J Plant Growth Regul (2005) 24:142–152
DOI: 10.1007/s00344-004-0007-5

J P G R
Journal of Plant Growth Regulation

Transgenic Tomato Plants with a Modified Ability to Synthesize Indole-3-acetyl-β-1-*O-D* -glucose

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Abstract

Esterification of indole-3-acetic acid (IAA) is thought to be an important component in the homeostatic regulation of the levels of this phytohormone. To better understand the role of the initial step in IAA esterification in the control of IAA levels, transgenic tomato plants were generated that either express maize *IAGLU* or have reduced levels of the enzyme IAA-glucose synthetase. These plants were obtained by expressing maize *IAGLU* in either sense or antisense orientation using the CaMV35S promoter. The maize *IAGLU* probe hybridized to two transcripts (1.3 kb and 2.5 kb) in wild-type tomato vegetative tissue and green fruit. The sense and antisense transformants exhibited distinct phenotypic characteristics. Sense transformants showed an almost complete lack of root initiation and development. Antisense transgenic plants, on the other hand, had unusually well developed root systems at early stages in development, and the amount of the endogenous 75 kDa *IAGLU* protein was reduced. *IAGLU* antisense plants also had reduced levels of IAA-glucose and lower esterified IAA.

Key words: Auxin conjugation; Glucosyltransferase; Indoleacetyl-glucose; *Lycopersicon esculentum*; Phytohormone; Transgenic tomato

Introduction

The plant hormone indole-3-acetic acid (IAA) is a natural auxin that controls diverse plant processes including shoot gravitropism, maintenance of apical dominance, differentiation of vascular tissue, cell wall extensibility, cell division, and fruit ripening (Normanly 1997). A large proportion of IAA in plants can be covalently linked to other compounds to form IAA conjugates (Cohen and Bandurski 1982). Auxins applied to plant tissues are rapidly conjugated and, in some cases, the conjugating enzymes appear to be induced by exogenous auxins (Venis 1972; Kowalczyk and others 1997; Staswick and others 2005). IAA-conjugate formation and the resultant ratio of free to conjugated forms is also thought to be tissue specific and developmentally regulated (Kleczkowski and Schell 1995; Ribnicky and others 2002). Conjugated forms of IAA play a role in hormone transport,

Received: 3 November 2004; accepted: 9 May 2005; online publication: 30 September 2005.
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in protection from peroxidation, and in storage of the hormone as an inactive form (Bartel 1997). In addition, IAA conjugates have been shown to be intermediates in IAA catabolism (Tsurumi and Wada 1990; Tuominen and others 1994; summarized in Woodward and Bartel 2005).

The free IAA level in cells is controlled by a series of bidirectional routes or ''metabolic shunts'' (Normanly and others 1995) involving hormone synthesis, degradation, transport, and conjugation. Viewed in this way, conjugation is an integral component of a sensitive mechanism to regulate the net level of free IAA in the cell (Normanly and Bartel 1999). Targeted changes in the genes involved in the IAA conjugation pathway thus provide the means to manipulate auxin metabolism and therefore the available levels of free IAA. Plants containing such genetic modifications should have a modified capacity for IAA regulation and would serve as valuable tools with which to study the metabolic pathways. Genetic approaches to modifying auxin metabolism offer several advantages over the traditional experimental methods for changing IAA levels, which require hormone application and are limited by uncertainties such as rates of entry, stability, dosage and timing of application (Bartel 1997). Previous work in which a bacterial gene for production of the conjugate IAA-ε-amino-L-lysine, *iaaL*, was used to alter IAA levels showed the general feasibility of this approach (Romano and others 1991), and additional molecular tools are becoming available with which it may be possible to alter IAA levels. Examples include IAA-amino acid conjugate hydrolase genes from *Arabidopsis* (Bartel and Fink 1995; Bartel 1997) or from bacteria (Chou and others 1998), and genes encoding enzymes responsible for the primary step in IAA esterification from maize (Szerszen and others 1994) and *Arabidopsis* (Jackson and others 2001).

We have targeted for genetic manipulation the indole-3-acetyl-β-1-*0*-D-glucose (IAA-Glc) synthase gene (*IAGLU*), the first cloned plant gene for an enzyme involved in IAA conjugate metabolism (Szerszen and others 1994). This gene encodes the enzyme that catalyzes the first reaction in the IAA conjugation pathway leading to the synthesis of IAA-Glc from UDP-glucose and IAA (Michalczuk and Bandurski 1982; Leznicki and Bandurski 1988a, 1988b). In addition to maize, tomato and other plants are also known to actively conjugate applied IAA to form IAA-Glc (Cohen and Bandurski 1982; Catalá and others 1992; Slovin and Cohen 1993). We show in this report that wild-ype tomato contains two transcripts that hybridize to maize *IAGLU*. We therefore used the maize gene to engineer tomato plants to either over-express *IAGLU* or to express the *IAGLU* in an antisense orientation. Antisense plants were analyzed for phenotype and gene expression, as well as for free IAA and IAA conjugate levels.

Materials and Methods

Plant Material and Growth Conditions

Tomato (*Lycopersicon esculentum* cv Ailsa craig) plants were grown in large clay pots with Jiffy-mix (Jiffy Products of America, Inc, Batavia, IL). The plants were grown and maintained in the greenhouse under ambient lighting supplemented with high-pressure sodium lamps. Plants were watered daily, fertilized once a week with Peters 20:20:20 plus micronutrients (Spectrum Group, United Industries, St. Louis, MO), and dolomitic lime was applied (approximately 100 g/plant) once a month. Leaves were harvested from greenhouse-grown plants at least 30 days post-germination or from rooted cuttings at least 20 days after removal from the mist bench. To maintain a population of clonal plants, 15–20 cm cuttings were taken from mature plants and rooted on a mist bench. Young leaves were less than 50% of their fully expanded size when harvested, and mature leaves were at greater than 90 % of their fully expanded size. Young shoots consisted of the terminal 3.0 cm of vegetative shoots.

