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Overexpression of Maize *IAGLU* in *Arabidopsis thaliana* Alters Plant Growth and Sensitivity to IAA but not IBA and 2,4-D

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Abstract

Overexpression of the *IAGLU* gene from maize (*ZmIAAGLU*) in *Arabidopsis thaliana*, under the control of the CaMV 35S promoter, inhibited root but not hypocotyl growth of seedlings in four different transgenic lines. Although hypocotyl growth of seedlings and inflorescence growth of mature plants was not affected, the leaves of mature plants were smaller and more curled as compared to wild-type and empty vector transformed plants. The rosette diameter in transgenic lines with higher *ZmIAGLU* expression was also smaller compared to the wild type. Free indole-3-acetic acid (IAA) levels in the transgenic plants were comparable to the wild type, even though a decrease in free IAA levels might be expected from overexpression of an IAA-conjugate–forming enzyme. IAA-glucose levels, however, were increased in transgenic lines compared to the wild type, indicating that the *ZmIAGLU* gene product is active in these plants. In addition, three different 35S*ZmIAGLU* lines showed less inhibition of root growth when cultivated on increasing concentrations of IAA but not indole-3-butyric acid (IBA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Feeding IAA to transgenic lines resulted in increased IAA-glucose synthesis, whereas the levels of IAA-aspartate and IAA-glutamine formed were reduced compared to the wild type. Our results show that IAA homeostasis can be altered by heterologous overexpression of a conjugate-forming gene from maize.

Key words: *Arabidopsis*; Auxin homeostasis; IAA conjugates; IAA-glucose synthase; *Zea mays*

Introduction

The concentration of free indole-3-acetic acid (IAA) within plant tissues is regulated by a number of

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processes including biosynthesis, degradation and/or conjugation to inactive forms (Bandurski and others 1995). Auxins can be conjugated to sugars, amino acids (Cohen and Bandurski 1982) or peptides (Bialek and Cohen 1986) and proteins (Walz and others 2002). Although the majority of IAA conjugates in vegetative tissues of dicots consists of IAA linked via an amide bond to amino acids or proteins, ester-bound conjugates are the predominant forms in the endosperm (Cohen and Bandurski 1982; Domagalski and others 1987). The regulatory mechanisms involved in conjugation suggest that conjugates are used for long-term storage, but it has also been postulated that they have an active role in short-term hormone responses (Oetiker and Aeschbacher 1997; Slovin and others 1999; Sztein and others 1999).

To date several genes involved in the hydrolysis of IAA conjugates have been cloned from *Arabidopsis thaliana* (Bartel and Fink 1995), *Arabidopsis suecica* (Campanella and others 2003), and bacteria (Chou and others 1998). Likewise, genes involved in conjugate biosynthesis have been isolated from *Pseudomonas syringae* ssp. *savastanoi* (Glass and Kosuge 1986; Roberto and others 1990), maize (*Zea mays* L.) (Szerszen and others 1994), and most recently, *Arabidopsis* (Jackson and others 2001; Staswick and others 2002, 2005).

Two possible mechanisms to reduce free IAA levels in plants involve the manipulation of genes responsible either for hydrolysis of conjugates or for the synthesis of these compounds. For example, overexpression of the IAA-ε-lysine synthase gene (*iaaL*) of *Pseudomonas syringae* ssp. *savastanoi* in tobacco and potato resulted in reduction of free IAA levels (Romano and others 1991; Spena and others 1991). All transformants exhibited leaf epinasty, reduced root growth, and increased side shoot formation. IAA-ε-lysine, however, has not been found as a natural conjugate in plants and may have limited utility for studies of the native systems for hormonal regulation. On the other hand, mutations in one of the IAA amide conjugate hydrolase genes of *Arabidopsis* did not alter the phenotype of the respective plants unless they were grown on media containing high levels of specific IAA-conjugates (Bartel and Fink 1995; Davies and others 1999). However, when three different IAA amino acid hydrolase mutations were combined in a single plant, levels of some IAA conjugates increased and free IAA levels decreased (Rampey and others 2004). The triple mutant has shorter hypocotyls and fewer lateral roots than the wild type. Transformation of *Arabidopsis* plants with a specific bacterial IAA-aspartate hydrolase resulted in plants with no significant difference from wild-type levels of free and total IAA, but with longer roots and hypocotyls and also an increase in rosette diameter (Tam and Normanly 2002). Thus the ability to impair IAA conjugation and hydrolysis may lead to a better understanding of the physiology of hormone homeostasis and to the development of methods to control plant growth. Perturbation of fruit ripening by overexpression of the maize gene encoding IAA-glucose synthase (*ZmIAAGLU*) in tomato accompanied an alteration of free IAA levels in these transgenic plants (Iyer and others 1999). Recently, the effect of overexpressing the endogenous IAA-glucose synthase gene (*UGT84B1*) from *Arabidopsis* was described (Jackson and others 2002). Our work is focused on the overexpression of *ZmIAAGLU* in *Arabidopsis* to determine its effect on the plant phenotype and IAA homeostasis. Our results now allow comparison in *Arabidopsis* of the effects obtained with overexpression of the *Arabidopsis* gene versus heterologous expression of *ZmIAAGLU*. The maize gene was selected for these studies because it has been studied in great detail and because, unlike the enzyme from the *Arabidopsis* gene (Jackson and others 2001), the maize gene lacks significant activity to other acyl donor molecules such as 2,4-dichlorophenoxyacetic acid (2,4-D) (Michalczuk and Bandurski 1982).

Materials and Methods

Plant Material

Arabidopsis. In this study the gl1 (glabrous = hairless) version of Columbia (Lehle Seeds) was used (for simplicity, the wild type will be referred to as Columbia). For the investigation of the phenotype of T2 transgenic *Arabidopsis* lines, the plants were cultivated either on Petri dishes containing full-strength MS medium (Murashige and Skoog 1962) in 1% (w/v) agar or on a mixture of compost:peat:sand (1:1:1) at 23°C, 60% humidity, and a light-dark regime of 16 h/8 h (280 μmol m^{-2} s^{-1}, Philips fluorescent lights TL55 daylight and TL32 Warmton de Luxe). The seeds were surface sterilized with 5% (v/v) commercial bleach (Clorox; a 5% solution of sodium hypoclorite) for 20 min, washed thoroughly, and planted on agar plates containing 50 μg ml^{-1} kanamycin as needed for selection and stratified for 24 h at 4°C before transfer to 23°C. Resistant plants were transferred to soil for further experiments.

Arabidopsis seedlings were also cultivated for 3–10 days as previously described (Ludwig-Müller and Hilgenberg 1992) in liquid shaking culture under continuous illumination using MS medium supplemented with 10 μg ml^{-1} kanamycin as needed for selection. Five-day-old seedlings were removed from the medium, washed with water, blotted dry on paper towels, weighed, and used for IAA and IAA-glucose determination.

Zea mays. Maize seeds (var. Lixis) were soaked under tap water for 1 h, and approximately 100 seeds were distributed on four layers of filter paper soaked with 90 ml of sterile water in trays of 17 × 27 cm (Ludwig-Müller and others 1995). Seedlings were cultivated under sterile conditions in the light at 23°C and 60% humidity and harvested after 5 days.

