expression of ACC deaminase behaved more like plants that constitutively expressed ACC deaminase than like non-transformed plants. For example, in growth pouches, 35*S* and *rolD* plants developed significantly longer roots than non-transformed plants. Similarly, in *Arabidopsis thaliana*, Roman et al. (1995) found that the root lengths of *eri-1* (ethylene root insensitive) and *etr1-3* (ethylene insensitive) mutants were greater than that of non-transformed plants.

Laser fluorescence spectroscopy and the pump-and-probe method of chlorophyll fluorescence measurement *in situ* are used for the detection of stress in plants, mutant selection and for the study of photosynthesis (Valentini et al., 1994; Agati et al., 1995), chlorophyll *a* being the most susceptible to stress. In both non-transformed and mutant tomato plants, the ratios of the fluorescence of different wavelengths provides an indication of the health, the presence of stress, the photosynthetic activity and the nitrogen nutrient status (Stober et al., 1992; Agati et al., 1996; Heisel et al., 1996; Ludeker et al., 1996). In this study, ACC deaminase transgenic plants showed higher

chlorophyll content and a higher fluorescence ratio than non-transformed plants. Apart from non-transformed plants, PSII activity in these plants was barely affected by the high level of silver accumulated by plant organs.

With some obvious restrictions, 35*S* plants may be viewed as being similar to antisense ACC oxidase plants. Under stress conditions the photosynthetic capability of ACC oxidase antisense transgenic plants exceeds that of non-transformed plants; John et al. (1995) did not observe a decrease in the F_v/F_m ratio in senescing tomato plants transformed with antisense ACC oxidase. Jensen and Veierskov (1998) observed a 50% decrease in chlorophyll in non-transformed tomato plants (cv. Ailsa Craig) and a small increase in leaf chlorophyll in antisense ACC oxidase plants when the photoperiod was extended from 16 to 23.5 h.

Ethylene initiates the events causing fruit ripening. During ripening the color of tomato fruit changes due to the transformation of chloroplasts into chromoplasts. In this study it was observed that the lycopene content in attached transgenic fruits was significantly higher than in non-transformed fruits.

The permeability of a plant's organs to ethylene is quite variable; ripening tomato fruit resembles a gas tank full of ethylene with about 90% percent of the ethylene released through the pedicel (De Vries et al., 1996). Tomato fruits rely heavily on their own ACC and seem to be affected by the entire plant health status. Thus, for example, here transgenic fruits were significantly larger than non-transformed fruits. Specific activity of ACC deaminase was low in ripening tomato fruits most likely because of the unfavorable pH for this enzyme. For efficient modulation of ACC levels in fruits, favorable kinetics of induction and expression, and a high steady state ACC deaminase

level is necessary. CamV35*S*, FMV35*S* and E8 (fruit specific and ethylene inducible) promoters were used to express ACC deaminase in tomato plants alone with antisense ACC oxidase and SAM decarboxylase to achieve a synergetic effect (Klee, 1992).

The results presented in this manuscript suggest that ACC deaminase transgenic plants exhibit a more healthy and productive phenotype compared to the non-transformed plants. If root-synthesized ACC is not important in normal plant growth and development, *rolD* and plants should behave similarly. On the other hand, if ACC of root origin is important in growth and development, then *rolD* plants are more likely to resemble 35*S* plants rather than non-transformed plants. For most of the characteristics tested, *rolD* and 35*S* plants showed the similar responses and phenotypes. More specifically, *rolD* and 35*S* plants had larger roots and shoots, a higher leaf chlorophyll content (*rolD* plants only), a higher level of leaf protein concentration, an increased average fruit weight, and an increased lycopene and β-carotene content in mature fruit. In addition, ACC deaminase transgenic tomato plants display increased resistance to several different phytopathogen (Lund et al., 1998; Robison et al., in preparation) and an increased resistance to the effects of flooding stress (Grichko et al., submitted for publication).