Seedling Liquid Culture

Tomato seeds were surface sterilized for 10 min in a solution of 0.5% sodium hypochlorite containing 400 μl/l Tween 80, rinsed with 5–6 changes of sterile water, and placed in a 125-ml Erlenmeyer flask containing 25 ml of sterile Murashige and Skoog salts medium (Life Technologies, Gaithersburg, MD) with 1% sucrose (Murashige and Skoog 1962). The seedlings were grown at room temperature (25° ± 3°C) for 10 days under cool white fluorescent lights (15 $\mu mol \cdot m^{-2} \cdot s^{-1}$) on an orbital shaker at 100 rpm. For root length analysis, seeds were germinated and grown for 4, 6, 8, or 10 days, harvested, and photographed, after which root lengths were measured. For all other studies, seedlings were harvested after 10 days.

Plasmid Constructs

The pBI121 vector (Clonetech, Palo Alto, CA) was modified to facilitate expression of *IAGLU* cDNA from a CaMV 35S promoter by deleting the GUS gene. The plasmid was first digested with *Sma*I followed by *Sst*I to release the GUS gene, then the

3′ overhang generated by *Sst*I was filled by T4 DNA polymerase. The vector was then re-ligated to yield plasmid vector pBI121ΔGUS.

Subcloning the IAGLU cDNA. The full-length maize *IAGLU* cDNA had been cloned (Szerszen and others 1994) in the *Eco*RI site of pBluescript SK- (Stratagene, La Jolla, CA). To obtain the *IAGLU*-containing fragment, the plasmid DNA was digested with *Eco*RI. The end of the *IAGLU*-containing fragment was blunted with Klenow (New England Biolabs, Beverly, MA) and ligated into a filled in *Xba*I site of pBI121ΔGUS, regenerating the *Eco*R1 site. Clones with *IAGLU* in either sense (pBI121ΔGUSS*IAGLU*) or antisense (pBI121ΔGUSAS*IAGLU*) orientations relative to the CaMV35S promoter were isolated as confirmed both by restriction mapping and partial DNA sequencing.

The constructs were used to transform *Agrobacterium tumefaciens* strain LBA4404 (Life Technologies) by electroporation, under conditions recommended by the supplier (Life Technologies).

Plant Transformation

Agrobacterium tumefaciens LBA4404 carrying either pBI121ΔGUSS*IAGLU* or pBI121ΔGUSAS*IAGLU*, or the vector alone, were co-cultivated with tomato cotyledons essentially according to the method of McCormick and others (1986). Briefly, seeds were germinated aseptically on Murashige and Skoog medium (Murashige and Skoog 1962). Cotyledons from 10–12-day seedlings were co-cultivated with the *Agrobacterium* cultures for 30 min with gentle shaking at room temperature. The transformants were plated on carbencillin (500 mg/l) and selected with kanamycin (100 mg/l). After 3–4 weeks, the kanamycin-resistant shoots were transferred to shoot elongation medium containing zeatin (0.1 mg/l). Shoots that had not formed roots were transferred to rooting medium. After a further 3–6 weeks, rooted plants were transferred to soil and grown in a green house. Individual T_0 plants representing independent transformation events (that is, derived from different cotyledons) were allowed to self-pollinate. The resulting seeds were germinated on a medium containing kanamycin (50 mg/l) and kanamycin-resistant seedlings selected for further analysis.

DNA Isolation and Hybridization

Total DNA was isolated from young shoot tips and leaves by the method of Dellaporta and others (1983). Total DNA (20 μg) was digested with *Eco*R1 or *Xba*1 (New England Biolabs, Beverly, MA), separated by electrophoresis on a 0.8% agarose gel and transferred by Southern blotting to a nylon membrane (DuPont NEN, Boston, MA) as described by Sambrook and others (1989). The pBluescript SK- plasmid containing maize *IAGLU* was digested with *Eco*RI, and the 1731-bp fragment encoding the full length gene was purified with a Geneclean II kit (BIO 101 Inc., La Jolla, CA) after agarose gel electrophoesis. An *IAGLU*-specific radioactive probe was generated by labeling with [^{32}P]dCTP using the Oligo labeling kit (Pharmacia, Piscataway, NJ). Hybridization was carried out at 55°C for 3 h with QuickHyb (Stratagene). The blots were washed with 0.2 × SSC (sodium chloride (150 mM)/sodium citrate (15 mM), pH 7.0), and 0.1% SDS at 50°C, then exposed to X-ray film (Action Scientific, Carolina Beach, NC).

RNA Isolation and Hybridization

To study *IAGLU* expression in wild-type plants, total RNA was isolated by the hot phenol method (Meier and others 1993) from 10-day-old seedlings, young shoot tissue (3-cm sections cut from the tips of rapidly growing shoots of greenhouse grown plants), young leaves, whole flowers, immature green fruit pericarp, mature green fruit pericarp, breaker stage fruit pericarp, and red ripe fruit pericarp. For studies of *IAGLU* expression in the transgenic plants, total RNA from young shoot tissue, young leaves, and fruit was isolated using Trizol (Life Technologies) or the hot phenol method. Total RNA (10 μg) was separated by electrophoresis on a 1.2% formaldehyde agarose gel and transferred to Hybond N (Amersham, Arlington Heights, IL) membranes as described by Sambrook and others (1989). RNA gel blots were hybridized at 65°C for 1 h using QuickHyb (Stratagene) containing 200 μg/ml of tRNA. Radiolabeled *in vitro* transcribed RNA probe was added at 1×10^6 cpm/ml of hybridization solution. Blots were washed with 0.1 × SSPE (sodium chloride (150 mM)/sodium phosphate (10 mM)/disodium EDTA (1 mM), pH 7.4), and 0.1% SDS at 60°C before autoradiography. After hybridization, radioactivity on the blot was imaged using a 4-h scan time with an AMBIS 4000 radioimaging system (Scanalytics, Inc., Billerica, MA). The radioactive bands were quantified and the data plotted as a percentage of total counts.