Vector Construction and Transformation of Plants

The full-length *ZmIAAGLU* cDNA (GenBank Accession number L434847) cloned into the *Eco*RI site of pBluescript SK (Stratagene) was provided by Dr. Robert Bandurski (Michigan State University). The cDNA was excised with *Eco*RI and cloned into the *Eco*RI site of pCGN1761ENX. This vector contains *Eco*RI, *Not*I, and *Xho*I cloning sites between a CaMV double promoter and a transcriptional terminator derived from the T-DNA *tml* gene. After identifying constructs with the sense orientation, the promoter–cDNA-terminator construct was excised with *Xba*I and cloned into the *Xba*I site of the binary T-DNA vector pCIB200. Transformation of *Arabidopsis* was performed by root transformation as described by Valvekens and others (1988). Putative transformants (T_0) were selected on MS agar containing 50 μg ml^{-1} kanamycin, then transferred to soil and allowed to set seed. All further experiments were performed with the kanamycin resistant homozygous T2 generation.

Phenotypic Analysis of Transgenic *Arabidopsis* Plants

Root and hypocotyl length of T_2 seedlings grown on agar plates as described above were recorded on days 7, 11, and 14 after sowing. For each time point, between 50 and 70 plants per line were analyzed. Inflorescence length was measured every 3 days after transplanting the seedlings to soil at 20 days after sowing. Rosette diameter was measured after 6 weeks on soil, and the plants were carefully removed from the soil with a spatula. The roots were cut at the hypocotyl intersection, washed, blotted dry between filter paper, and the root fresh weight was determined.

Root Inhibition Assay with IAA, IBA, and 2,4-D

T2-seedlings were sterilized and sown on agar plates containing 0.1 to 10 μM of IAA, indole-3-butyric acid (IBA) or 2,4-D. Root length of 30 individuals per treatment was scored 14 days after sowing. Results for 5 μM hormone concentration are shown.

RNA Extraction and RNA Blotting

Total RNA was isolated from seedlings grown on agar for 19 days using TRIzol reagent (Gibco BRL) according to the manufacturer's instructions. A biotinylated (bio-dUTP, Boehringer Mannheim) 1400 bp cDNA probe was used for RNA blot hybridization (prepared according to Löw and Rausch 1994). The probe was synthesized by polymerase chain ractions (PCR), using the *ZmIAAGLU* cDNA as template and the following primers: (1) sense primer: 5′-ACCTA ACATCCATTGTTGCAA-3′, (2) antisense primer: 5′GAGTTCGTGCAGTTTGTG CG-3′. Polymerase chain reaction was performed according to standard procedures, using the following program: Initial denaturation at 94°C for 90 s, followed by 40 cycles of 94°C/30 s–50°C/30 s–72°C/90 s, and final elongation at 72°C for 4 min. The probe was hybridized to the correct size RNA from 5-day-old maize seedlings. Non-radioactive RNA blots were performed according to Löw and Rausch (1994). Signals were detected using horseradish peroxidase coupled to streptavidin with subsequent incubation with luminol (Pierce) as substrate according to the manufacturer's instructions, and the blot was exposed to X-ray film (Kodak X-omat, Bechtold, Kelkheim, Germany). Equal sample loading (20 μg total RNA) was confirmed by staining the blot prior to hybridization with methylene blue (incubation for 10 min at RT in 5% acetic acid, then 5 min in 0.5 M NaAc, pH 5.2 containing 0.04% methylene blue, washing with H_2O until bands are clearly visible).

Immunoblot Analysis

Frozen plant material was pulverized in liquid nitrogen and subsequently extracted with 10 mM Tris, pH 8.0, containing 7.5 mM β-mercaptoethanol and 1 mM PMSF to prepare crude cytosolic protein extracts. The homogenate was spun at 14,000 × *g* for 20 min at 4°C. The clear supernatant was mixed with 3× sample buffer (Laemmli 1970), and the protein

concentration was determined according to Peterson (1977). Proteins were separated on a 12% denaturing polyacrylamide gel according to Laemmli (1970). The proteins were blotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore) as previously described (Sambrook and Russell 2001), and the membrane was blocked with 5% (w/v) nonfat dried milk in PBS buffer. IAGLU was detected using the Western-Star (Tropix) chemiluminescence kit according to the manufacturer's instructions with antibodies against *ZmIAAGLU* overexpressed in *E. coli* (obtained from M. Kowalczyk, Umeå, Sweden). Equal sample loading (10 μg protein per lane) was confirmed by amido black staining.

Determination of Free IAA

Frozen or freeze dried *Arabidopsis* 5-day-old seedlings grown in liquid culture were used for the analysis of free IAA. The plant material was ground in liquid nitrogen, extracted, and purified as described elsewhere (Chen and others (1988). One advantage of this procedure, important for samples high in sugar esters, is that it quickly separates neutral conjugates from the free acid by ion-exchange solid-phase extraction on an amino resin. [$^{13}C_6$]-IAA (Cohen and others 1986) was used as internal standard. GC-MS analysis (Hewlett Packard 6890 GC/5973 MSD) was performed in triplicate. For IAA, the molecular and quinolinium ions at m/z 189/195 and m/z 130/136, respectively, were monitored (molecular and quinolinium ions deriving from endogenous and [$^{13}C_6$]-IAA, respectively), and endogenous concentrations of free IAA were calculated by isotope-dilution analysis as given in Ilić and others (1996).

Determination of IAA-Glucose

For determination of IAA-glucose, freeze-dried 5 day-old liquid-grown seedlings (5 g fresh weight) were homogenized in an extraction buffer containing 65% 2-propanol and 35% 200 mM imidazole buffer, pH 7.0. As a radiotracer and isotope standard for quantification of IAA-glucose, mixed isotopic [^3H]/[$^{13}C_6$]-IAA-glucose (10 ng) was added. Synthesis of mixed [^3H]/[$^{13}C_6$]-labeled IAA-glucose was performed enzymatically using IAA-glucose synthase from maize endosperm as described by Tam and others (2000). The homogenate was kept at 4°C for 2–4 h, then filtered through a Whatman filter, after which the filtrate was washed once with extraction buffer and reduced to about 20 ml. The pH was adjusted to 2.5 with 1 N HCl, the sample centrifuged for 10 min at 12,000 × g, and the supernatant extracted three times with equal volumes of ethyl acetate. The aqueous phase was reduced to about 2 ml for Sephadex LH-20 column chromatography. The aqueous extract was applied to the column equilibrated with 50% 2-propanol and eluted with the same solvent. IAA-glucose was traced by scintillation counting. Radioactive fractions were pooled, brought to pH 7.0 with 1 N $NaHCO_3$, evaporated to dryness, and acylated using a mixture of 250 μl of 1% dimethyl amino pyridine (Aldrich) in pyridine and 250 μl of acetic anhydride (Supelco) at 60°C for 1 h. The pyridine and residual acetic anhydride were then evaporated and the residue was resuspended in 100–200 μl of 10% acetonitrile in water. The acylated IAA-glucose was further purified by high performance liquid chromatography (HPLC) on a C_{18} UltraSphere-ODS 25 cm × 4.6 mm column, with acetonitrile (A) and water (B) used as solvents. The program was 10%–100% solvent A in 1 h; flow rate was 1 ml min^{-1}. Under these conditions, pentaacyl-IAA-glucose eluted at 44–52 min. Radioactive fractions were pooled, evaporated to dryness, and resuspended in ethyl acetate for GC-MS analysis. The GC-MS conditions for analysis of pentaacyl-IAA-glucose were the same as for IAA determination. The retention time of pentaacyl-IAA-glucose was 15.3 min. For selected ion monitoring, the molecular and quinolinium ions at 505/511 and 130/136, respectively, were monitored (ions from endogenous and [$^{13}C_6$]-IAA-glucose).