4.3. Potential of transgenic plants tested for use in phytoremediation

Transgenic tomato plants *Lycopersicon esculentum* (Solanaceae) cv. Heinz 902 expressing the bacterial gene 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, under the transcriptional control of either two tandem 35*S* cauliflower mosaic virus promoters (constitutive expression), the *rolD* promoter from *Agrobacterium rhizogenes*

(root specific expression) or the pathogenesis related PRB-1*b* promoter from tobacco, were compared to non-transformed plants in their ability to accumulate Cd, Co, Cu, Mg, Ni, Pb, or Zn. Parameters that were examined include metal concentration and ACC deaminase activity in both plant shoots and roots, root and shoot development, and leaf chlorophyll content. In general, transgenic tomato plants expressing ACC deaminase, especially those controlled by the PRB-1*b* promoter, acquired a greater amount of metal within the plant tissues, and were less subject to the deleterious effects of the metals on plant growth than were non-transformed plants.

It was previously observed that the growth of canola and tomato seedlings treated with an ACC deaminase-containing plant growth-promoting bacterium was partially protected from inhibition by nickel (Burd et al., 1998). In this case, the bacterial plant growth-promoting effect was attributed to the ability of the bacterium to lower the level of stress ethylene induced by the nickel (Burd et al., 1998). Recently, considerable attention has been directed toward the possibility of using plants to remove heavy metals from the environment (Raskin et al., 1997; Salt et al., 1998). Phytoremediation, which may be defined as the use of plants to remove, destroy or sequester hazardous substances from the environment, is considered to be an attractive, although unproven, alternative to the approaches that are currently in use for dealing with heavy metal contamination. While considerable effort has been directed toward identifying plants that are metal tolerant and accumulate large amounts of metal from the soil and might therefore be useful in phytoremediation, an attractive alternative might include the genetic engineering of plants in an effort to specifically increase their usefulness in phytoremediation. As a first step in this direction, we have examined the ability of

transgenic tomato plants with ACC deaminase placed under the control of three different promoters, i.e. 2x 35*S* (Christopher et al., 1987), *rolD* (Elmayan and Terfer, 1995) and PRB-1*b* (Eyal et al., 1992), to take up different metals from the environment.

In general, the expression of ACC deaminase, which is aimed both at reducing the level of stress ethylene in plants and assisting in the amelioration of some of the consequences of stress caused by toxic metals, resulted in: i) stimulation of plant growth leading to a higher total amount of metal accumulated; ii) an increase in metal uptake; iii) an increase in the shoot/root metal ratio. Based on the results of the study reported here, transgenic tomato plants with ACC deaminase controlled by the PRB-1*b* promoter appear to have greatest potential of the three constructs tested for use in phytoremediation.

Tomatoes are neither hyperaccumulators of heavy metals nor is it likely that tomatoes will be used directly in the phytoremediation of metal contaminated soil. Nevertheless, the response of transgenic tomatoes to the presence of various heavy metals suggests approaches that could be useful in engineering other plants to be more effective in the phytoremediation of heavy metals in the environment. In this regard, the transgenic tomato plants that were used in this study, originally constructed as part of an effort to develop phytopathogen tolerant cultivars, may be viewed as a model system and the approaches that were successful here should be successful with other ethylene sensitive plants including *Brassica juncea* (Indian mustard). In addition to the obvious scientific advantages to obtain commercially-significant phytoremediation performance, regulatory and public acceptance barriers are currently substantially less severe for the use of transgenic plants than they are for engineered microorganisms in the environment.

4.4. Increased tolerance of ACC deaminase transgenic plants to flooding stress

For plants, lack of oxygen in the roots is the main consequence of flooding (Kozlowski, 1984). In tomato and other plants, flooding stress causes deleterious symptoms such as epinasty, leaf chlorosis, necrosis, and reduced fruit yield. Flooding increases the synthesis of ethylene, which causes the development of these symptoms (Abeles et al., 1992). The molecular basis for the increase in ethylene production observed in flooded tomato plants is the induction of 1-aminocyclopropane-1-carboxylate (ACC) synthase, a key enzyme in ethylene biosynthesis (Olson et al., 1995). ACC, which is synthesized in roots, is transported to shoots, where it is converted to ethylene (Else and Jackson, 1998). Tomato plant roots express different ACC synthases at various times, each of which performs overlapping functions during flooding stress (Shiu et al., 1998). An anaerobiosis-responsive box has been identified in the promoter of the tomato LE-ACS3 (ACC synthase) gene (Olson et al., 1995); hybridization analysis of RNA isolated from the roots of flooded tomato plants revealed the rapid production of two specific ACC synthase transcripts which are not present in leaf mRNAs. In tomato, early flooding-induced ethylene production based on ACC delivery from root-to-shoot occurs within six hours of the onset of flooding (Bradford and Yang, 1980). The promoter of the ACC synthase gene expressed earliest after flooding, *LE-ACS7*, is normally tagged by a Sol3 transposon which may be part of the mechanism of communication between root and shoot in flooded tomato plants (Shiu et al., 1998). This ACC synthase, *LE-ACS7*, is also involved in the early wound response of tomato leaves (Shiu et al., 1998). *LE-ACS2* transcripts appear eight hours after flooding, while polyadenylated *LE-ACS3* pre-mRNA remains unspliced under anaerobic stress (Olson et al., 1995). Recently the molecular