In Vitro Transcription

Strand-specific probes were synthesized with an *in vitro* transcription kit, mMessage (Ambion, Austin, TX). Plasmid pBS (SK-) containing the full-length *IAGLU* cDNA was linearized with *Hind*III and transcribed with T3 RNA polymerase to generate a sense strand–specific probe. For an antisense

strand–specific probe, the plasmid was digested with *Bam*HI and transcribed using T7 RNA polymerase.

Protein Extraction and Immunoblotting

Seedling tissue was homogenized on ice in a buffer containing 0.5M Tris-HCl pH 7.8, 1.0% v/v β-mercaptoethanol, and 1 mM PMSF. Cell debris was eliminated by centrifugation at 13,000 × g for 10 min at 4°C. Protein concentrations were determined with a micro Bradford assay (Bradford 1976). The samples were mixed with NuPAGE sample buffer (Novex, San Diego, CA), denatured at 70°C for 15 min, and separated on a 4%–12% NuPAGE polyacrylamide gel (Novex). The gel was blotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) according to the method of Towbin and others (1979). To confirm uniform loading and transfer, membranes were reversibly stained with BLOT-faststain (Gene Technology Inc., St. Louis, MO). The membrane was blocked in PBS and 5% fat free dry milk, then incubated with *IAGLU* peptide antibody (1:2000 dilution), followed by a 1:5000 dilution of the secondary antibody, anti-rabbit alkaline phosphatase conjugate (Sigma, St. Louis, MO). The cross-reacting bands were detected with the Western-star Chemiluminescent detection system (Tropix, Bedford, MA).

A multiple antigenic peptide antibody to the IAGLU protein was raised in rabbit to the peptide sequence EAASAARKAAGEWRDRARAAV (Research Genetics, Inc., Huntsville, AL). The peptide sequence consisted of amino acid residues 427–447 from maize IAGLU. No cross-reacting bands were detected with the preimmune serum. Proteins recognized on immuno-blots using this peptide antibody were the same as those recognized by two IAGLU antibodies provided by Dr. M. Kowalczyk (Umeå, Sweden): a polyclonal antibody prepared to the purified maize protein; and a polyclonal antibody prepared to the recombinant maize protein expressed in *Escherichia coli.*

Preparation of Isotope-labeled IAA-Glc Internal Standard

The liquid endosperm of sweet corn (*Zea mays* L. cv. ''Silver Queen,'' obtained from a local market at the fully developed table-ready stage, approximately 25–30 days after pollination) was collected by cutting the rows of kernels with a razor blade and pressing out the kernel contents against the rim of a beaker chilled on ice. The liquid endosperm tissue was then squeezed through two layers of cheesecloth, and the resulting endosperm suspension was frozen immediately after collection in liquid nitrogen and stored at –80°C for later use. To obtain the crude enzyme preparation, 60 g of the frozen maize liquid endosperm was thawed on ice. Next, 50 ml of 50 mM Tris buffer, pH 7.6, and $(NH_4)_2SO_4$ to 85% saturation (610 g/l) were added while stirring, with pH adjustment as required to maintain pH 7.6. The preparation was centrifuged at 10,000 × g for 10 min and the resulting pellet suspended in 10 mM Tris buffer, pH 7.1. The sample was dialyzed (12,000–14,000 M.W. cut-off, Spectra/Por 4, The Spectrum Co., Gardena, CA) against water overnight with constant stirring at 3°C. Following dialysis, the dialysis bag was placed in a dry beaker and covered with anhydrous flake polyethylene glycol (M.W. 20,000, Aquacide III, Calbiochem, La Jolla, CA) for 5 h. The concentrated solution was centrifuged at 10,000 × g for 10 min, and the supernatant, which contained the IAA-Glc synthetase enzyme activity, was used to produce the required standard (Michalczuk and Chisnell 1982). The reaction was carried out by mixing 0.5 ml of the enzyme preparation, 0.3 ml of buffer (50 mM Tris, 8.3 mM $MgCl_2$, and 8.3 mM glutathione, pH 7.6), 10 mg UDP-Glc (Sigma, St. Louis, MO), 100 μg [$^{13}C_6$]-indole-3-acetic acid, and 25 μCi of [^{3}H]-indoleacetic acid. The reaction was incubated for 24 h at 35°C; then 2 ml of isopropanol was added to stop the reaction. The reaction product was purified on a 1 × 30 cm column of Sephadex LH-20, equilibated and run in 50% isopropanol/water. The concentration of IAA-Glc was determined by hydrolyzing an aliquot of [$^{13}C_6$/^{3}H]IAA-Glc (estimated to be 500 ng) in the presence of 250 ng of IAA in 4 ml of 1 N NaOH for 1 h at room temperature. After hydrolysis, the sample was brought to pH 2.5 and purified by C_{18}-HPLC, methylated, and analyzed by GC-MS in the selected ion mode (Tam and others 1998). The ion intensities at m/z 136 and 130 were used to calculate the amount of [$^{13}C_6$/^{3}H]IAA-Glc relative to the peak of unlabeled IAA standard.

