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**Metabolism of Auxins during Fruit Development and
Ripening**

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BARD Final report

3. Abstract***Metabolism of auxins during fruit development and ripening***

We had proposed to look at several aspects of auxin metabolism in fruit tissues: 1) IAA biosynthesis from tryptophan and IAA biosynthesis via the non-tryptophan pathway; 2) changes in the capacity to form conjugates and catabolites of auxin at different times during fruit development; and 3) the effects of modifying auxin metabolism in fruit tissues. The latter work focused primarily on the maize *iaglu* gene, with initial studies also using a bacterial gene for hydrolysis of IAA-aspartate. These metabolic and molecular studies were necessary to define potential benefits of auxin metabolism modification and will direct future efforts for crop improvement by genetic methods.

An *in vitro* system was developed for the production of tomato fruit in culture starting from immature flowers in order to ascertain the effect of auxin modification on fruit ripening. IAA supplied to the fruit culture media prior to breaker stage resulted in an increase in the time period between breaker and red-ripe stages from 7 days without additional IAA to 12 days when 10^{-5} M IAA was added. These results suggest that significant changes in the ripening period could be obtained by alteration of auxin relationships in tomato fruit.

We generated transgenic tomato plants that express either the maize *iaglu* gene or reduced levels of the gene that encodes the enzyme IAA-glucose synthetase. A modified shuttle vector pBI 121 expressing the maize *iaglu* gene in both sense and antisense orientations under a 35S promoter was used for the study. The sense and antisense plants exhibited distinct phenotypic characteristics. The sense plants showed total lack of root initiation and development. The antisense transgenic plants, on the other hand, had unusually well developed root systems at early stages in development. Analysis showed that the amount and activity of the endogenous 75 kDa IAGLU protein was reduced in these plants and consequently these plants had reduced levels of IAA-glucose and lower overall esterified IAA.

Objectives of the original research proposal: The primary objective was to determine the effect of auxin application and/or genetic modification on endogenous and applied auxin metabolism in fruit tissue. Specifically, we had planned to:

- Determine the pathway for auxin production in fruit tissue and study the effect of applied auxins on the pathway and rate of native auxin production;
- Determine the interaction between conjugation of applied compounds and conjugation of naturally occurring auxins;
- Produce transgenic plants with modifications in IAA biosynthesis and conjugation in fruit and evaluate the biochemical and agriculturally important effects of such modifications;
- Determine the changes in key enzyme activities and metabolic pathways during fruit development and ripening in both normal and transgenic plants.

Relevant data, methodology, results and discussion

The amount of IAA in tomato fruit from greenhouse and storage at various stages of maturation.

The amount of IAA was determined in tomatoes which were either picked directly from the greenhouse at various stages of maturation, or kept in storage at 25C until they reached the desired maturation stage. We found free, ester- and amide-linked IAA in all the tomatoes tested (Figure 1). Large differences were found in the level of amide bound conjugates between the two treatments. In the greenhouse tomatoes the level ranged from 2.3 ng/g FW in the red ripe stage to 6.3 ng/g in the breaker stage. With tomatoes matured in storage, however, the range was from 3.5 ng/g in mature green to 61.6 ng/g in the red ripe stage. Levels of esterified IAA were also somewhat higher in tomatoes from storage. In the greenhouse tomatoes the level of free IAA dropped from 4.2 ng/g in immature green tomatoes to 2.4 ng/g in the mature green stage and 0.7 ng/g in the breaker stage. A drop in IAA levels was well correlated with and may be required for the maturation

and ripening stages. No such changes were observed in tomatoes ripened in storage.

Metabolism of IAA by pericarp sections of tomatoes during various stages of maturation.

In previous work on the metabolism of exogenous IAA by tomato pericarp it was shown that most of the IAA was converted to IAA-glucose (Slovin and Cohen, 1993). This prior work was done using greenhouse (GH) tomatoes and the incubation period was for 24 h. To further understand the role of IAA in tomato maturation we extended the study to include tomatoes ripening in storage (ST). Storage consisted of greenhouse tomatoes kept at 20°C and that were removed for analysis at various stages of maturation (mature green, breaker, pink, red and ripe red). Sections of pericarp were incubated with ^{14}C -IAA for different lengths of time. Similar experiments were performed with greenhouse tomatoes ripened on the vine as a control. After incubation, the tissue was washed with water, blotted dry and extracted in acetone. The acetone extract was chromatographed on TLC plates (E. Merck) with solvent A (5:3:1:1; ethyl acetate:methyl ethyl ketone:ethanol:water) and the radioactivity of the spots was detected using an AMBIS radioactivity imaging system. We found (Figure 2) that in all stages of maturation IAA was converted very rapidly to IAA glucose. The level of ^{14}C -IAA dropped rapidly and after 48 h free IAA disappeared completely (Note: Oxidation of IAA by enzymes on the cut surface of the tissue was probably a major cause for the decrease in the applied IAA level). Following 6 h of incubation, the level of IAA-glucose in mature and breaker stages was almost equal in GH & ST fruits (from 10% to 20% of cpm recovered). During the pink stage IAA-glucose in GH was double that of ST while at the red stage IAA-glucose was almost three times higher in ST than in GH. Considerable amount of ^{14}C -IAA were still present in the tissues after 6 h of incubation and contributed significantly to the total cpm recovered. We therefore recalculated the percent of IAA-glucose in the tissues after excluding cpm of IAA from the total.