mechanism of early induction of ACC oxidase in shoots of tomato plants was clarified as well; in ethylene signalling genetic pathway, the EIN3 protein is both DNA-binding protein and a transcriptional regulator; the tomato early ethylene-response gene *LE-ACO1* was shown to have an EIN3 binding site (Chao et al., 1997).

ACC which is the immediate precursor of ethylene in plants (Abeles et al., 1992) can be metabolized by the microbial enzyme ACC deaminase (Honma and Shimomura, 1978). Transgenic tomato plants expressing ACC deaminase may be constructed by using a variety of different promoters; however, to date all such transgenic tomato plants have utilized the 35*S* promoter (Klee et al., 1991). On the other hand, either a promoter that responds to stress (such as the PRB-1*b* promoter) or a promoter that is both root specific and anaerobically inducible (such as the *rolD* promoter) would appear to be ideal. Considering the complex and not always well understood responses of tomato plants to flooding, we employed three different ACC deaminase transgenic genotypes to study the possibility of amelioration of damage to plants caused by flooding stress.

Ethylene directs the response to flooding stress in tomato plants; hypoxia that accompanies flooding causes an increase in the synthesis of ACC (the immediate precursor of ethylene in plants) in roots, as the expression of ACC synthase genes is induced and ACC oxidation in roots is arrested (Abeles et al., 1992; Shiu et al., 1998). During flooding, the concentration of ACC in roots increases; given the high K_m that ACC deaminase has for ACC this results in a rate of cleavage of ACC that increases in parallel to the concentration of ACC. At the same time, as a consequence of an increase in the uptake by the plant of metals and radical scavengers, the oxidation of ACC to ethylene by ACC oxidase may be inhibited (Nilsen and Orcutt, 1997; Pirrung, 1998). In

this way, the conversion of ACC to α-ketobutyrate and ammonia is favored over its oxidation to ethylene despite the fact that ACC oxidase binds ACC with a higher affinity than does ACC deaminase.

The transgenic tomato plants tested in this study exhibited a higher tolerance to flooding than did the non-transformed plants. In this regard, plants expressing ACC deaminase under the control of the *rolD* promoter were the most resistant to flooding stress. The *rolD* plants display a statistically significant difference from the non-transformed plants in shoot fresh and dry weight, leaf chlorophyll content, ethylene production by petiolar tissue, and epinastic curvature (Tables 16 & 17; Figs. 25 & 28). Transgenic tomato plants that expressed ACC deaminase constitutively (i.e., 35*S* plants) were less efficient than the *rolD* plants at resisting the effects of flooding stress, perhaps as a consequence of the metabolic load caused by a relatively high level of foreign gene expression in these plants (Glick, 1995b). PRB-1*b* plants responded to flooding stress by producing a similar level of shoot growth and leaf chlorophyll content as 35*S* plants (Tables 16 & 17). However, petiolar epinasty was the most pronounced in PRB-1*b* plants compared to all other plants including the non-transformed plants (Fig. 26) and ethylene production in petioles was not affected by the ACC deaminase genotype (Fig. 28). Because the PRB-1*b* promoter can be induced by different factors at different times (Eyal et al., 1993), we assume that ACC deaminase gene transcription controlled by the PRB-1*b* promoter was insufficient to prevent the development of epinasty at the beginning of the flooding experiment. The PRB-1*b* promoter requires light and is induced by ethylene in 2-3 days after the onset of flooding (Eyal et al., 1993).