IAA-Glc Analysis

Levels of IAA-Glc in tomato tissues were determined by isotope dilution analysis using the [$^{13}C_6$/^{3}H]IAA-Glc as the internal standard. Tomato seedlings were obtained by growing wild-type, antisense, and vector control seedlings in sterile liquid culture. The tomato seedlings (10–20 g) were frozen in liquid nitrogen, ground in a liquid nitrogen chilled mortar and pestle, and extracted with 60% isopropanol/200 mM imidazole buffer, pH 7.0, containing 200 ng [$^{13}C_6$/^{3}H]-IAA-Glc. The extract was purified with ethyl acetate partitioning at pH 2.5 to remove lipids and organic

acids, followed by chromatography on a 1.5 × 45 cm column of Sephadex LH-20 (Pharmacia) equilibrated and run with 50% isopropanol/water at approximately 1 ml/min. IAA-Glc eluted between 46 and 52 ml. The sample was then adjusted to pH 7 with NaOH, brought to dryness *in vacuo*, and acylated with a 1:1 mixture of [1% dimethylaminopyridine in prydine] and acetic anhydride at 60°C for 1 h (Chisnell 1984). The solvents were removed *in vacuo*, and the acylated IAA-Glc was redissolved in 200 µl of 10% acetonitrile/water. The sample was purified by C_{18} HPLC on a 25 cm × 4.6 mm UltraSphere column (Beckman, Berkeley, CA) with a programmed linear gradient of 10%–100% acetonitrile/water over a 1-h period with a flow rate of 1 ml/min. Under these conditions, the acylated IAA-Glc had a retention volume of 44–52 ml. The acylated IAA-Glc fractions were pooled, reduced to dryness, and resuspended in 25 µl ethyl acetate for GC-MS analysis on a Hewlett Packard 5973 system (Hewlett Packard, Wilmington, DE), using the selected ion mode and monitoring ions at m/z 130, 136, 505, and 511. Gas chromatography conditions were: HP-5 column (30 m, 0.25 mm i.d., 0.25 µm film thickness, Hewlett Packard) using He as carrier gas at 1 ml/min; the injector temperature was 280°C, and the column was initially equilibrated at 70°C for 2 min, followed by a temperature program of 20°C/min to 280°C and followed by 5 min at 280°C. Under these conditions the acylated IAA-Glc had a retention time of 10.1 min.

Free, free plus ester, and total IAA was determined using [$^{13}C_6$]IAA as internal standard for GC-MS analysis as described by Cohen and others (1986). Free IAA was purified from 0.1 g (fresh weight) of 10 day-old seedling tissue after extraction with 60% isopropanol/200 mM imidazole buffer, pH 7.0. The extract was reduced to the water phase *in vacuo*, pH adjusted with HCl to 2.5, and partitioned against ethyl acetate. After evaporation *in vacuo* and resuspension of the sample in 100 µl 50% methanol/water, HPLC was carried out on a 50 × 4.6 mm Phenomenex (Torrance, CA) Ultracarb 305 µ ODS column. The samples were eluted isocratically at 1 ml/min using a mobile phase of 25% methanol/water containing 1% acetic acid (v/v). The IAA fraction was methylated with ethereal diazomethane and analyzed by selected ion monitoring GC-MS (Tam and others 1998). In recognition of the potential for problems with IAA-glucose hydrolysis resulting in overestimates of free IAA levels (Baldi and others 1989), we developed extraction procedures that minimize inadvertent hydrolysis (Ludwig-Mueller and others 2005). In particular, the extraction time was short and the extraction solvent was buffered. In addition, IAA and IAA-glucose were separated early in the purification procedure. Using the ethyl acetate partitioning method, we found less than 5% of added radioactivity from IAA-glucose tracer was found in the ethyl acetate phase. The majority of these radioactive counts were extracted back into neutral buffer upon two subsequent partitioning steps, indicating that these counts were from IAA-glucose, consistent with what we previously reported for IAA-glucose stability and partitioning during short-term analyses at pH 7.0 (Baldi and others 1989). Measured hydrolysis was consistently well below the biological variation noted and thus should not be considered a significant source of error in these studies. Hydrolysis of IAA-glucose during the extraction and purification procedure for IAA-glucose would not have altered the quantitative data because of the use of the [$^{13}C_6$]-labeled internal standard.

Total IAA was determined essentially by the method of Chen and others (1988). The extract was hydrolyzed with 7 N NaOH at 100°C for 3 h with oxygen free nitrogen (Bialek and Cohen 1989). IAA was purified on a 3 ml J. T. Baker (Phillipsburg, NJ) C_{18} solid phase extraction column followed by HPLC prior to analysis by selected ion monitoring GC-MS. Free plus ester IAA values were obtained in the same way, except that hydrolysis was performed with 1 N NaOH for 1 h at room temperature (Cohen and others 1986).

Results

Generation and Analysis of CaMV35S*IAGLU* Transgenic Plants

Although kanamycin-resistant shoots developed on tomato cotyledons co-cultivated with *Agrobacterium* containing the maize *IAGLU* sense construct, these shoots failed to establish roots and therefore could not be propagated. Several attempts were made to induce rooting, including treating the shoots with commercially available rooting powder (0.3% IBA on talc, Hormex rooting powder No. 3, Brooker Chemical, North Hollywood, CA); transferring the shoots to media supplemented with varying concentrations of IBA (0.1 mg/l to 10 mg/l) as well as combinations of IBA and NAA (NAA concentrations of 0.1 mg/l to 1 mg/l); and transferring the shoots to medium supplemented with indole-3-butyryl-N-L-phenylalanine (IBA-phe) (2mg/l) and indole-3-butyryl-N-L-alanine (IBA-ala) (2 mg/l), conjugates of IBA. However, no rooting was observed under any of these conditions, and attempts to graft the sense shoots to wild-type plants were also unsuccessful.