The results were as follows: (percent of total cpm - cpm of IAA)

	Mature	Breaker	Pink	Red
Greenhouse	38.1	46.6	55.9	28.7
Storage	44.4	54.2	43.3	37.7

Very small differences are noticed in the amount of IAA-glucose at the different stages between GH and ST tomatoes. After 24 h of incubation two times more IAA was converted IAA-glucose in tissues of GH than ST tomatoes during mature and breaker stages. The difference grew smaller during the pink and red stages and in the red ripe stage two times more IAA-glucose was found in the ST tomatoes. In a second experiment, we incubated pericarp from only red ripe tomatoes from one week of storage for 3, 6, 24 and 48 hrs with ^{14}C -IAA. A new compound, probably IAA aspartate (IAA_{Asp}), was detected after the short periods of incubation. Amounts of metabolites was calculated from the AMBIS results. For the short incubation periods (3 h and 6 h) we calculated the percent of cpm of the metabolites after deducting the amount of IAA from the total:

	3h	6h	24h	48h
Origin	7.8	8.9	23.3	42.8
IAA _{Asp}	10.7	11.2	---	---
IAA-glucose	43.0	45.3	42.2	29.8

It is clear that the radioactivity of a compound in the origin of the plate was increased dramatically after 24 h and 48 h of incubation, while the IAA-glucose amount reached very high levels already after 3 h of incubation, but started to decline after 48 h. The compound from the origin was eluted from the silica gel by stirring with 50% aqueous iso-propanol. Two ng of $^{13}\text{C}_6$ -IAA was added, and one half of the extract was hydrolyzed with 1N NaOH for 1 h at room temperature and the other half was hydrolyzed with 7 N NaOH for 3 h at 100°C. Following C_{18} SPE column chromatography and HPLC on C_{18} column, the extract

was methylated with diazomethane and analyzed by GC-MS. We found that mild hydrolysis had some effect on the unknown compound, and 7 N NaOH hydrolysis increased the amount of free IAA considerably. We assume therefore that the unknown compound at the origin is an amide conjugate of IAA, probably with a peptide.

Figure 1

Figure 2

Studies of IAA metabolism in *Cucumis melo*

In order to compare auxin metabolism in tomato with another ripening fruit system, a series of studies were done in muskmellon. These experiments are described in the following publication.

Transgenic tomato plants with modified ability to make IAA-glucose (Manuscript to be submitted to Plant Physiology).

The plant hormone indole-3-acetic acid (IAA) is a natural auxin which controls diverse plant processes ranging from shoot gravitropism, maintenance of apical dominance, differentiation of vascular tissue, cell wall extensibility, cell division to fruit ripening (Normanly, 1997). Free IAA is detectable in plants only in picomolar quantities while a large proportion of IAA is found to be covalently linked to other compounds to form IAA conjugates. Exogenous auxins are also rapidly conjugated (Cohen and Bandurski, 1982). IAA conjugate formation is thought to be tissue specific and developmentally regulated (Kleczkowski and Schell, 1995). Conjugated forms of IAA have been shown to play a necessary role in several processes such as transport, protection from peroxidation, storage of the hormone as an inactive form, and as intermediates in IAA catabolism (Slovin and Cohen, 1993; Bartel 1997). It is likely that free IAA levels *in-vivo* are controlled by a series of such bidirectional routes or 'metabolic shunts' (Cohen and Badurski, 1982). Viewed in this way, conjugation is potentially a component of a sensitive mechanism to regulate the net level of free IAA in the cell (Slovin and Cohen, 1993). Previous work on alteration of IAA metabolism by use of the bacterial gene for production of IAA- ϵ -amino-L-lysine has shown the general feasibility of alteration of IAA metabolism by such methods for decreasing steady-state levels of IAA (Romano et al., 1991). More recently, plants mutants resistant to IAA-amino acids have been isolated (Bartel and Fink 1995; Bartel 1997) and this work has resulted in the isolation of a group of genes encoding proteins with IAA-amino acid conjugate hydrolase activity.

Targeted changes of genes involved in the IAA conjugation pathway could be an important tool with which to manipulate auxin metabolism and therefore the available levels of free IAA. Generation of transgenic lines with modified capacity for IAA regulation would also be valuable tools to study the metabolic pathways themselves. These genetic approaches to modify auxin metabolism offer several advantages over the traditional application methods that are limited by uncertainties such as rates of entry, stability, dosage and timing of application (Cohen 1984).