The significant differences between non-transformed and ACC deaminase transgenic tomato plants in leaf chlorophyll content following flooding (Table 17) may be partly explained by an increase in magnesium uptake from the soil, which is generally higher for all metals studied so far in transgenic plants than in non-transformed plants (Grishko, V.P., Filby, B. and Glick, B.R., submitted for publication). Interestingly, both shoot fresh and dry weight and chlorophyll content were stimulated by magnesium in *rolD* plants while 35*S*, PRB-1*b* and non-transformed plants showed a more complex response. Because the temporal curves for germination of *rolD* and 35*S* seeds are identical in the presence of Mg (Fig. 29A), these promoters apparently affect seed germination and growth in young plants in the same way. Seeds for PRB-1*b* tomato plants germinate very slowly so that they are not at all comparable to the other transgenic tomato seeds used in this study.

ACC deaminase activity results in production of ammonium ion and α-ketobutyrate and, hence, leucine and isoleucine (Honma and Shimomura, 1978). Tomato plants are adapted to relatively high-light conditions and may readily reduce nitrate to ammonium in the chloroplast utilizing photosynthetically produced energy (Nilsen and Orcutt, 1997). In shoots, ammonium ion accumulation could result in an increase in pH and a shift to reducing conditions that may change a plant's sensitivity to ethylene at the receptor level.

The compartmentalization within a plant of the enzymes that are involved in stress ethylene biosynthesis (i.e., ACC synthase in roots and ACC oxidase in shoots) is most compatible with the manipulation of plant ethylene levels using the root-specific *rolD* promoter to control ACC deaminase gene expression. In this way, much of the ACC

that is overproduced in roots in response to flooding stress is cleaved before it can be transported to the shoots where it would otherwise be converted to ethylene. Thus, while all three of the promoters tested offered some degree of protection against the effects of flooding stress, the *rolD* promoter is preferred in this instance.

4.5. Amelioration of flooding stress by FNR transcriptionally regulated ACC deaminase containing plant growth-promoting bacteria

Seeds of non-transformed tomato plants *Lycopersicon esculentum* (Solanaceae) cv. Heinz 902 were inoculated either with *Enterobacter cloacae* UW4, *E. cloacae* CAL2, *Pseudomonas putida* ATCC17399/pRKACC or *P. putida* ATCC17399/pRK415, the first three of these bacterial strains carrying and expressing the gene for ACC deaminase. When they were 55 days old, tomato plants were flooded for nine consequtive days before a number of physiological and biochemical parameters were assessed. Characteristics that were observed include root and shoot growth, epinastic curvature in leaf petioles, ACC deaminase activity, ethylene production, and leaf chlorophyll concentration. Tomato plants that were grown from seeds bacterized with organisms expressing ACC deaminase showed a substantial tolerance to flooding stress. Plant growth-promoting bacteria are free-living soil bacteria that can facilitate plant growth and development in two different ways: indirectly or directly (Glick, 1995a; Glick et al., 1999). The indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism (usually a fungus) by any one or more of several different mechanisms (Glick and Bashan, 1997). On the other hand, the direct promotion of plant growth by plant growth-promoting bacteria

generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment. The major direct mechanisms of plant growth promotion include: the solublization of phosphorus, nitrogen fixation, sequestering of iron from the soil by siderophores, production of phytohormones such as auxin, cytokinin, and gibberelin, and the enzymatic lowering of plant ethylene concentrations (Glick, 1995a; Glick et al., 1999). Relatively recently it was discovered that a number of plant growth-promoting bacteria contain the ACC deaminase (Jacobson et al., 1994; Glick et al., 1994; Shah et al., 1997; Glick et al., 1998; Shah et al., 1998) and that this enzyme can cleave the plant ethylene precursor ACC (Yang and Hoffman, 1984; Abeles et al., 1992), and thereby lower the level of ethylene in a developing or stressed plant. When plant growth-promoting bacteria that contain the enzyme ACC deaminase are bound to the seed coat of a developing seedling, they may act as a mechanism for insuring that the ethylene level does not become elevated to the point where root growth is impaired. By facilitating the formation of longer roots, these bacteria may enhance the survival of some seedlings, especially during the first few days after the seeds are planted (Glick et al., 1998). Moreover, ACC deaminase-containing plant growth-promoting bacteria can, in some instances, protect a plant from the deleterious effects of environmental stress (Glick et al., 1997; Burd et al., 1998). In flooding, ACC, which is synthesized in roots, is transported to plant shoots where it is converted to ethylene by ACC oxidase (Bradford and Yang, 1980; Else and Jackson, 1998). The molecular basis for the increase in ethylene production observed in shoots of flooded tomato plants is an increase in the activity of both ACC synthases in the submerged roots and ACC oxidase in shoots (Olson et al., 1995; Chao et al., 1997; Shiu et al., 1998). Since the production