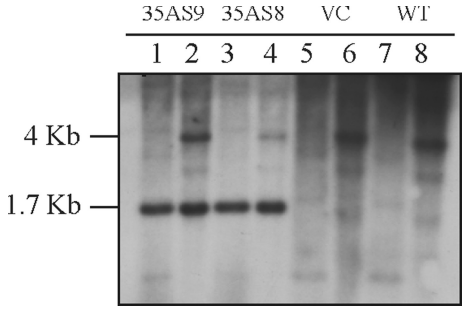

Figure 1. Southern blot analysis of total DNA from young leaves harvested from untransformed, vector control and transformed plants 35AS8 and 35AS9. DNA was digested with *Eco*R1 (lanes 2, 4, 6, and 8) and *Xba*1 (lanes 1, 3, 5, and 7) and hybridized with the full-length maize *IAGLU* cDNA as described in *Materials and Methods*.

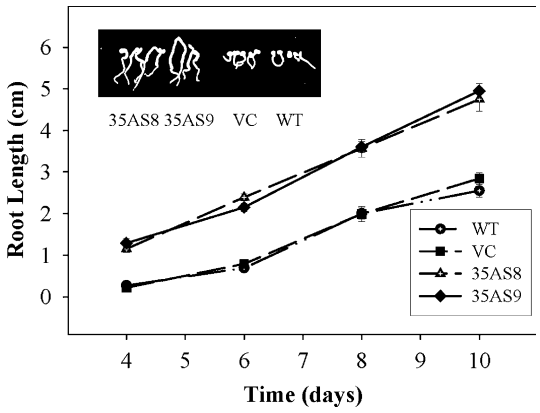

Figure 2. Root length was measured on tomato seedlings grown in liquid culture from WT, non-transformed Alisa craig seedlings; VC, vector control T_2 generation seedlings, as well as T_2 generation seedlings from antisense transformants 35AS8 and 35AS9 from day 4 to 10 days in culture. Representative seedlings at day 6 are shown in the insert. Data are the mean from 12 seedlings harvested at each time point ± SE.

In contrast, transgenic plants expressing *IAGLU* in the antisense orientation developed numerous well-formed roots in the medium routinely used for initial shoot formation that contains high cytokinin (zeatin, 1 mg/l). Transgenic shoots carrying the vector alone did not develop roots on this medium but developed normal roots when transferred to medium containing lower cytokinin (zeatin, 0.1 mg/l). Two lines transformed with the antisense construct, and shown by slot blot analysis to contain the *NPT*II gene, were designated 35AS8 and 35AS9 and used for further analysis.

Gene copy reconstruction analysis of 35AS8 and 35AS9 DNA, using maize *IAGLU* as the probe, confirmed that the two lines contain from 1 to 5 copies of the transgene. Under moderately stringent hybridization and wash conditions, a 4 kb *Eco*R1 fragment (lanes 2, 4, 6, 8) could be seen in DNA from transgenic plants, vector controls, and the wild-type plants, and probably represents tomato *IAGLU* (Figure 1). An additional *Xba*1 or *Eco*R1 band of about 1.7 Kb, was seen only in the antisense lines (lanes 1–4).

35AS8 and 35AS9 plants grown to maturity in the greenhouse were indistinguishable from the wild-type and vector control plants. Fruit set as well as ripening also appeared to be unaffected by the presence of the antisense transgene. In contrast, 6-day-old seedlings of 35AS8 and 35AS9 grown in liquid culture exhibited increased root growth as compared to wild-type and vector control seedlings (Figure 2). The average root length of the two transgenic lines was 31 mm and 32 mm, whereas that of the wild-type and vector control was 14 mm and 15 mm, respectively (Figure 2).

Analysis of *IAGLU* Expression in Transgenic Plants

On total RNA blots from Ailsa craig (wild-type) vegetative tissue and green fruit, the maize *IAGLU* probe hybridized under stringent conditions to two transcripts (1.3 kb and 2.5 kb), but hybridized to only a 1.3-kb band on total RNA blots from pericarp after the breaker stage (Figure 3). Expression of the antisense CaMV35S*IAGLU* gene in transgenic plants was also analyzed on RNA blots (Figure 4). With the *IAGLU* sense riboprobe, antisense transcripts of approximately 1.8 kb were detected in leaves of 35AS8 and 35AS9 plants (Figure 4A). Wild-type plants and plants transformed with vector alone did not show any transcripts hybridizing with this probe (Figure 4A). When the blot was re-probed with the antisense riboprobe, which detects endogenous tomato *IAGLU* transcripts, the hybridization patterns were the same for 35AS8, 35AS9, vector control, and wild-type plants (Figure 4A). Quantification of the hybridizing bands from three separate experiments showed no significant differences between antisense transformants and control plants (Figure 4B).

IAGLU protein accumulation was analyzed by immunoblotting. Total protein from young shoots of greenhouse-grown plants was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblots were analyzed with the maize IAGLU peptide antibody. Two cross-reacting proteins, at 42 kDa and 75 kDa, were

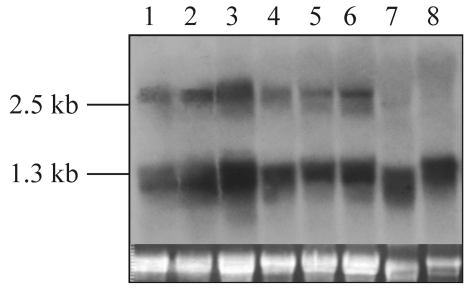

Figure 3. Total RNA (10 μg) from 10 day old wild type Alisa craig seedlings (lane 1); young shoots (lane 2); young expanding leaf tissue (lane 3); whole flowers (lane 4); immature green fruit pericarp (lane 5); mature green fruit pericarp (lane 6); breaker stage fruit pericarp (lane 7); and red ripe fruit pericarp (lane 8) was analyzed on RNA blots probed with a full length maize *IAGLU* riboprobe (top). The lower panel shows the gel stained with ethidium bromide to show RNA loading.