Metabolism of IAA

Pairs of leaves from each of two 20-day-old plants of several independent transformed lines were incubated in glass vials (45 × 15 mm; RPI, Chicago, IL) containing 200 μl MS medium with or without 500 μM IAA, as described elsewhere (Barratt and others 1999). After a 24-h incubation, the metabolites were extracted with methanol containing 100 mg l^{-1} butylated hydroxytoluene (Sigma, St. Louis, MO) for HPLC analysis as described in Barratt and others (1999) using a Jasco BT8100 HPLC equipped with an AS1550 autosampler and a MD-915 photo diode array detector (Jasco GmbH, Groß-Umstadt, Germany). Separation was on a Lichrosorb Reverse phase C_{18} column (Knauer, Berlin, Germany) using a gradient system with 1% aq. acetic acid and 100% methanol as solvent. IAA-conjugates were quantified based on the HPLC peak area at 280 nm. A standard curve of known amounts of IAA was used to convert peak areas of metabolites to ng IAA equivalents (Barratt and others 1999). Metabolites found in this study were previously identified by HPLC with fluorescence detection and GC-MS (Barratt and others 1999).

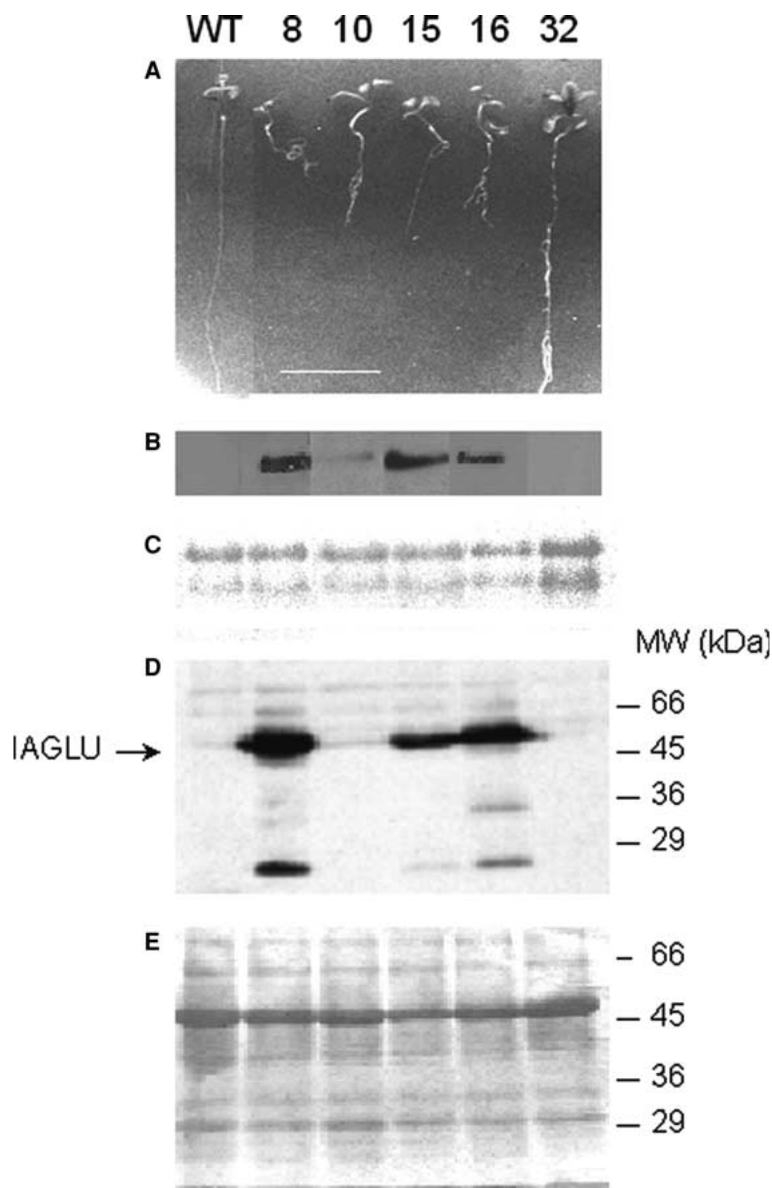

Figure 1. Analysis of transgenic lines (8, 10, 15, 16) constitutively overexpressing *ZmIAAGLU* compared to empty vector transformant (32) and wild type (WT). (**A**) Phenotype of wild-type and 35S*ZmIAAGLU* transformed seedlings on agar after 19 days of growth. The bar represents 1 cm. (**B**) RNA blot of wild-type, 35S*ZmIAAGLU* transformants and vector control hybridized with a homologous *ZmIAAGLU* probe. (**C**) Loading control showing the RNA on the blot stained with methylene blue. (**D**) Protein immunoblot analysis of wild-type, 35S*ZmIAAGLU* and vector control transformed plants. (**E**) Total protein patterns on the same blot stained with amido black.

Results

Expression of *ZmIAAGLU* mRNA and Protein in *Arabidopsis*

ZmIAGLU was overexpressed in *Arabidopsis* under the control of a double CaMV 35S promoter. About 40 independently transformed plants were initially selected on 50 µg ml^{-1} kanamycin, and the seeds of individual plants were harvested. In a second round of selection from these kanamycin-resistant putative transformants, 12 lines (T_2) were recovered and used for further analysis. Of 12 putative transformants analyzed, 4 lines were selected that expressed the *ZmIAAGLU* transgene to different extents (Figure 1). As determined by RNA and immunoblot analysis, line WD10 showed only moderate *ZmIAAGLU* expression, whereas a considerable amount of the maize gene product accumulated in lines WD8, WD15, and WD16 (Figure 1B, 1D). Although there is a native *IAGLU* in *Arabidopsis*, as well as several related glucosyl transferase sequences (Graham and Thornburg 1997; Jackson and others 2001), DNA blot analysis using a cDNA probe homologous to *ZmIAAGLU* did not detect any signal in either the wild type or an empty vector transformant (WD32) (data not shown). The amount of *ZmIAAGLU* mRNA correlated with the amount of IAGLU protein detected in the transgenics using an antibody made to the