To investigate the role of auxins and auxin conjugates, we have targeted the IAA-glucose synthase gene (*iaglu*), the first plant gene to be cloned for an enzyme involved in IAA conjugate metabolism (Szerszen et al., 1994). This gene encodes for the enzyme IAA-glucose synthetase that catalyzes the first reaction in the maize IAA conjugation pathway leading to the synthesis of 1-O- β -D-indole-3-acetyl-glucose (IAAgluc) from UDP-glucose and IAA. Transgenic tomato plants that either over express the maize *iaglu* gene or express the maize *iaglu* gene in an antisense orientation have been analyzed. Transgenic tomato plants over expressing the maize *iaglu* gene failed to establish roots and therefore could not be propagated. Transgenic plants with significantly lower levels of IAA-glucose synthetase and of IAA-glucose, as a result of antisense expression, exhibited 'over rooting'. Transgenic antisense plants were grown in the green house and were, as mature plants, almost indistinguishable from the wild type and vector control plants.

Materials and Methods

Plasmid constructs: A modified pBI121 vector was constructed to facilitate expression of *iaglu* cDNA from a CMV 35S promoter. The vector was first modified to delete the GUS gene. Plasmid pBI121 was digested with SmaI followed by SstI to release the GUS gene. The 3' overhang generated by SstI was filled by T4 DNA Polymerase and the vector was religated to yield plasmid vector pBI121 Δ GUS.

Subcloning the *iaglu* cDNA: The maize *iaglu* cDNA on a EcoRI fragment was converted to a blunt ended fragment and subcloned into a filled in XbaI site of pBI121 Δ GUS. Transformations E.coli competent cells were selected on Luria agar plates containing Kanamycin (50 mg/ml. Clones with *iaglu* gene in both sense (pBI121 Δ GUSS*iaglu*) and antisense (pBI121 Δ GUSAS*iaglu*) orientations relative to the 35S promoter were isolated. Gene orientations were confirmed both by restriction mapping and partial DNA sequencing.

The constructs were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Life Technologies) by electroporation under conditions recommended by the manufacturer.

Stable transformation: *Agrobacterium tumefaciens* LBA4404 carrying either *iaglu* sense,

iaglu antisense plasmid constructs or the vector alone was introduced into tomato cotyledons (McCormick). Tomato seeds (cv. Ailsa Craig) were surface sterilized in 10% bleach for 10 minutes and washed with several volumes of sterile distilled water to remove excess bleach. The seeds were germinated aseptically in Murashige and Skoog medium (Murashige and Skoog, 1962). Cotyledons (10-12 day old) were cocultivated with the *Agrobacterium* clones and selected on carbencillin (500 mg/ml) and kanamycin (100 mg/ml) plates. After 3-4 weeks, the kanamycin resistant shoots were transferred to shoot elongation medium containing zeatin (0.1 mg/L). After 3-6 weeks the transformed plants were then transferred to soil. Seeds from the T₀ plants were collected and screened for kanamycin resistance. Two lines from the antisense constructs were selected based on the level of *iaglu* expression as detected by Northern analysis.

Seed germination: Seeds from untransformed, vector control and transgenic plants were germinated in MS liquid media with 1% sucrose. The seeds were surface sterilized in 0.5% sodium hypochlorite for 10 minutes. They were subsequently washed with 5-6 changes of sterile water to remove all traces of residual sodium hypochlorite. Seeds were allowed to germinate and grow for 4-5 days and their root lengths were measured for a comparative analysis. For all other studies, seedlings were harvested after 10 days.

DNA Isolation and hybridizations: Total DNA was isolated from young stems and leaves by the method of Dellaporta et al. (1983). Total DNA (20 µg) was digested with several restriction enzymes and separated by electrophoresis on a 0.8% agarose gel. It was then transferred by Southern blotting to a Nylon membrane (DuPont NEN) as described by Sambrook et al. (1989). An *iaglu* specific radioactive probe was generated with labeled [³²P]dCTP using the Oligo labeling kit (Pharmacia). Hybridization was carried out at 55°C using QuickHyb (Stratagene, La Jolla, CA) for 3 h. The blots were washed at medium stringency (0.2X SSC and 0.1% SDS) at 50° C and exposed to X-Ray film for autoradiography.

RNA Isolation and Hybridization: Total RNA from vegetative tissue and green fruits was isolated using Trizol (Gibco-BRL). Total RNA (10 µg) was analyzed by electrophoresis

through a 1.2% formaldehyde agarose gel and transferred to Hybond N (Amersham) membranes as described by Sambrook et al. (1989). Northern blots were hybridized at 65° C using QuickHyb (Stratagene, La Jolla, CA) containing 200 mg/ml of tRNA for 1 h. Radiolabeled ribo probe was added at a concentration of 1×10^6 cpm/ml of hybridization solution. Blots were washed at high stringency (0.1 X SSPE and 0.1% SDS) at 60° C before autoradiography.