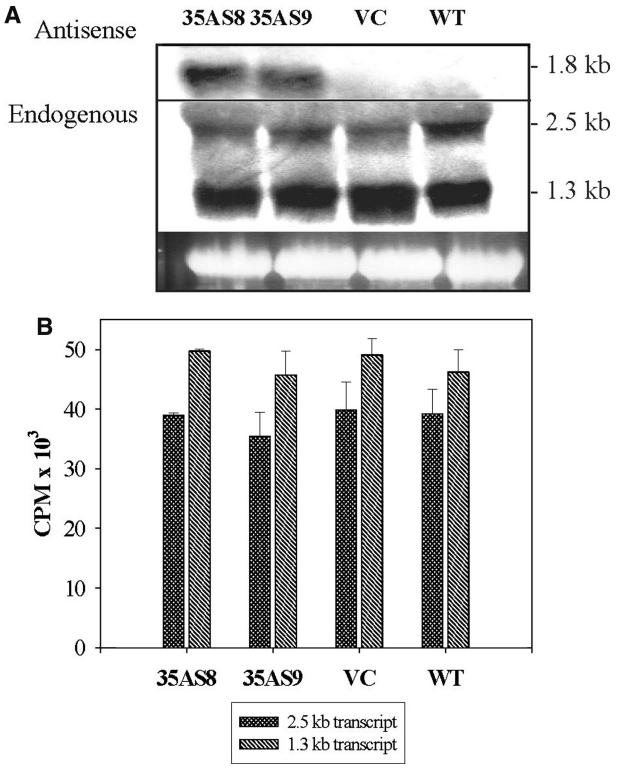

Figure 4. RNA blot analysis was used to assess expression of endogenous tomato *IAGLU* and the antisense *IAGLU* construct in leaves of wild type (WT), vector control (VC), and antisense transformed plants (35AS8, 35AS9). Total RNA (10 μg) was loaded on each lane and the blot was probed with a sense strand riboprobe (A, Antisense). Approximately equal amounts of RNA were loaded in each lane, as confirmed by ethidium bromide staining of the gel (A, Endogenous). The blot was re-probed with an antisense riboprobe and the hybridizing endogenous transcripts in the vegetative tissue were quantified using an AMBIS radioactivity imaging system (B). Bars indicate the mean ± SE from analysis of three independent gels.

detected in seedlings of wild-type and vector control plants (Figure 5). In the antisense transgenic plants, the cross-reacting protein at 75 kD was conspicuously absent, whereas there was little or no difference in the level of the 42 kDa protein in the transgenic lines when compared to wild-type and vector control.

Auxin Levels in Seedlings

To determine the effect of *IAGLU* antisense expression on IAA metabolism, the steady-state levels of free, ester, and amide-linked IAA were determined in 35AS8, 35AS9, wild-type, and vector control seedlings (Figure 6, insert). Total IAA levels were not significantly different from wild-type in vector control and 35AS8, but they appear to be slightly decreased in 35AS9. This decrease is reflected in the levels of free IAA. Levels of amide-linked IAA are essentially unchanged across all lines. However, the levels of ester IAA were consistently lower in both antisense lines measured relative to wild-type and vector control lines.

The product of the gene, IAA-glc, constitutes a part of the ester IAA pool. To specifically determine the effect of antisense expression on IAA-Glc levels, we synthesized a [$^{13}C_6$]-labeled standard and used it for isotope dilution analysis of the steady-state levels of IAA-Glc in 35AS8 seedlings (Figure 6). The levels of IAA-Glc were reduced approximately fourfold in the antisense line 35AS8 relative to vector control and wild-type seedling at the same age. In all three lines measured, IAA-Glc accounted for approximately 50% of the total ester pool.

Discussion

Even though IAA-Glc synthetase has been shown to catalyze the first reaction in the IAA ester conjugation pathway in maize (Leznicki and Bandurski 1988a; Michalczuk and Bandurski. 1982; Szerszen and others 1994), the role of this enzyme in the regulation of plant IAA levels is not as straightforward as might be apparent at first. The reaction catalyzed by IAA-Glc synthase,

IAA + UDP-Glc $\Longleftrightarrow$ IAA-Glc + UDP,

yields a product in which the acyl alkyl acetal bond between IAA and the aldehydic oxygen of glucose is approximately 1.4 kcal above that of the bond between Glc and UDP (Leznicki and Bandurski 1988b). In maize, this energetically unfavorable

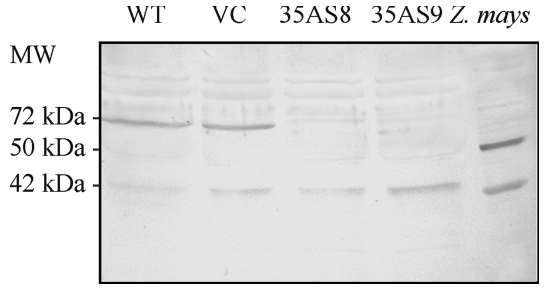

Figure 5. An immunoblot of total proteins (100 μg/lane) from young shoots of untransformed wild type (lane 1), vector control (lane 2) and *IAGLU* transgenic tomato plants 35AS8 (lane 3) and 35AS9 (lane 4) was analyzed for IAGLU expression using the maize IAGLU peptide antibody. Lane 5 is an endosperm extract from maize loaded as a positive control. Molecular weight standards are indicated on the left.