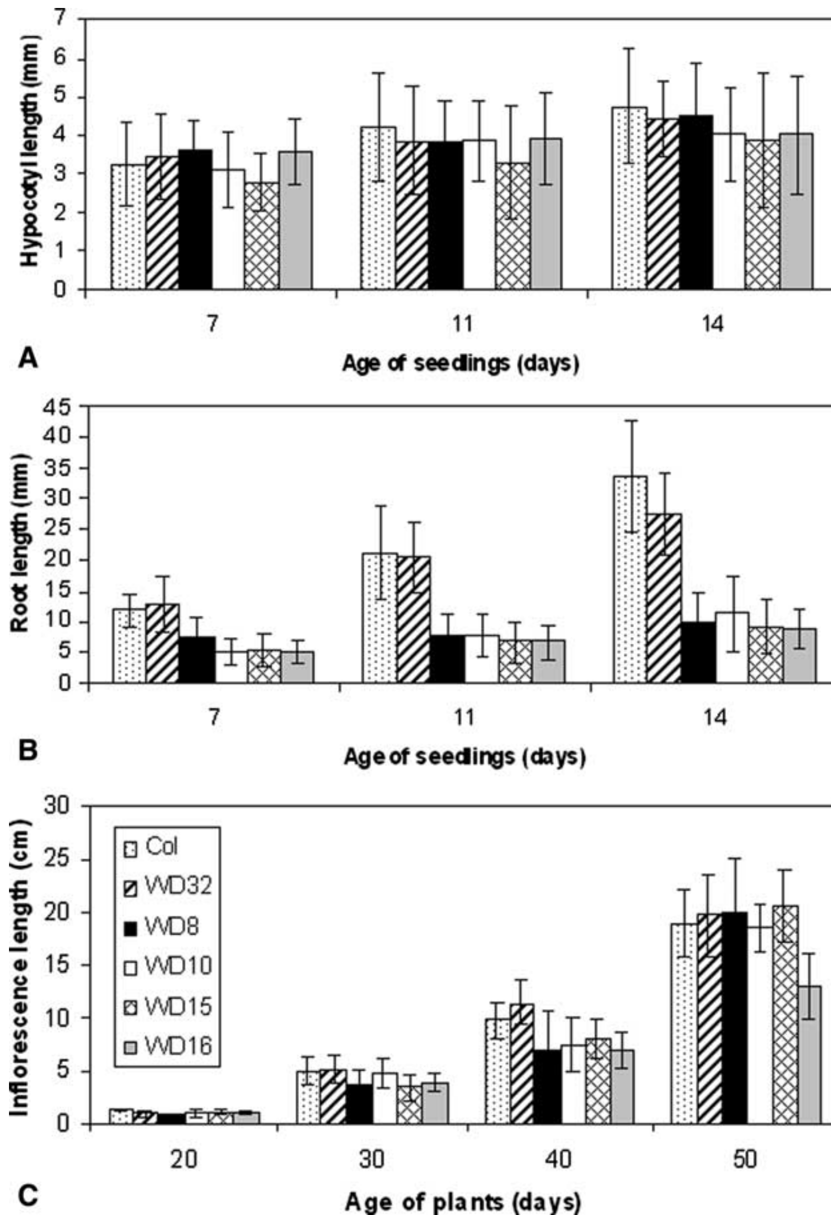

Figure 2. Growth of 35S*ZmIAAGLU* transformant lines WD8, WD10, WD15, WD16, empty vector transformant line WD32, and the corresponding wild type. (**A**) Hypocotyl length of seedlings grown on agar, (**B**) root length of seedlings grown on agar plates, (**C**) inflorescence length of mature plants grown in soil. Only those plants are shown that contained elevated levels of *ZmIAAGLU* mRNA.

maize IAGLU protein overexpressed in *E. coli* (Figure 1D). The lines containing high levels of *ZmIAAGLU* mRNA accumulated relatively higher amounts of the respective protein. Only very low levels of IAGLU protein were detected in WD10. All of the seedlings with detectable levels of *ZmIAAGLU* mRNA displayed shorter roots compared to wild-type and vector control plants; however the hypocotyls and leaves of the seedlings did not show any differences at this developmental stage (Figure 1A). The roots of line WD8, which exhibited the highest *ZmIAAGLU* expression, were curled, suggestive of an agravitropic phenotype (Simmons and others 1995).

Growth Analysis of Seedlings and Mature Plants

More detailed analysis of WD8, WD10, WD15, and WD16 seedlings grown on agar showed that root length was reduced by about 50%–70% compared to the wild type and empty vector transformant over the entire developmental period investigated (7–14 days after germination) (Figure 2B), whereas hypocotyls were not influenced (Figure 2A). Inflorescence length, monitored in 22 – 50-day-old plants growing in soil, was not affected in WD8, WD10, and WD15 as compared to wild type and vector control (Figure 2C). WD16 showed reduced

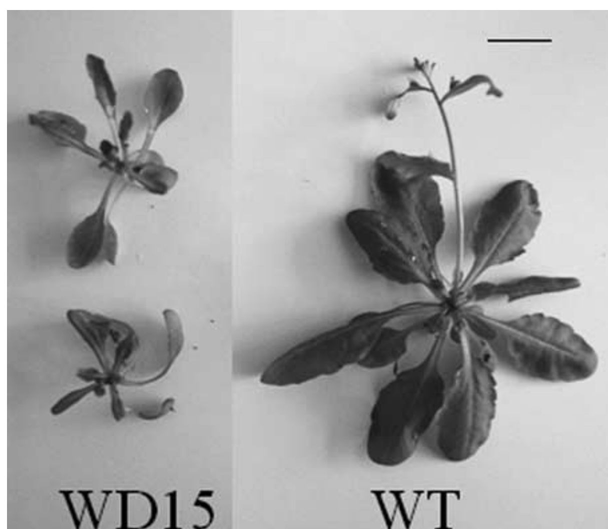

Figure 3. Phenotype of line WD15 and Columbia wild type 24 days after sowing in soil. The bar represents 1 cm.

inflorescence length, but only at 50 days after germination. Several other differences were observed in the transgenic lines, in particular a remarkable change in rosette and cauline leaf shape. All leaves were smaller and showed a curled phenotype (Figure 3). The rosette diameter after 6 weeks of growth in soil was smaller for WD8, WD15, and WD16, whereas WD10 did not differ in this respect from the wild type and empty vector transformant (Table 1). Root fresh weight was determined for plants grown in soil 6 weeks after transplanting (Table 1). In contrast to the results obtained for root growth of seedlings, the root biomass of mature plants was not reduced in the transgenic lines.

IAA and IAA-Glucose Content in Transgenic *Arabidopsis* Plants

To determine whether the maize IAGLU protein is active *in planta*, the concentrations of free IAA and IAA-glucose were determined in 5-day-old seedlings of WD8 and 15, the wild type, and the empty vector control (Figure 4). IAA-glucose concentrations were considerably higher in WD8 and WD15 than in controls. IAA-glucose in WD8 plants increased by about 40-fold compared to the wild type, whereas in WD15 the increase in IAA-glucose was about 14-fold (Figure 4). The amount of IAGLU protein detected by immunoblot analysis (Figure 1D) appears to correlate directly with the *in vivo* enzyme activity, as suggested by the measured levels of IAA-glucose. The elevated levels of IAA-glucose in the 35S *ZmIAAGLU* transformants also suggests that the *in planta* level of UDP-glucose is not rate-limiting, even though thermodynamic considerations requires that high levels of UDP-glucose be present to inhibit the back reaction (Slovin and others 1999). No significant differences in free IAA levels were found for WD8 and WD15 as compared to controls.

It should be noted that in the work reported by Jackson and others (2002) the methods for purification and analysis of IAA and IAA-glucose were significantly different from those we employed. In particular, in the previous work, free IAA and IAA-glucose were both isolated together, and thus the authors found it important to use two differently labeled internal standards to correct for observed hydrolysis of IAA-glucose during extraction and purification. In recognition of the potential for problems with IAA-glucose hydrolysis resulting in overestimates of free IAA levels (Baldi and others 1989), our procedures were designed to minimize inadvertent hydrolysis during IAA analysis. Specifically, the extraction time was short and the sample was buffered. In addition, IAA and IAA-glucose were separated early in the purification procedure. To confirm that these methods were sufficient, we analyzed test samples using the radioisotopic tracer in our prepared IAA-glucose to optimize the procedures and avoid conditions resulting in hydrolysis. In three test runs, the hydrolysis of IAA-glucose during the free IAA analysis procedure was found to be less than 0.1%. Out of 50,000 dpm of $[^{3}H]/[^{13}C_6]$-IAA-glucose added, less than 50 dpm above background were measured in the acidic eluent from the amino column, consistent with what we previously reported for IAA-glucose stability during short-term analyses at pH 7.0 (Baldi and others 1989). 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.