In vitro transcription: Strand specific probes were synthesized using an *in vitro* transcription kit, mMessage (Ambion). Plasmid pBS (SK-) containing the full length iaglu cDNA was linearized with Hind III and transcribed using T3 RNA polymerase to generate a sense strand specific probe. For an antisense strand specific probe, the plasmid was digested with BamHI and transcribed using T7 RNA polymerase

Protein Extraction and Immunoblotting: Young vegetative tissue was homogenized on ice in a buffer containing 0.5M Tris-HCl pH 7.8, 10% v/v β -mercaptoethanol and 1 mM PMSF. Cell debris was eliminated by centrifugation at 13,000 x g for 10 minutes. Protein concentrations were determined. The samples were denatured at 70°C for 15 minutes and separated on a 4-12% polyacrylamide gel using the NuPAGE system (Novex,CA) . The gel was blotted onto nitrocellulose membrane (Schleicher &Schuell) by electrotransfer in transfer buffer (25mM Tris, 192 mM Gly, and 20 % methanol) at 120 ma for 1 hour. To confirm uniform loading and transfer, membranes were reversibly stained using BLOT-faststain (Gene Technology Inc., St. Louis, MO). The membrane was blocked in PBS and 5% fat free milk, incubated with iaglu peptide antibody (1:2000 dilution) followed by 1:5000 dilution of the secondary antibody, antirabbit alkaline phosphatase conjugate. The cross reacting bands were detected using the Western-star Chemiluminescent Detection System (Tropix).

IAA-glucose Analysis: Preparation of isotope labeled internal standard. The liquid endosperm of sweet corn (*Zea mays* L. cv Silver Queen, obtained from a local market at the full 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 liquid was frozen immediately after collection in liquid nitrogen and stored at -80°C for later use. Sixty g of frozen corn endosperm was thawed on ice. Fifty ml of 50 mM Tris buffer, pH 7.6, and $(\text{NH}_4)_2\text{SO}_4$ to 85% saturation (610 g/l) were added while stirring and with pH adjustment. The preparation centrifuged at $10,000 \times g$ for 10 min and the resulting pellet suspended in 0.01M Tris buffer, pH 7.1. The sample was dialyzed against water at 3°C overnight with constant stirring. 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 $10,000 \times g$ for 10 min and the supernatant, which contained the IAA-glucose synthetase enzyme activity, was used to produce the required standard (Michalczyk and Chisnell, 1982). The reaction was carried out by mixing 0.5 ml of the enzyme preparation, 0.3 ml of buffer containing 50 mM Tris, 8.3 mM MgCl_2 and 8.3 mM glutathione, pH 7.6, 10 mg uridine-5-diphosphoglucose (Sigma), 100 μg $[\text{C}_6^{13}]$ -indole-3-acetic acid and 25 μCi of $[\text{H}^3]$ -indoleacetic acid. The reaction was incubated for 24 h at 35°C and then 2 ml of isopropanol was added to stop the reaction. The reaction product was purified on a 1 x 30 cm column of Sephadex LH-20, equilibrated and run in 50% isopropanol/water. The quantity of IAA-glucose in the final solution from the pooled fraction was determined by hydrolyzing an estimated 250 ng of $[\text{C}_6^{13}]/[\text{H}^3]$ IAA-glucose in the presence of 250 ng of IAA in 4 ml of 1 N NaOH for 1 h at room temperature. Following 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 et al., 1998). The ion intensities at m/z 136 and 130 were used to calculate the amount of $[\text{C}_6^{13}]/[\text{H}^3]$ IAA-glucose relative to the peak of unlabeled IAA standard.

Levels of IAA-glucose in tomato tissue was determined using the $[\text{C}_6^{13}]/[\text{H}^3]$ IAA-glucose internal standard. Tomato seedlings were obtained by growing wild type, antisense and vector control seedling in sterile liquid culture. Tomato seeds were surface sterilized in 50 ml of a solution of 0.5% sodium hypochlorite containing 20 μl Tween 80, rinsed with sterile distilled water and placed in a 125 ml flask containing 50 ml of sterile Murashige and Skoog salts medium (Life Technologies) with 1% sucrose. The seedlings