of ethylene in the shoots of flooded tomato plants is responsible for the phenotype that is observed following flooding (Jackson, 1997), tomato seeds were inoculated with ACC deaminase-containing plant growth-promoting bacteria in an attempt to decrease the concentration of flooding stress ethylene. It was reasoned that these bacteria might act as a sink for ACC, thereby ameliorating some of the damage to plants caused by flooding (Kozlowski, 1984; Nilsen and Orcutt, 1997).

The rationale of the approach that was taken in this study is based on lowering the amount of ACC available for oxidation to ethylene in the shoots of flooded tomato plants by the bacterization of seeds and the subsequent colonization of roots by plant growth-promoting bacteria expressing an ACC deaminase gene, which is transcriptionally controlled by an anaerobically regulated promoter.

As a consequence of flooding, ACC synthase genes are induced in the roots of tomato plants while ACC oxidation (to ethylene by ACC oxidase) is arrested because of root hypoxia (Abeles et al., 1992; Shiu et al., 1998). ACC is therefore transported by the xylem to shoots (Jackson, 1997) where it is oxidized to ethylene. As a part of their response to the flooding stress, plants exude ACC into the surrounding water (Fig. 30) which may stimulate the proliferation of facultative anaerobes with ACC deaminase activity.

To a first approximation, the results observed in this study agree well with the above mentioned predictions. Tomato plants treated with ACC deaminase-containing bacteria exhibited a much higher tolerance to flooding than did either non-bacterized control plants or plants treated with bacteria that do not contain ACC deaminase activity. Protection against flooding stress which was achieved as a result of the treatment of seeds

with ACC deaminase-containing bacteria resulted in statistically significant differences in overall plant growth, leaf chlorophyll content and substantially decreased ethylene production in leaf petiolar tissue (Tables 19 & 20; Fig. 34).

Contrary to expectations, petiolar epinasty was lowest in plants grown from seeds inoculated with *P. putida*/pRK415 which was the only bacterial strain tested that did not show any ACC deaminase activity compared to all other strains (Fig. 33). This observation might be explained as follows: colonization of plant roots with the bacteria containing ACC deaminase activity may result not only in the lowering of ACC levels in plant tissues (Penrose et al., submitted for publication) but also in a change in a plant's sensitivity to ethylene at the receptor level. More than 80% of plant growth-promoting bacteria, as well as other free-living soil bacteria, produce indoleacetic acid (Patten and Glick, 1996), in addition to other compounds that can moderate plant growth responses (Glick, 1995a; Glick et al., 1999). It is possible that some of these compounds may alter the concentration of functional ethylene receptors or change the stability of the ethylene-metal bond through the ligand trans-effect. Since the native promoter of (at least two different) ACC deaminase genes needs at least 10-12 h for induction (Honma and Shimomura, 1978; Jacobson et al., 1994) it is likely that the effect of bacterial ACC deaminase activity on treated plants can not be seen until several days after the stress.

The surprising results with epinastic curvature notwithstanding, the compartmentalization of the enzymes involved in stress ethylene biosynthesis within a plant allows for the efficient lowering of the stress ethylene level that results from flooding using root-associated bacteria expressing ACC deaminase activity

transcriptionally controlled by anaerobically-regulated promoters such as FNR (Guest, 1992).