Sensitivity of Roots to IAA, IBA, and 2,4-D

The sensitivity of *Arabidopsis* wild-type and transgenic seedlings to IAA, IBA, and 2,4-D was evaluated. IAA and 1-NAA are known to be good substrates for the maize IAGLU protein in vitro; however, both IBA and 2,4-D have been reported to be poor substrates (Michalczuk and Bandurski 1982). We have recently found that IBA is a weak substrate for the maize enzyme expressed in *Escherichia coli* (J. Ludwig-Müller and A. Wiepning, unpublished results), and we were therefore interested in the substrate specificity of the maize IAGLU protein *in planta*. Transgenic lines WD8 and WD15 were significantly more tolerant to 5 μM IAA in the

Table 1. Rosette Diameter and Root Fresh Weight of *Arabidopsis* Plants Transformed with *ZmIAAGLU*

Line	Root fresh weight (mg plant^{-1})	Rosette diameter (cm)
WD8	2.09 ± 0.23 (123)	2.7 8 ± 0.95 (87)
WD10	2.23 ± 0.41 (132)	3.21 ± 0.64 (101)
WD15	2.66 ± 0.33 (157)	2.13 ± 0.25 (67)
WD16	1.87 ± 0.15 (110)	1.54 ± 0.39 (48)
WD32 (vector control)	1.85 ± 0.21 (109)	3.30 ± 0.47 (103)
Columbia (WT)	1.69 ± 0.18 (100)	3.19 ± 0.97 (100)

Roots were harvested from 6-week-old plants, and at the same time point the rosette diameter was measured (from the leaf tips of opposite rosette leaves). Numbers in parentheses: percent of wild type.

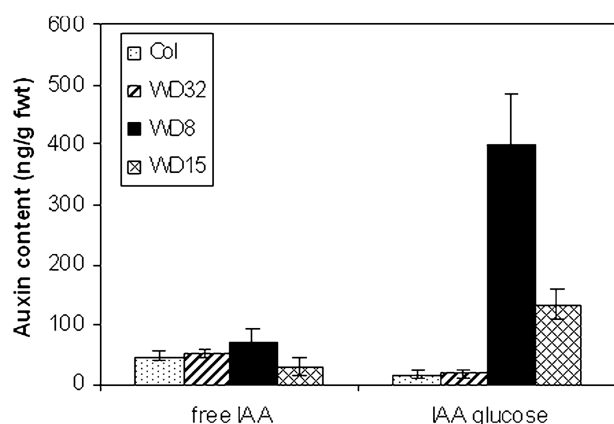

Figure 4. Determination of free IAA and IAA-glucose in two 35S*ZmIAAGLU* lines (WD 8 and WD15), empty vector transformant line WD32 and wild type (Col). The values represent means of three independent experiments. The measurements were done with 5-day-old seedlings grown in liquid culture.

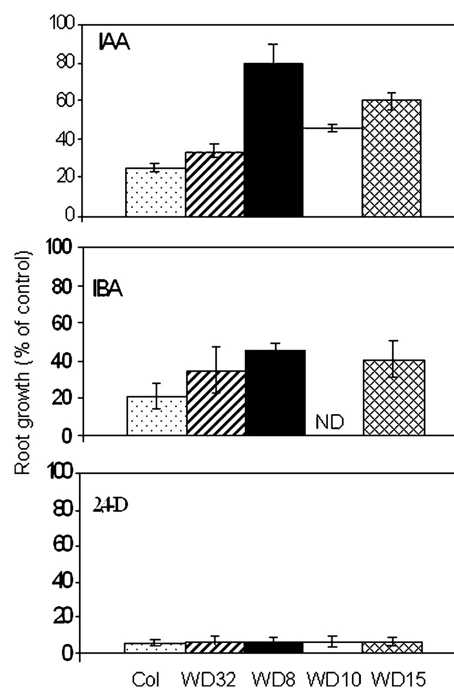

Figure 5. Root sensitivity to 5 µM exogenous IAA, IBA, and 2,4-D of 35S*ZmIAAGLU* transformants, WD8, WD10, and WD15, as well as the vector control line WD32 and the wild type, Col, 14 days after sowing. Root growth is given as percent of control without IAA (=100%). ND: not determined.

root inhibition assay than either the wild type or the vector control, 14 days after sowing (Figure 5). Similar results were seen with 0.1–1 µM IAA (data not shown). WD10 showed the least difference in sensitivity to IAA compared to the wild type and vector control, as might be expected from its low level of expression of the transgene (Figure 1). Although the average root growth of WD8 and WD15 suggests some resistance to IBA, this difference was not statistically different when compared to the empty vector transformant WD32 (Figure 5). No difference in 2,4-D resistance was observed at 5 µM (Figure 5) or at 0.1–1 µM (not shown).

IAA Metabolism

Kesy and Bandurski (1990) showed that IAA-glucose is the indole acyl donor for the synthesis of other IAA ester conjugates. In addition, IAA-glucose is able to transfer the indole acyl moiety to amide groups, suggesting a potential alternative route to amide conjugate formation in addition to adenylation of IAA (Staswick and others 2002, 2005). As a first step in exploring the potential role of IAA-glucose in amide conjugate formation, we analyzed IAA conjugate biosynthesis in several of the transgenic lines. Seeds from the T_2 transformants were

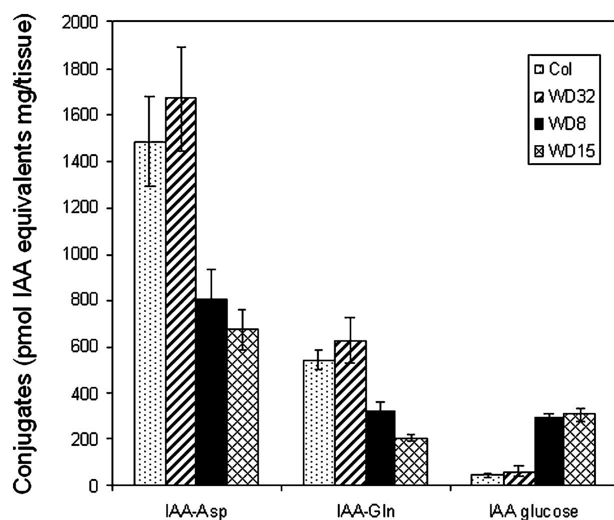

Figure 6. Metabolism of exogenous IAA in 35S*ZmIAA-GLU* transformants of *Arabidopsis*. Pairs of leaves from 20-day-old plants were incubated for 24 h with 500 μM IAA. For quantification of IAA conjugates, the extracts were analyzed by reverse phase high performance liquid chromatography (HPLC). The results from wild-type, vector control, and two different transgenic lines (WD8, WD15) are shown as the mean of two independent determinations, each consisting of three replicates (expressed in pmol of IAA equivalents per mg leaf tissue).

germinated and grown on MS medium. Pairs of leaves from 20-day-old plants were incubated in MS liquid medium with 500 μM IAA, and the metabolites were extracted 24 h later for HPLC analysis and quantitation. The results from two transgenic lines compared to the wild-type and vector control line are shown in Figure 6 as the mean of three independent determinations (expressed in pmol of IAA equivalents per mg leaf tissue). WD8 and WD15 accumulated significantly more IAA-glucose than wild-type and vector control WD32 after a 24-h incubation period. These same two lines showed reduced accumulation of the amide conjugates IAA-aspartate and IAA-glutamine.

