December 2006

Comparative Terpenoid Metabolism in Artemisia annua Seedlings

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Project Number PZW-BD06

Comparative Terpenoid Metabolism in *Artemisia annua* Seedlings

A Major Qualifying Project Report:

submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

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Date: December 14, 2006

Approved:

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Abstract

This MQP compares the effects of sugars on growth and terpenoid production in the plant *Artemisia annua*. It also includes a review of hexokinase locations in plants. Seedlings were grown in media containing sucrose, glucose, or fructose. The growth of the seedlings was measured in biomass and number of leaves per plant. Terpenoids were measured by a TLC profile made from extractions of the seedlings. This MQP furthers the study of hexokinase localization and terpenoid production and regulation in plants.

Acknowledgements

We would like to thank everyone that helped in making the completion of this project possible:

Our project advisor, Dr. Pamela J. Weathers, we are greatly appreciative of the time, patience, and guidance you took to help us through this project.

Thank you Dr. Melissa Towler, for your help with finding lab supplies, giving culture donations, and showing us how to properly use lab equipment and do lab procedures.

Thanks to Patrick Arsenault and all those in GH 06 for supplying us with seeds for our experiments again and again.

Thank you very much Josyln Foley, your patience and dedication saw us through this project. We couldn't have done it without you.

Yi Wang, thanks, for helping us with the seed sterilization procedure.

Most of all, we thank our parents for your support and encouragement.

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1. Introduction

1.1 Significance of Artemisinin

Malaria is a disease that causes over one million deaths each year, putting 40% of the world population at risk (WHO, 2004). There are four parasites that cause malaria but the one that causes the most illness and death is *Plasmodium falciparum*. This is because some strains have become resistant to many of the affordable current treatments including, chloroquine, quinine, mefloquine, and primaquine (Balint, 2001).

An antimalarial drug that has been used as a folk remedy since 168 B.C. in China was first purified and its molecular structure found in 1972. It was named qinghaosu, but it is now better know as artemisinin, a sesquiterpene from the plant *Artemisia annua L.* (Meshnick *et al*, 1996). Artemisinin and its derivatives have been found to be effective against all stages of resistant strains of *P. falciparum* (Balint, 2001).

Although artemisinin has been found to be a useful medicine, its production is very low in comparison with what is actually needed. The World Health Organization, WHO, estimated that 130 million treatments would be needed in 2006, requiring 330 tons of artemisinin (WHO, 2004). This presents a problem because of the very low production levels of artemisinin in the native plant. One ton of dry *A. annua* leaves produce only 6 kg of artemisinin. Considering that the drug also can not be economically produced by organic synthesis (Abdin *et al*., 2003) researchers have been trying to increase artemisinin production in *A. annua* plants.

1.2 Chemistry and Source of Artemisinin

Artemisinin is an endoperoxide sesquinterpene lactone in the terpenoid family of secondary metabolites (Figure 1). Its molecular formula is $C_{15}H_{22}O_5$. The key part of its

structure is its endoperoxide bridge. It has been proposed that the endoperoxide bridge is cleaved by a heme group to form a free radical that causes selective alkylation of parasite proteins, leading to parasite death (Pandey *et al*.1999). Pandey *et al*. (1999) also proposed that artemisinin forms a complex with heme that interrupts the parasite's hemoglobin catabolism.

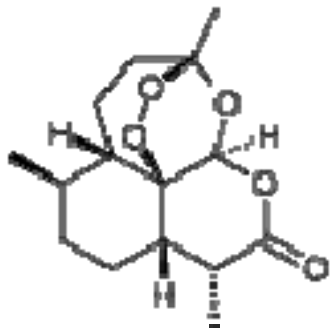

Figure 1. The chemical structure of artemisinin.

There have been several attempts to synthetically make artemisinin, however the results have been inefficient and costly. Although these attempts were impractical at a large enough scale to be useful, it should be noted that artemisinic acid was a major intermediate. This is interesting because some strains of *A. annua* produce eight to ten times more artemisinic acid than artemisinin (Abdin *et al*., 2003).

1.3 Biochemical Pathway

Artemisinin is a sequiterpenoid synthesized from five units of isopentenyl diphosphate (IPP); IPP is produced in the cytoplasm, mitochondria, and plastids. Although it is uncertain if IPP can be transported between all of these cellular compartments, the pathways for the synthesis of terpenoids include the mevalonate pathway, in the cytoplasm, and the non-mevalonate pathway in the plastid (Figure 2).