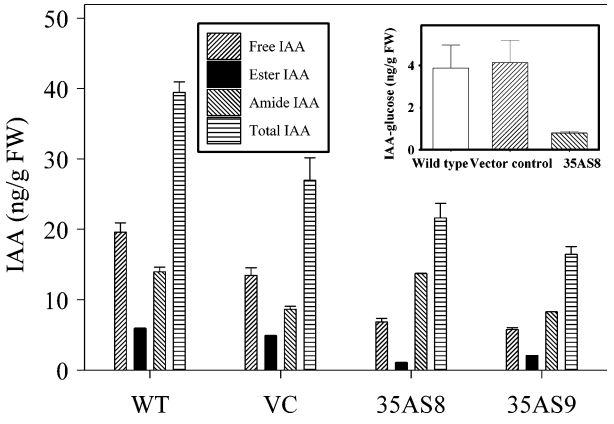

Figure 6. [$^{13}C_6$]-IAA was used to determine the levels of free IAA, ester IAA (IAA present following 1 N NaOH 25°C hydrolysis minus the free IAA value), amide IAA (total IAA minus the free plus ester value) and total IAA in 10 day old seedlings as described in Materials and Methods. Values are the means ± SE from three determinations. Isotope dilution, selected ion monitoring GC-MS analysis with a [$^{13}C_6$]IAA-Glc internal standard was also used to measure IAA-Glc levels in 10 day old seedlings of non-transformed (WT), vector control (VC) and antisense *IAGLU* tomato lines. Data shown are the mean ± SE from three determinations. Note, in comparing the abscissae of the insert and the main figure, the molecular mass of IAA (175) is about half that of IAA-glucose (337). Thus, for example, 5 ng of IAA-glucose is 15 pmol and contains 2.6 ng of the IAA moiety that would be released by base hydrolysis.

reaction is pulled in the direction of conjugate formation by the energetically favored transacylation of the IAA moiety from IAA-Glc to *myo*-inositol (Leznicki and Bandurski 1988b; Szerszen and others 1994). The rate of IAA-Glc formation is thus probably controlled by the rate of formation of the second conjugate in the series, and also potentially by the availability of UDP-Glc. Thus, the effect of plant transformation with *IAGLU* may well depend on the availability of excess UDP-Glc and a suitable transacylation reaction in the transformed plant. The potential exists for aspartate or another amino acid to serve as an alternative substrate for such transacylations from IAA-Glc or, as recently proposed (Jakubowska and Kowalczyk 2004), IAA-peptides or IAA-proteins (Walz and others 2002) could result from such a acyl transfer mechanism. Although proposed, such reactions have never been demonstrated i*n vivo*. The fact that the antisense tomato seedlings showed a change in the levels of IAA-Glc and in ester IAA pools but no corresponding alteration in the levels of amide conjugates suggests that these two routes of conjugation are not linked via a common acyl transfer intermediate of IAA-Glc, and that amino acids may not, in this instance, serve as suitable indole-acyl acceptors. Thus, although dicots as well as monocots form IAA-Glc (Zenk 1961; Slovin and Cohen 1993; Jackson and others 2001) and some dicots contain IAA-*myo*-inositol (Domagalski and others 1987), the potential IAA-acyl acceptor in most dicots remains unknown.

IAA conjugate levels in plants are usually analyzed as families based on chemical linkage (ester and amide). Their levels are determined by measurement of the amount of free IAA present following treatment to selective hydrolysis conditions (Cohen and others 1986). Results from our analysis of the tomato lines showed that ester IAA levels are reduced to 25%–30% of wild-type and vector control levels in seedlings of both *IAGLU* antisense lines studied (Figure 6, insert). This type of analysis tells us that ester-linked IAA is reduced, but it does not specify the chemical identity of the ester form. Specific conjugates can be measured by isotope dilution analysis. Although such analyses are very accurate, the method has been used infrequently because of the requirement for a suitable internal standard (see Cohen 1982; Cohen and Ernstsen 1991; Jakas and others 1993; Tam and others 2000). Isotope dilution GC-MS analysis with an internal standard of [$^{13}C_6$/^{3}H]IAA-Glc showed that IAA-Glc accounted for approximately half of the ester class of conjugates in wild-type, vector control, and antisense transformed tomato seedlings. This is unusual because in other plants where IAA-Glc is known to occur, it is a transient intermediate in the formation of more stable ester forms, such as IAA-*myo*-inositol, which in maize then form the major portion of the lower molecular weight IAA-ester pool (Bandurski and others 1995). Our results may

indicate that acyl transferase activity is the rate-limiting step in IAA ester formation in tomato.

The results presented here demonstrate the use of a heterologous gene to alter the metabolism of IAA and the level of an important IAA conjugate. Expression of the maize *IAGLU* antisense RNA inhibited protein accumulation, resulting in reduced levels of IAA-Glc, and it had significant effects on the phenotype of the transformed tomato lines. Of the 10 independent transformants we recovered using the sense overexpression construct, only one transformant developed some lateral roots. However, that plantlet failed to survive when transferred to soil, probably as a result of extremely reduced root development. Although we were unable to analyze hormone levels in these transformants, the reduced root formation may be due to the overexpression of the *IAGLU* gene and an increase in IAA-conjugation. These changes would lead to an alteration in the ability of the plant to regulate free IAA levels during critical stages of organogenesis. This idea is supported by related work we have done in cooperation with Ludwig-Müller (unpublished), where sense *IAGLU Arabidopsis* transformed lines could be obtained using vacuum infiltration of reproductive tissue without differentiation from callus. The viability and growth of the transformed embryos is predictable from previous measurements showing very high levels of free IAA and low conjugate pools during the critical early stages of embryogenesis (Ribnicky and others 2002).