were grown for 10 days under cool white fluorescent lights ($25 \mu\text{mol m}^{-2}\text{s}^{-1}$) on a shaker (1000 rpm, Junior Orbit, Labline). The tomato seedlings (10-20 g) were extracted with 60% isopropanol/200 mM imidazole buffer, pH 7.0 containing 200 ng [$^{13}\text{C}_6/^3\text{H}$]-IAA-glucose. The sample was purified using first ethyl acetate partitioning at pH 2.5 to remove lipids and organic acids, followed by chromatography on a 1.5 x 45 cm column of Sephadex LH-20 run in 50% isopropanol/water. IAA-glucose eluted between 46-52 ml. The sample was then adjusted to pH 7, brought to dryness and acylated using a 1:1 mixture of [1% dimethylaminopyridine in pyridine] and acetic anhydride at 60°C for 1 h (Chisnell, 1984). The solvents were removed and the acylated IAA-glucose redissolved in 200 μl of 10% acetonitrile/water. The sample was purified by C_{18} HPLC on a 25 cm x 4.6 mm UltraSphere column using a programmed linear gradient of 10%-100% acetonitrile/water over a 1 h period. The acylated IAA-glucose had, under these conditions, a retention volume of 44-52 ml. The acylated IAA-glucose fractions were pooled, reduced to dryness and resuspended in 25 μl ethyl acetate for analysis using GC-MS, using the selected ion mode and monitoring ions at m/z 130, 136, 505 and 511. GC conditions were: HP-5 column, 0.21 mm x 30 m; the injector was at 280°C, and the column was initially at 70°C for 2 min, followed by a temperature program at 20°C/min to 280°C and a 5 min hold at 280°C. Under these conditions the acylated IAA-glucose had a retention time of 10.1 min.

Free, free plus ester and total IAA was determined using [$^{13}\text{C}_6$]-IAA as internal standard for GC-MS analysis as described by Cohen *et al.* (1986). Free IAA was purified from a buffered 60% isopropanol extract of 0.1 g of tissue (fresh weight) using ethyl acetate for partitioning for fruit tissue. HPLC was carried out on a 50 mm x 4.6 mm Phenomenex Ultracarb 30 5 μ ODS column. The samples were eluted isocratically using a mobile phase of 25% methanol/water containing 1% acetic acid (v/v). The IAA fraction was methylated using diazomethane and analyzed by GC-SIM-MS (Tam *et al.*, 1998).

Total IAA was determined essentially by the method of Chen *et al.* (1988). The extract was hydrolyzed with 7 N NaOH at 100°C for 3 h using oxygen scrubbed nitrogen (Bialek and Cohen, 1989). IAA was purified on a 3 ml C_{18} SPE column and further purified by HPLC prior to analysis by GC-MS. Free plus ester IAA values were obtained in the same way, except hydrolysis was with 1 N NaOH for 1 h at room temperature (Cohen *et*

al., 1986).

RESULTS

Genetic analysis of 35S*iaglu* transgenic plants: The maize *iaglu* gene was cloned in sense and antisense orientation under the 35S promoter. Transgenic tomato plants were generated using agrobacterium strains expressing either the vector alone or *iaglu* gene constructs. The T₀ sense transformants overexpressed the *iaglu* gene, consistently failed to establish roots. In order to propagate these plants several methods were attempted to induce rooting: (a) grafting the sense shoots to wild type tomato Ailsa craig plants (b) treating the shoots with the commercially available root media (c) 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 0.1mg/l to 1mg/l) (d) transferring the shoots to media supplemented with IBA-lys (2mg/L) and IBA-ala (2 mg/L), conjugates of IBA. However no rooting was observed under any of these conditions.

Transgenic plants were also analyzed that expressed *iaglu* in the antisense orientation. These plants exhibited well formed roots in the media with high cytokinin that induces shoot formation. Transgenic plants carrying the vector alone developed normal roots. Slot blot analysis of DNA extracted from the leaves of the T₀ plants indicated presence of the kanamycin gene (data not shown). We selected two lines 35S:AS8 and 35S:AS9 for further analysis. Based on the hybridization we estimated the copy number to be between 1-5 (data not shown). Seeds from the two transgenic lines, the wild type and vector control were germinated in liquid media. After 6 days the seedlings were harvested and the length of the roots of 18 seedlings from each of the transgenic lines, the wild type and the vector control was measured. There was an remarkable difference in the length of the roots developed by 35S:AS8 and 35S:AS9 when compared to the wild type and vector control seedlings. The average length of roots of the two transgenic lines was 3.1cms and 3.2cms. while that of the wild type was 1.4 cms. and the vector control was 1.47 cms. Southern analysis on the DNA extracted from subsequent generation (T₁) of antisense plants is shown in Figure 1 using the *iaglu* gene as the probe. In addition to the endogenous fragment of 4 kb fragment (lanes 1,3,5,7) seen in the transgenic plants, vector controls and the wild type Ailsa craig plants an additional band of 1.7 Kb was seen only in

the 35S:AS plants, lines 8 and 9 (lanes 75-8). This band was absent in the vector control and wild type Ailsa craig DNA (Lanes 1-4). These results confirm the two lines to be positive as well as show that the full length cDNA has been integrated.