In the cytoplasm the pathway begins at acetyl-CoA and is converted into 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). HMG-CoA is converted into mevalonic

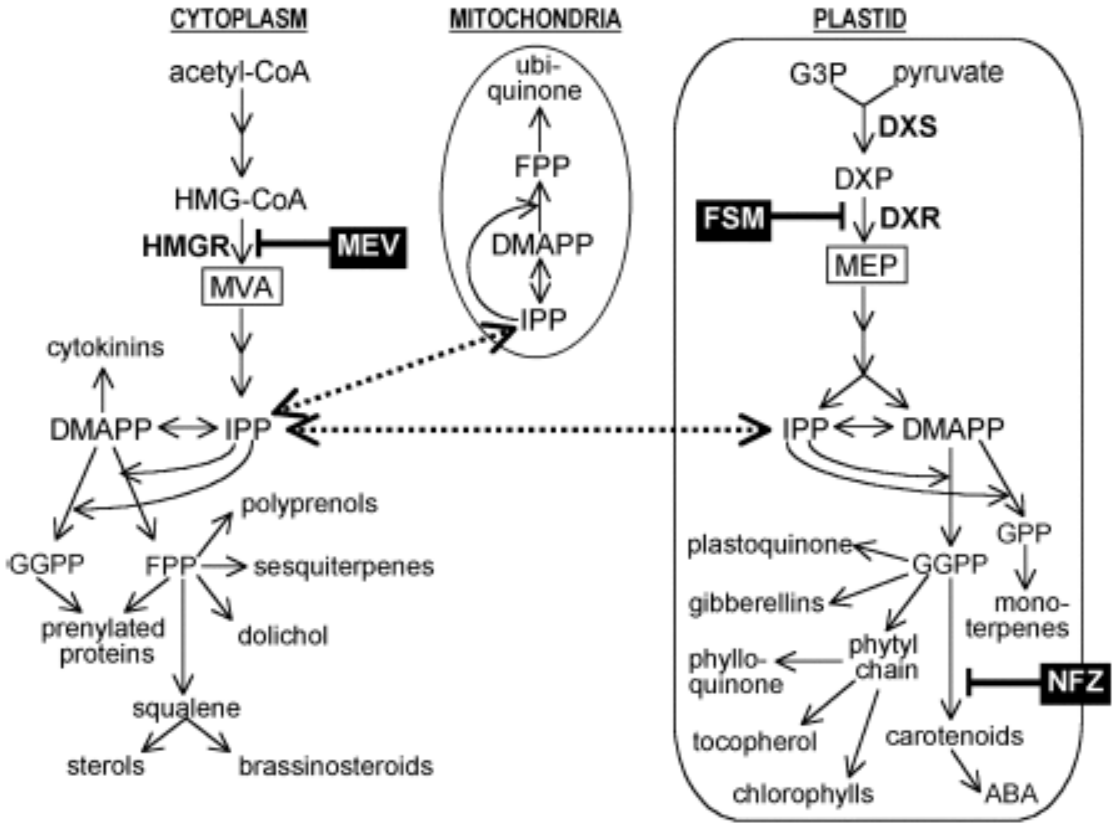

Figure 2. Terpenoid biosynthesis pathways.

DMAPP, dimethylallyl diphosphate; DXP, deoxyxylulose 5-phosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; G3P, glyceraldehyde3-phosphate; GPP, geranyl diphosphate; HMG-CoA, hydroxymethylglutaryl CoA; IPP, isopentenyl diphosphate; MEP, methylerythritol4-phosphate; MVA, mevalonic acid.; DXR DXP reductoisomerase; DXS, DXP synthase; HMGR, HMG-CoA reductase. Inhibitors in black boxes: MEV, mevinolin; FSM, fosmidomycin and NFZ, norflurazon. (Taken from Rodríguez-Concepción et al., 2004).

acid (MVA) by HMG-CoA reductase (HMGR) (Rodriguez-Concepcion *et al*., 2004). This step can be inhibited by mevinolate (MEV) (Alberts et al., 1980). MVA is converted into IPP and IPP's isomer dimethylallyl diphosphate (DMAPP) (Rodriguez-Concepcion *et al*., 2004). From these two molecules farnesyl diphosphate (FPP) and

geranylgeranyl diphosphate (GGPP) can be made. Sesquiterpenes and other terpenoids are made from FPP.