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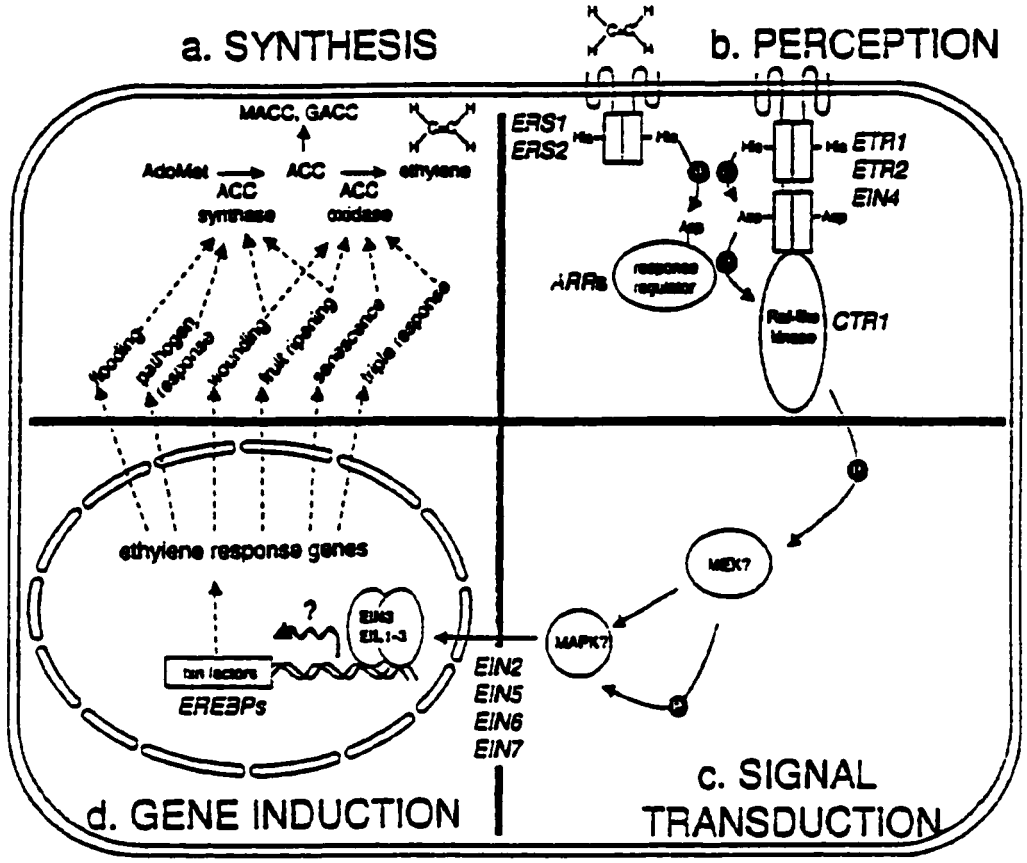
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6. APPENDIX

The ethylene response circuit. The figure was adapted from Johnson and Ecker (1998).



The ethylene response circuit. (*a*) The first committed step in ethylene biosynthesis is conversion of S-adenosyl methionine to ACC by the enzyme ACC synthase. ACC is efficiently converted to ethylene by ACC oxidase. Both enzymes are encoded by multigene families whose members are differentially expressed upon external stimuli such as flooding, pathogenic infection, or wounding, as well as internal stimuli such as seedling triple response, fruit ripening, and senescence. Some of these processes act through other hormones, such as auxin or cytokinin. (*b*) Ethylene binds to membrane spanning regions in the sensor domain of receptors. Presumably, ligand binding inhibits a kinase cascade, originated by autophosphorylation of a histidine residue in the histidine kinase domain. The phosphate group can be transferred to an aspartate residue in the receiver domain of the receptor, or it might be shuttled to an aspartate residue in a separate protein, the response regulator. Receptor activity regulates the CTR1 kinase. (*c*) CTR1 is most similar to Raf, a kinase whose substrate is MEK, which modifies MAPKs. Multiple MEKs and MAPKs exist in plants, but none has been directly implicated in ethylene signaling. Epistasis analysis places EIN2 downstream of CTR1, so it is situated after MAPK to preserve the canonical kinase cascade. EIN5, EIN6, and EIN7 also act after CTR1, but their relationship to EIN2 is not yet apparent. (*d*) EIN3 activity is induced by the hormone and is necessary and sufficient for eliciting ethylene responses, presumably through the activation of transcription factors such as the EREBPs. EREBPs induce specific subsets of genes that contribute to the phenotypic responses elicited by the stimuli in *Panel a*.