Discussion

A gene for an enzyme that catalyzes the formation of IAA-glucose using UDPG as a donor (UDP-glucose:indole-3-acetic acid-β-D-glucosyltransferase) was first cloned from maize (Szerszen and others 1994). This gene (*ZmIAAGLU*) encodes a cytosolic protein of about 54 kDa (Szerszen and others 1994). In maize, the reaction to form IAA-glucose is followed by the energetically favored transacetylation of IAA from glucose to *myo*-inositol (Kesy and Bandurski 1990). Several putative *Arabidopsis IAGLU* homologs have been described (Graham and Thornburg 1997; Yamagishi and others 1998; Jackson and others 2001), whose sequences encode proteins that are approximately 30% identical (43% similar) to the maize IAGLU protein. However, of these only UGT84B1 (Accession No. AAB87119) has been demonstrated to have IAA-glucose synthase activity, and it is likely that the others encode glucosyl transferases with other substrate specificity (Jackson and others 2001). The *Arabidopsis* IAA-glucosyl transferase UGT84B1 was identified by analysis of the substrate specificity of a large number of UDP-glucosyl transferase genes individually expressed in *E. coli* (Jackson and others 2001). At the protein level, UGT84B1 shows only dispersed and limited similarity to maize IAGLU (Figure 7).

Although a considerable amount of IAA in *Arabidopsis* is amide conjugated, the ester IAA-glucose is also present and accounts for about 10% of the total extracted conjugate pool (Tam and others 2000). A substantial amount of another endogenous auxin, IBA, is ester conjugated in *Arabidopsis* as well (Ludwig-Müller and others 1993). An enzymatic activity catalyzing the sugar transfer from UDP-glucose to IBA was previously described (Ludwig-Müller and others 1993), and this transfer was shown to be catalyzed by UGT84B1 (Jackson and others 2001).

Four transformed lines showing different degrees of overexpression of *ZmIAAGLU* were produced and examined for morphometric and biochemical properties (Figure 1). All plants expressing the *ZmIAAGLU* accumulated IAGLU protein, and all had smaller roots at the seedling stage, although the hypocotyls and leaves of seedlings appeared to be unaffected. Even the low levels of *ZmIAAGLU* expression in WD10 were sufficient to result in the observed root phenotype. The observation that minor changes in IAA-glucose synthase expression have a noticeable effect on root development may be a reflection of the unusual thermodynamic requirement for an extreme excess of UDP-glucose, as discussed in Slovin and others (1999). *Arabidopsis* plants overexpressing the native gene, *UGT84B1*, displayed a seedling root phenotype with curled roots, and the severity of curling correlated with transcript level (Jackson and others 2002). The high relative levels of enzyme present in WD8 and WD15 resulted in less pronounced phenotypes than those of the *Arabidopsis* seedlings transformed with *UGT84B1*. This is somewhat surprising, because overexpression of *ZmIAAGLU* in tomato severely affected development of the root system. Shoots from tomato plants overexpressing *ZmIAAGLU* did not develop primary roots, and only one of several transformed shoots developed adventitious roots

(Iyer and others 2005). These differences may reflect the complexity of auxin metabolism in different plant systems, which in turn influences auxin regulation of development (Cohen and others 2003). The similarities and differences between *Arabidopsis* overexpression of the endogenous gene or *ZmIAAGLU* are summarized in Table 2.

Although Michalczuk and Bandurski (1982) had found that maize IAGLU did not conjugate 2,4-D *in vitro*, inhibition experiments indicated that 2,4-D inhibited conjugation of IAA (Leznicki and Bandurski 1988). Our results, showing increased resistance to root inhibition by IAA but not by IBA and 2,4-D in WD8, WD10, and WD15 (Figure 5), are consistent with these *in vitro* observations (Leznicki and Bandurski 1988; Michalczuk and Bandurski 1982). The ability of *Arabidopsis* UGT84B1 to use IAA but not 2,4-D as a substrate is also consistent with its *in vivo* activity, as demonstrated by root growth inhibition assays (Jackson and others 2002).

Mature *Arabidopsis* plants expressing *ZmIAAGLU* did not show significant differences in inflorescence length (Figure 2) or inflorescence branching (data not shown). Only the inflorescence of WD16 was shorter compared to the other plants, and only at 50 days after transplanting to soil. The root fresh weight of mature plants was also the same for the wild-type and transgenic lines (Table 1). However, mature plants displayed a smaller rosette diameter (Table 1, Figure 3), with curled rosettes and cauline leaves. The smaller leaf phenotype resulted in a less compact phenotype (data not shown) and correlated with the degree of mRNA and IAGLU protein expression in the different lines. Overexpression of *UGT84B1* in *Arabidopsis* also resulted in a smaller rosette phenotype, and the leaves were severely wrinkled; however, these plants also showed a significant reduction in height as well as a high degree of inflorescence branching (Jackson and others 2002). This phenotype closely resembles that of tobacco plants overexpressing *iaaL* from *Pseudomonas savastanoi*. These transgenic tobacco plants exhibited several distinct phenotypes including: (1) reduced apical dominance, (2) reduced rooting, (3) wrinkled leaves, and (4) inhibition of vascular differentiation (Romano and others 1991). In another study, tobacco plants transformed with *iaaL* showed an increased nastic curvature (epinasty) of their leaves that correlated with an increased content of IAA conjugates in the leaf blade (Spena and others 1991). These transgenic tobacco plants also showed reduced root growth and increased side shoot formation. Potato plants transformed with *iaaL* also showed leaf epinasty and reduced root growth, and in addition they displayed longer and thinner internodes (Spena and others 1991). *Arabidopsis* transformed with *iaaL* showed a similar phenotype to *UGT84B1* overexpressing plants in terms of plant size and number of shoots (Jackson and others 2002). Overall, transformation with genes that encode IAA conjugating enzymes results in alterations in leaf phenotypes and alterations in root growth. However, the effects observed can be specific to the gene and the plant species.

The free IAA content in the *iaaL* transgenic tobacco plants was reduced compared to the wild type (Romano and others 1991). This was not the case with 35S*ZmIAAGLU Arabidopsis* plants, even though the content of IAA-glucose was markedly increased in the transgenic lines (Figure 4). However, IAA levels differ in various tissues and through development (Ljung and others 2001). Thus, the examination of IAA and conjugate levels in seedlings, where IAA levels in general correlate with cell elongation (Gray and others 1998), might be expected to be more diagnostic than in leaves, where IAA regulation of cell expansion is known to be complex (Keller and others 2004). Another explanation for these results may be the reversible nature of the reaction catalyzed by IAA-glucose synthase (Szerszen and others 1994), as compared to the formation of IAA-lysine (LeClere and others 2002). Alternatively, IAA-glucose could be hydrolyzed by a different enzyme in *Arabidopsis*, as is the case in maize and other plants (Jakubowska and others 1993; Ludwig-Müller and others 1996). The lack of an effect on IAA levels suggests that, unlike the case with amide conjugates such as IAA-aspartate (Östin and others 1998), there is no direct catabolic pathway from IAA-glucose.