The tomato antisense transgenic lines exhibited a distinct phenotype in the primary transformants, with abundant root development on shoots that were growing on medium containing high cytokinin levels. One might have expected that a decrease in the capacity to form IAA-Glc would increase tissue IAA levels; however, this is not what is found in 35AS8 and 35AS9 (Figure 6, insert). This observation suggests a complex role for IAA-Glc in establishing the ''set point'' for free IAA levels in plant tissues. The antisense seedlings grown in liquid culture exhibited increased primary root length, as would be expected from a lower free IAA set point. This phenotype is distinct from the increase in the number of lateral roots that is indicative of increased levels of free, active IAA, as measured in the *rty* mutant of *Arabidopsis* (King and others 1995).

Using the maize *IAGLU* cDNA as a probe, we looked for expression of the native tomato *IAGLU*. In seedlings and in vegetative tissues of tomato plants, two mRNA species corresponding to 2.5 kb and 1.3 kb were detected (Figure 3). The predicted molecular weights of proteins encoded by these mRNAs would correspond to about 75 kDa and 42 kDa, and proteins of these molecular weights were immunologically detectable in untransformed and vector control plants (Figure 5). In the antisense transgenic lines analyzed, the 75 kDa protein levels decreased substantially; however, the 42 kDa protein levels remained essentially the same. The relative importance of the 75 and 42 kDa forms for the production of IAA-Glc is unknown; however, the levels of IAA-Glc in the antisense plants reflect the decrease in the 75 kDa protein. Surprisingly, we observed no significant difference in the levels of either of the two apparent endogenous messages in the vegetative tissue (Figure 4) or fruit (data not shown) of transgenic, untransformed, or vector control plants. There have been other reports of heterologous antisense RNA not being highly effective in inhibiting accumulation of the sense mRNA, yet resulting in significantly decreased protein levels. For example, Oliver and others (1993) introduced antisense cucumber NADH-hydroxypyruvate reductase DNA into tobacco and observed no reduction in the levels of the native mRNA, although all the antisense transgenic plants displayed an apparent reduction in the hydroxypyruvate reductase protein levels and enzyme activity. Another example where antisense inhibition, using a heterologous antisense RNA, appears to occur at the level of translation was reported by Temple and others (1993). No significant reduction in the level of the endogenous mRNA was observed when an alfalfa glutamine synthase gene was introduced into tobacco. The observed antisense effect appears to differ mechanistically from the model where the target gene is inhibited by the formation of a duplex between the sense and the antisense transcript, which is then susceptible to degradation by dsRNA-specific RNases (Bird and Ray 1991). This mechanism has been observed in some more thoroughly investigated systems where homologous antisense RNA was used (Smith and others 1990). In cases where a heterologous gene is introduced in an antisense orientation, the sequence of the endogenous message and that of the introduced gene may not fully overlap. In such cases, the steady-state levels of the endogenous RNA may not be altered if the RNA double helix is stabilized by terminal unpaired bases (Eguchi 1991). Temple and others (1993) suggested that the stable RNA duplex would not be transported from the nucleus to cytoplasm, and the mRNA is therefore inaccessible to the translation machinery. This mechanism appears to be contradictory to the discovery that small RNAs can trigger degradation of homologous mRNAs in the cytoplasm (see Matzke and others 2001);

however, the distinct phenotype and biochemical changes of the 35AS8 and 35AS9 plants is consistent with the idea that our understanding of the mechanisms by which antisense expression inhibits protein synthesis is still evolving. Our results show that the expression of the antisense RNA is sufficient to block the expression of the larger protein and to cause a significant reduction in the IAA-Glc levels.

When we transformed tomato with both sense and antisense CaMV35S-constructs of maize *IAGLU*, we observed that the tomato sense transformants failed to produce roots, despite a variety of treatments and grafting methods that were used in an effort to get functional roots. Antisense plants rooted, even in the presence of the high levels of cytokinin normally used to induce shoot formation. Thus, transformation with *IAGLU* might prove useful for alteration of rooting behavior of some difficult-to-root plant species or cultivars. The ability to transform plants with either sense or antisense constructs of *IAGLU* should be an important tool for development of strategies for changing IAA metabolism in plants. Such attempts, however, are not as straightforward as might at first appear, and they require a full recognition of the energetics and intermediates of the reactions involved. The availability of plants with altered capacity to synthesize the key compound necessary for the synthesis of IAA esters should, however, be a valuable tool for determining the role of these compounds in plant growth and the regulation of developmental events.

ACKNOWLEDGMENTS

We thank Dr. Robert S. Bandurski, Michigan State University, for providing maize *IAGLU* and authorizing its use in our studies, and for helpful advice during the early stages of this work. We also thank Dr. Harry J. Swartz (University of Maryland, College Park) for his help with tomato grafting and Dr. M. Kowalczyk (Umeå, Sweden) for the gift of the two IAGLU antibodies. We thank Drs. Jennifer Normanly (University of Massachusetts) and Jutta Ludwig-Müller (TU-Universität, Dresden, Germany) for their critical reading of the manuscript and helpful comments. This paper is dedicated to the memory of our friend and colleague, Dr. Jedrzej B. Szerszen, who cloned maize *IAGLU*, thus making the current work possible. This research was supported by U.S.–Israel Binational Research and Development (BARD) Fund grant US-2498-94, U.S. Department of Energy grant DE-FG02-00ER15079, and U.S. National Science Foundation grant IBN 0111530.

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