Analysis of the *iaglu* expression in the transgenic plants: Expression of the 35Siaglu gene in transgenic plants were analysed by northern analysis (Figure 2). Transcripts approximately 1.8 kb was detected using *iaglu* as a probe. Expression was also confirmed using strand specific probes. When the blots were probed with the sense strand, antisense transcripts in the vegetative and fruit tissue from 35S:ASiaglu, lines 8 and 9 were detected (Figure 2). As expected plants transformed with vector alone and wild type Ailsa craig did not show any transcripts hybridizing with this probe. However, when the blot was probed with the antisense specific strand that detects endogenous RNA, there was no difference in the hybridization signal in the vegetative and fruit tissue of 35S:ASiaglu line 8 and 9, compared to the wild type Ailsa craig plants or vector alone transformants. This was further confirmed when the band was quantitated using the AMBIS (Figure 2). This indicates that expression of the antisense gene does not alter the stability and lower the steady state levels of the endogenous *iaglu* mRNA. The unaltered levels of the endogenous message may be also due to low expression levels of the antisense transcript and is dependent on the degree of homology between the antisense RNA and the endogenous RNA (Oliver et al., 1993; Temple et al., 1993 and references cited therein).

As the transgenic plants did not produce any demonstrable effect on the endogenous *iaglu* RNA levels it was important to determine its influence at the level of protein expression. For this total proteins from stems and leaves of trees in the green house were separated on a SDS-PAGE gel and immunoblotted with the peptide antibodies raised to maize IAGLU protein. In the tomato vegetative tissue there are two immunologically detectable bands at 75 kDa and 42 kDa. These forms are evident in the wild type and the vector control extracts. However, in the transgenic plants the cross reacting band at 75 kD is conspicuously absent. There is however, no significant change in the levels of the 42 KDa protein in the transgenic lines when compared to wild type and vector control. This leads us to postulate that in the antisense transgenic plants translation of the endogenous *iaglu* protein at 75 kDa could be impaired.

The relative importance of the 75 and 42 kDa forms for the production of IAA-glucose is unknown. In order to determine the effect of antisense expression, the steady state levels of IAA-glucose as well as levels of free, ester and amide IAA was determined in seedlings (Figure 3). As expected, the levels of IAA-glucose were significantly reduced in the antisense line #8. In addition, the levels of ester IAA were lower in both antisense lines measured relative to wild type and vector control lines. While each line showed some differences in the levels of free, amide and total IAA, no consistent pattern emerged. For example, although antisense line #9 had reduced levels of ester IAA relative to wild type and vector control lines, it had almost twice the level of ester IAA as did antisense line #8. While antisense line #8 had free, amide and total IAA levels very similar to that found in control lines, antisense line #9 had noticeably reduced levels of free, amide and total IAA. These results indicate that the 75 kDa gene product must have a significant role in the biosynthesis of IAA-glucose in tomato seedlings.

Discussion

Although several genes have been altered IAA metabolism in plants (Klee and Estelle, 1991), *iaglu* was the first plant gene to be cloned that encodes an enzyme involved in IAA conjugate metabolism. The *iaglu* gene encodes the enzyme, IAA-glucose synthase, which catalyzes the first reaction in maize for the IAA ester conjugation pathway (Szerszen *et al.*, 1994). The role of *iaglu* in the possible regulation of IAA levels in plants is not as straight forward as might be at first apparent. The reaction catalyzed by IAA-glucose synthase,

yields a product where the acyl alkyl acetal bond between IAA and the aldehydic oxygen of glucose is approximated 1.4 kcal above that of the bond between glucose and UDP. This energetically unfavorable reaction is "pulled" in the direction of conjugate formation, by the energetically favored transacylation of the IAA moiety from IAA-glucose to *myo*-inositol (Szerszen *et al.*, 1994). The rate of IAA-glucose 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-glucose. Thus, the effect of plant transformation with *iaglu* should depend on the availability of excess UDP-glucose and of 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-glucose, although this has never been demonstrated. The fact, however, that the antisense tomato seedlings showed a change in the levels of IAA-glucose 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-glucose and that amino acids do not, in this instance, serve as suitable indole-acyl acceptors.

The results presented here demonstrate the use of a heterologous antisense *iaglu* RNA to alter the metabolism of IAA and the level of an important IAA conjugate. Expression of the maize *iaglu* antisense RNA inhibited the protein as well as reduced the levels of IAA-glucose. Alteration of *iaglu* had significant effect on the phenotype of the transformed lines of tomato. For example, the sense transformants failed to root. Of the 10 independent transformants that exhibited this low rooting phenotype, only one transformant was obtained that developed some lateral roots. However, probably as a result of reduced root development, that plant failed to survive when transferred to soil. We did not analyze in detail any of the sense transformed plants since they were all primary transformants and thus were likely to be chimeric. We tentatively conclude, however, that this reduced root formation is likely due to the overexpression of the *iaglu* gene which may lead to an increase in IAA-conjugates and potentially a decreased free IAA levels. On the other hand, the transgenic lines with the antisense gene exhibited a 'rooty' phenotype which was distinctly observed in the T₀ plants, the primary transformants, and was also seen as an increase in root growth when the seeds from the transgenic plants were germinated.