Arabidopsis plants overexpressing a bacterial IAA-aspartate hydrolase gene, *IAAspH*, also failed to show significant changes in IAA content, but they did show increases in rosette diameter, as well as root and hypocotyl length (Tam and Normanly 2002). As with the *ZmIAAGLU* overexpressors, *IAAspH*-overexpressing plants showed growth phenotypes that were developmentally transient in some cases and not expressed throughout the life of the plant.

Tomato fruit from plants transformed with E8 promoter–driven antisense *ZmIAAGLU* had lower IAA-glucose levels and lower levels of free IAA as compared to control. Fruit from tomato plants expressing E8-driven *ZmIAAGLU* had slightly increased free IAA concentrations, in addition to the expected increase in IAA-glucose (Iyer and others 1999). These results, together with our findings, suggest that IAA-glucose formation is part of a homeostatic mechanism for regulation of phyto-

hormone levels that allows plants to adjust IAA levels as required by other aspects of cellular metabolism.

Much remains to be studied concerning the role of IAA-glucose in auxin regulation, but from studies of transgenic plants it is clear that the role of IAA-glucose is significantly different from that of IAA-amino acid conjugates. Jackson and others (2002) observed that the typical relationship of auxin levels in younger and older leaves was reversed in the transformants. In the same plants, the levels of both IAA-glucose and free IAA were higher in the transgenic *UGT84B1* plants than in the wild type, and the highest levels of free IAA were found in the transgenic lines that also produced the most IAA-glucose (Jackson and others 2002). Although our *ZmIAAGLU* transgenic seedlings had increased IAA-glucose levels, they did not show significant differences from the wild type in the levels of free IAA. Together, these results suggest that the increases in free IAA levels noted in *UGT84B1* transformants was not a simple metabolic consequence of increased IAA-glucose levels.

The higher levels of IAA-glucose in 35S*ZmIAAGLU* seedlings indicates that the maize enzyme was active in *Arabidopsis* and could affect the rooting phenotype, even when expressed at low levels. Activity of the maize enzyme is consistent with the observed sensitivity to IAA, IBA, or 2,4-D (Figure 5). The root growth inhibition by IAA lies in the range of that reported elsewhere, for example, by Campanella and others (1996). A decrease in root growth inhibition by IAA in 35S*ZmIAAGLU* seedlings is consistent with increased IAA-glucose synthase activity, because excess IAA would be removed from the active pool by conjugating it to glucose.

The metabolism of IAA in plants treated with higher levels of IAA differs significantly from the metabolism of IAA by untreated plants (Cohen and Bandurski 1982). However, in analyzing the effect of transgenes, such experiments can be of diagnostic value. In our studies, although IAA treatment of WD8 and WD15 resulted in an increase in IAA-glucose formation, the formation of two major amide conjugates, IAA-aspartate and IAA-glutamine, was lower in these two lines (WD8 and WD15) that showed an altered phenotype (Figure 6). Similar observations were made by Jackson and others (2002), who found lower endogenous levels of several IAA amino acid conjugates after feeding [^{14}C]-IAA to their transgenic plants overexpressing the analogous *Arabidopsis* gene, *UGT84B1*. These results suggest an inverse relationship between the amounts of IAA-glucose and the amide conjugates, which would not be expected if IAA-glucose were a direct precursor for IAA amide conjugates, as was originally postulated by Kesy and Bandurski (1990). Indeed, recent findings by Staswick and others (2002, 2005) show IAA-amide conjugates originating by an adenylation mechanism.

Some of the phenotypic characteristics observed in *ZmIAAGLU* transformants (for example, wavy root phenotype, smaller rosette) were also seen when the native *Arabidopsis* gene, UGT84B1, was overexpressed (Jackson and others 2002), but other phenotypes such as shorter seedling roots and normal stature of mature plants were not. Thus, there are notable differences in the phenotypes of *Arabidopsis* plants overexpressing maize and *Arabidopsis* IAA glucosyltransferases, enzymes with apparently similar functions. Comparison of the protein sequences of *Arabidopsis* UGT84B1 gene product and maize IAGLU revealed only 34.7% identity in a 464 residue overlapping region (Figure 7A). Although identical residues were clustered and not evenly distributed throughout the sequence, the hydrophilicity/hydrophobicity map and the distribution of prominent protein structural motifs reflected no obvious structural relationships between the two proteins (Figure 7B,7C). Even within the region identified as being involved in glucosyl transferase activity, there appears to be significant heterogeneity both in amino acid sequence and physical properties of the two proteins (Figure 7).

The differences in the physical properties of the resultant protein could alter interactions with other cellular proteins or with microenvironments within the cell. In addition, the sequence and resultant physical differences must account for the differences in substrate utilization of the two enzymes. For the maize enzyme, IBA was a poor substrate, and 2,4-D was not esterified (Michalczuk and Bandurski 1982), and this substrate specificity was reflected in the results from the root growth inhibition experiments we conducted. Although there has not been a direct comparison of the substrate requirements of the two enzymes, AtUGT84B1 appears to have less stringent requirements for the aromatic acid substrate (Jackson and others 2001) as compared to maize IAGLU. Because direct enzymatic measurements were not conducted with the *ZmIAAGLU* transformants, the protein levels detected by immunoblotting cannot be directly attributed to actual enzyme activity. However, indirect evidence such as the increased IAA-glucose content (Figure 4) and the increased IAA-glucose accumulation after feeding of IAA (Figure 6), supports the idea that the expressed protein has enzymatic activity.

A

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Atugt   1   MGSSEGQETHVLMVTLPFQGHINPMLKLAKHLSLSSKNLHINLATIESARDLLSTVEKPRYPVDLVFFSD
iaglu   1   MAP------HVLVVPFPGQGHMNPMVQFAKRL--ASKGVATTLVT---TRFIQRTADVDAHPAMVEAISD

Atugt   71  GLPKEDPKAPETLLKSLNKVGAM---NLSKIIEEKR-----YSCIISSPFTPWVPAVAASHNISCAILWI
iaglu   71  GHDEGGFASAAGVAEYLEKQAAAASASLASLVEARASSADAFTCVVYDSYEDWVLPVARRMGLPAVPFST

Atugt   141 QACGAYSVYYRYYMKTNSFP-----DLEDLNQ-----TVELPALPLLEVRDLPSFMLPSGGAHFYNLMA-
iaglu   141 QSCAVSAVYYHFSQGRLAVPPGAAADGSDGGAGAAALSEAFLGLPEMERSELPSFVFDHGPYPTIAMQAI

Atugt   211 -EFADCLRYVKWVLVNSFYELESEIIESMADLKPVIPIGPLVS-PFLLGDGEEETLDGKNLDFCKSDDCC
iaglu   211 KQFAHAGKD-DWVLFNSFEELETEVLAGLTKYLKARAIGPCVPLPTAGRTAGANGRITYGANLVKPEDAC