We have looked for the expression of the tomato native *iaglu* gene and have found that in the seedlings and vegetative tissues of tomato plants there are two hybridizing bands corresponding to 2.5 Kb and around 1.3 Kb (unpublished data). Predicted molecular weights of polypeptides translated from these RNA would correspond to around 75 kDa and 42 kDa. Proteins of these molecular weights were indeed detected by western analysis of untransformed and vector control plants (Figure 4). However, in the antisense

transgenic lines analyzed there was a great reduction only in the protein at 75 kDa and the levels of the protein at 42 kDa remained essentially the same. It is interesting that the protein levels of the 75 kDa form is specifically vulnerable to down regulation by antisense expression. A possible reason could be the difference in mismatch annealing of the endogenous mRNA(s) to the introduced maize antisense gene. As expected, there have been reports of heterologous antisense RNA not being as effective as the homologous antisense RNA (Oliver et al., 1993; Temple et al., 1993). Thus the effect we see as differential suppression may be due to the use of a heterologous antisense RNA and its differential interaction with the two size forms of putative tomato *iaglu*. Antisense methods could probably be enhanced for use in tomato, once the native genes are available, by the use of homologous RNA. We also observe no significant difference in the endogenous message between the transgenic plants and the untransformed or vector control plants when the total RNA is probed with the antisense specific strand. Experiments with the antisense RNA in plants have not yet resolved the mode or site of antisense action. The principle of antisense RNA is that the target gene is inhibited in some way by the formation of the duplex formed between the sense and the antisense transcript and this is susceptible to degradation by dsRNA specific RNAases (Bird and Ray, 1991). This has been observed in more thoroughly investigated systems where the homologous antisense RNA was used (Smith et al., 1990). In a case where a gene from a heterologous plant is introduced in an antisense orientation, the expression of the endogenous message and that of the introduced gene under the control of the 35S promoter may not overlap. Hence the steady state levels of the endogenous RNA is not altered. The RNA duplex formed interferes with the transport from the nucleus to cytoplasm and is inaccessible to the translational machinery and the inhibition occurs at the level of translation. We observe, however, that the expression of the antisense RNA is sufficient to block the translation of the larger protein and cause a significant reduction in the IAA-glucose levels.

When we transformed tomato with both sense and antisense 35S-constructs of the maize *iaglu* gene we observed that the tomato sense transformants failed to produce roots, although a variety of compound treatments and grafting methods were used to attempt to induce root formation. Antisense plants rooted even in the presence of the high levels of

cytokinin 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 straight-forward as might first appear and 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.

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Figure 1. Southern blot analysis of *iaglu* antisense plants. Total DNA from untransformed, vector control and transformed plants 35S:AS8 and 35S:AS9 (T2 generation) were digested with EcoR1 (Lanes 1,3,5 and 7) and Xba1 (Lanes 2,4,6 and 8) and hybridized with the full length maize *iaglu* cDNA as described in Materials and Methods.

Figure 2. Northern blot analysis of *iaglu* antisense transformed plants. Total RNA (10 μ g) from the antisense plants 35S:AS8 and 35S:AS9 as well as untransformed plant and vector control was analysed on an agarose-formaldehyde gel and blotted onto a Nylon membrane.

(A) The blot was probed with sense specific riboprobe

(B) Equal amount of RNA was loaded in each lane as determined by ethidium bromide staining of the gel.

(C) The Blot was probed with the antisense specific riboprobe and the endogenous transcript that hybridized were quantified using an AMBIS radioactivity imaging system and plotted as percentage of total counts. Bars indicate the mean $\pm$ SE from three independent gels analyzed.

Figure 3. Levels of free, ester, and total IAA in wild type, vector control and antisense seedlings as determined by quantitative GC-MS analysis. IAA-glucose levels were also determined using GC-MS with an internal standard of [$^{13}\text{C}_6$]IAA-glucose.

Figure 4. Western blot analysis of transgenic plants. Total proteins (100 μ g) from untransformed wild type plant (Lane 1), vector control (Lane 2) as well as total proteins from transgenic plants with maize *iaglu* cDNA under the 35S promoter in antisense orientation, 35S:AS8 (lane 3) , 35S:AS9, (lane 4), were loaded onto a SDS-polyacrylamide gel and immunoblotted using the peptide antibody against the maize IGS. Lane 5 is the endosperm extract from maize loaded as the positive control. Molecular weight markers in kilodaltons are indicated on the left.

Additional molecular studies

To study the effect of auxin conjugating enzymes specifically on fruit ripening we have obtained transformants with plasmid constructs expressing the *laglu* gene under a fruit specific promoter, E8, in both sense and antisense orientation. We obtained 6 antisense transformants and 5 sense transformants which have been successfully grown to the T₁ stage and seed obtained. Currently they are being grown in the greenhouse and evaluated in terms of ripening characteristics. A southern blot analysis using two probes E8laglu gene and laglu gene alone on all the transformants has confirmed the presence of the gene.