Atugt   281 MEWLDKQARSSVVYISFGSMLETLENQVETIAKALKNRGLPFLWVIRPKEKAQNVA-VLQEMVKEGQGVV
iaglu   281 TKWLDTKPDRSVAYVSFGSLASLGNAQKEELARGLLAAGKPFLWVVRASDEHQVPRYLLAEATATGAAMV

Atugt   351 LEWSPQEKILSHEAISCFVTHCGWNSTMETVVAGVPVVAYPSWTDQPIDARLLVDVFGIGVRMRNDSVDG
iaglu   351 VPWCPQLDVLAHPAVGCFVTHCGWNSTLEALSFGVPMVAMALWTDQPTNARNVELAWGAGVRARRDAGAG

Atugt   421 ELKVEEVERCIEAVTEGPAAVDIRRRAA-ELKRVARLALAPGGSSTRNLDLFISDITIA
iaglu   421 VFLRGEVERCVRAVMDGGEAASAARKAAGEWRDRARAAVAPGGSSDRNLDEFVQFVRAGATEK
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B

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Atugt   1   --------EHEEEEE--------HHHHHHHHHHH-----EEEH-HHHHHHHHH----------EEEE---
Iaglu   1   -----EE-----------HHHHHHHHH-H---EEEEEEHHHE----------HHHE------------HH

Atugt   71  ----------HHHHHHHHHHHHHHHH--HHHH---EEEE---------HH-----HHEHEEEEH----EE
Iaglu   71  -HHHHHHHHHHHHHHHHHHHHHHHHH-------EEEE------HHHHHH-H------------EEEEEEE

Atugt   141 EEEEE--------------H-------HH---------------HHHHHHHHHHHHHHHHHEEEE---HH
Iaglu   141 ----------------------HHHHHHHH-------H-----EE--------HHHHHHHHHHH-----H

Atugt   211 HHHHHHHHHH----------------------HHH-------------HHHHHHHH---EEEEEE----H
Iaglu   211 EEH---HHHHHHHHHHHHHHH----------------------EEEE---------H-H----------E

Atugt   281 HHHHHHHHHHHHHH-------EEE--------HHHHHHHHHHH---EEEE-----HHE------EEEEE-
Iaglu   281 EEEEE--E------HHHHHHHHHHHH---HHEEH---------HHHHHHHHHH---EE--------H---

Atugt   351 ------HEEE----EE------------HEEEEHH---EEE----------HHHHHHHHHHH------HH
Iaglu   351 -----EEEE------HHH------HEHH-----------HHHHHH-H--H--H-----EEE---HHHHHH

Atugt   421 HHHHHHHHHHHHHHHH----------HEEE------
Iaglu   421 HHH---HHHHHHHHH--HHHHHHH-E-----------HHHHHHHH------
```

C

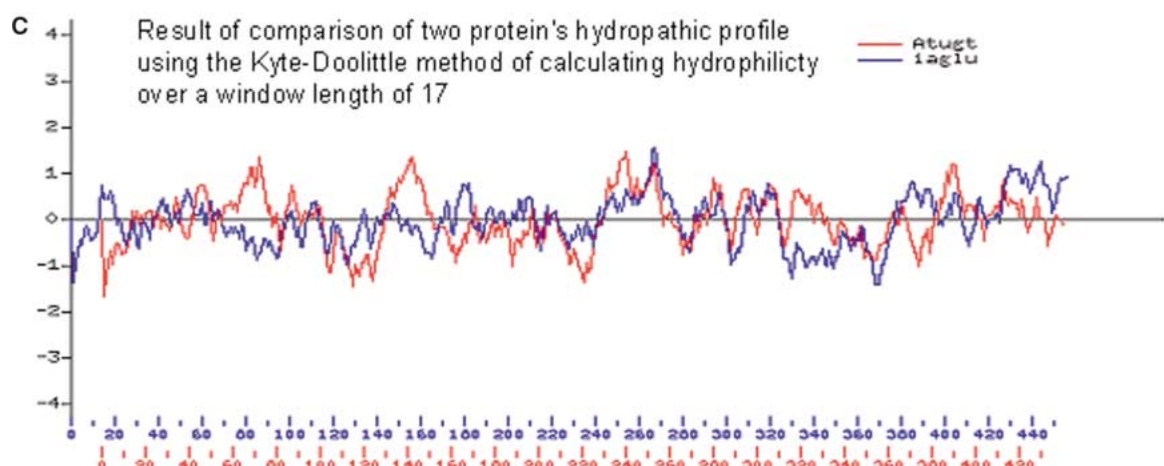

Table 2. Comparison of Homologous (UGT84B1; see Jackson and others 2002) and Heterologous (*ZmIAAGLU*; this paper) Expression of an IAA-glucose Synthase Gene in *Arabidopsis* Compared to Wild-Type Features

Feature	*ZmIAAGLU* overexpression	UGT84B1 overexpression
Seedling root length	Strongly reduced	Slightly reduced
Curled roots on agar	Yes	Yes
Seedling hypocotyl length	Not affected	Not affected
Plant height	Slightly reduced, only in one transgenic line	Shorter
Leaf size	Smaller	Smaller, shorter petioles
Leaf shape	Slightly curled leaves, otherwise no change in leaf shape	Rounded, wrinkly, curling, often disruption of midrib
Apical dominance	Not altered	Increased degree of branching
Sensitivity to IAA	Yes	Yes
IBA	No	ND
2,4-D	No	No
Free IAA content	Not altered in seedlings	Increased in total plant tissue
IAA-glucose content	Increased	Increased
IAA amide conjugate content	Decreased (data not shown)	Decreased
Metabolism to IAA-glucose	Increased	Increased
Metabolism to IAA amide conjugates:		
IAA-alanine	ND	Increased
Other amide-conjugates	Reduced	Reduced

ND = not determined.

In conclusion, overexpression in *Arabidopsis* of a gene involved in IAA homeostasis resulted in no measurable change in free IAA levels, but an increase in the internal levels of an ester conjugate. Yet the presence of the transgene affects both root and leaf phenotypes. Clearly, a full understanding of the role of metabolic activity in defining growth patterns in plants will require a detailed analysis of the function of genes involved in the complex processes that regulate auxin availability at the cell and whole plant levels. Transformation with native and heterologous genes is a promising method for uncovering new relationships between plant morphology and its regulatory controls.

Figure 7. **(A)** Sequence comparison of *Arabidopsis* UGT84B1 (Atugt) and maize IAGLU (iaglu) protein sequences. Identical amino acids are highlighted in red. Green boxes highlight the regions associated with glucosyltransferase activity (identified by a domain search using the Conserved Domain Database of NCBI; http://www.ncbi.nlm.nih.gov/Structure/ cdd/cdd.shtml). **(B)** Secondary structure comparison between *Arabidopsis* UGT84B1 (Atugt) and maize IAGLU (iaglu) proteins using NNPREDICT (http://www.cmpharm.ucsf. edu/~nomi/nnpredict.html; Kneller and others 1990). The predicted structure is: ''H'': a helix element; ''E'': a beta strand element, or ''-'': a turn element. **(C)** Comparison of the hydropathic profile of *Arabidopsis* UGT84B1 (Atugt) and maize IAGLU (iaglu) proteins. Analysis was done using the Kyte-Doolittle method of calculating hydrophilicity (Kyte and Doolittle 1982) from the Weizmann Institute of Science Genome and Bioinformatics Web server page (http//:bioinformatics.weizmann.ac.il/hydroph/).

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