Attempts to isolate the tomato homolog of the maize *laglu* gene are being made by screening tomato c-DNA and genomic libraries under different hybridization conditions and using a PCR based method. Currently we are in the process of sequencing a likely partial clone for the tomato gene.

Finally, we have produced transgenic tomato carrying the IAA-aspartate hydrolase gene (Chou et al., 1998). Plant transformed with this gene under control of the CMV35S promoter were very weak and did not survive outside of tissue culture. Plants transformed with the E8 construct appear more vigorous and are currently nearing the stage where we will transfer them to the greenhouse.

Reference:

Chou J-C, Mulbry WW and Cohen JD (1998) The gene for indole-3-acetyl-L-aspartic acid hydrolase from *Enterobacter agglomerans*: molecular cloning, nucleotide sequence and expression in *Escherichia coli*. *Molecular and General Genetics* 259:172-178

Evaluation of the research achievement with respect to the original research proposal and its objectives:

- o Determine the pathway for auxin production in fruit tissue and study the effect of applied auxins on the pathway and rate of native auxin production. *This work has*

progressed well and we are currently doing our final series of experiments on the change in the pathway during fruit maturation. Current work adds the extra concern of the role of ripening in the change in pathway and this is being addressed by doing the in vitro studies using both Alsia craig and Never ripe lines.

- *Determine the interaction between conjugation of applied compounds and conjugation of naturally occurring auxins. Our studies on conjugation were directed mainly at the process with native auxins, primarily due to the relationship to the transformation studies underway in the lab that went very well throughout the grant period. We did several studies to look at the interaction of various auxins on the induction of both IAA-glucose formation and IAA-aspartate formation. These studies did not develop into a major theme of this proposal mainly because the reviewers had suggested the IBA and other non-IAA work should have lower priority.*
- *Produce transgenic plants with modifications in IAA biosynthesis and conjugation in fruit and evaluate the biochemical and agriculturally important effects of such modifications. These studies progressed very well, as detailed above in the report.*
- *Determine the changes in key enzyme activities and metabolic pathways during fruit development and ripening in both normal and transgenic plants. We developed new ways to study auxin metabolism using fruit organ culture and have used this to characterize auxin metabolism in fruit. Studies with the transgenics has not progressed beyond the initial analysis stage, but we did develop a very fine assay for IAA-glucose that has been used in the work. Clearly we did a lot here, but also more work needs to be done and we are continuing with this aspect of the work.*

Both the production of transgenic plants and the studies of IAA metabolism, in general, progressed ahead of the research plan in the original proposal. Proposed experiments on IBA in tomato was strongly critiqued by the reviewers and because the other aspects of the study went well we let success direct our efforts. We had hoped to use one additional fruit specific promoter in the proposal, but were not able within the time frame of the proposal to do this. In part this is due to the fact that when we applied for a use permit for 2A11 the

documents were never signed by Calgene although they assured us of their willingness for us to use the promoter. This has not, however, had any serious effect on our progress since the use of 35S and E8 were our primary interest and that work has turned out well.

Description of cooperation

Our cooperative work was aided by the fact that Dr. Epstein spent extended periods in the Beltsville laboratory. Thus, during the first two years, Dr. Epstein was in Beltsville for almost half the time. This made it possible to advance our studies at a good rate and had eliminated any potential duplication of effort and facilitated technology transfer in both directions. Dr. Epstein retired from his position in Israel during the end of the second year of the project and we were fortunate to be able to have him join the US laboratory. This allowed us to work closely on evaluation of the transgenic material as it became available and we intend to continue the work for at least another year.

Conclusions

Our research clearly showed that 1) IAA is important for fruit ripening and that changes in fruit IAA or in the capacity to form IAA-glucose can be used to extend the ripening period (shelf life) of tomato, 2) that novel methods for manipulation of endogenous IAA metabolism have great potential for the agronomic alteration of plant hormone metabolism, 3) that the biosynthetic pathway to IAA changes during fruit maturation, and 4) that hormone metabolism during fruit maturation and ripening is a dynamic process that requires more detailed investigations. We have also advanced the field significantly by development of two techniques that should be useful to other researchers: a new method for quantitative analysis of IAA-glucose and an in vitro system to study fruit ripening.

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Manuscripts in preparation:

Iyer M, Slovin JP, Epstein E and Cohen JD Transgenic tomato plants with modified ability to make IAA-glucose. (To be submitted to Plant Physiology)

Iyer M, Slovin JP, Epstein E and Cohen JD Tomato plants transformed with sense and antisense constructs of *iaglu* under control of the fruit specific promoter E8 show altered rates of fruit ripening. (To be submitted to Plant Journal)

No patents are expected from this work.