In the plastid 1-deoxy-D-xylulose 5-phosphate (DXP) is made from pyruvate and glycerol dehydyde 3-phosphate (G3P) using DXP synthase (DXS) (Rodriguez-Concepcion *et al*., 2004). DXP is converted into methylerythritol phosphate (MEP) by DXP reductase (DXR). This step can be inhibited by fosmidomycin (FSM) (Steinbacher et al., 2003). MEP is converted into IPP and DMAPP which can be converted into GGPP or geranyl diphosphate (GPP) (Rodriguez-Concepcion *et al*., 2004). These terpenoid precursors are made in the plastid. Note that some of these precursors are made both in the cytosol and the plastid, for example, GGPP (Figure 2). Norflurazon (NFZ) can inhibit GGPP's conversion into carotenoids. IPP can also be used in the mitochondria to eventually be converted into ubiquinone (Rodriguez-Concepcion *et al*., 2004).

1.4 Factors Affecting Production

Many factors can affect production of artemisinin and many attempts have been made to improve production yields. These include precursor feeding, where precursors, such as mevalonic acid, are added to plant media (Woerdenbag *et al*., 1993). There have also been attempts to influence the biosynthetic pathway with inhibitors. For example, the sterol inhibitor miconazol inhibits sterol demethylase, which is the first regulatory step leading to sterols which in turn coordinately up regulates production of sesquinterpenes (Abdin *et al*., 2003). However, there is much about the regulation of these pathways that is still unknown and being studied.

1.4.1 Sugars

1.4.1.1 Sugars as a food source

Through photosynthesis plants use light energy, carbon dioxide, and water to produce sugars. These sugars are stored in the plant to be used as a food source. Cellular respiration takes the energy rich chemical bonds of a sugar like glucose, and converts it into energy that can be used by the plant. Sugars also coordinate internal regulators and environmental cues that have an influence on the growth and development of plants (Koch, 1996; Sheen *et al*., 1999; Smeekens, 2000).

1.4.1.2 Sugars alter artemisinin production

Recent experiments done by Yi Wang for her MS thesis at Worcester Polytechnic Institute showed that sugars may act as signaling molecules to regulate the production of artemisinin in *A. annua* seedlings. Her experiments further showed that in *A. annua* plantlets artemisinin production was also significantly increased when glucose was fed to plantlets in comparison to sucrose. Fructose on the other hand showed an inhibitory affect, lowering the artemisinin production in those plantlets (Wang, 2006). Weathers et al. (2004) studied the effects of glucose, fructose and sucrose on hairy roots of *A. annua*. They studied growth and artemisinin production of roots grown in both combinations of glucose, fructose, and sucrose and these sugars alone. Combinations including glucose, such as glucose with sucrose, and glucose with fructose and sucrose, compared to sugar combinations without glucose, such as sucrose and fructose, showed an increase in growth. The only change seen in artemisinin production was in the experiment with the fructose and sucrose combination, which greatly inhibited artemisinin production. When each of the three sugars was used alone glucose reduced growth, but increased artemisinin production (Weathers et al., 2004).

1.4.1.3 Sugars as signaling molecules

Recently it has been shown that besides serving as carbon sources, sugars can be sensed in plants, and can induce signals that affect metabolism and development. Sugar sensing is the interaction between sugar molecules and sensor molecules, which are usually proteins (Smeekens, 2000). Genetic analyses have shown that although there are widespread interactions between sugars and plant hormone signaling, the enzyme hexokinase (HXK) plays a central role in the sensing of glucose. It has been proposed that glucose activates both HXK-dependent and HXK-independent pathways (Rolland *et al*., 2006). Further, glucose also uses different molecular mechanisms to control transcription, translation, protein stability, and enzymatic activity (Rolland *et al*., 2006).

1.4.1.4 Sugars as regulators of plant secondary metabolites

There is little information about sugars having an effect on the production of plant secondary metabolites. In 1998 Larronde *et al.* grew *Vitis vinifera* cell cultures for twelve days in IM2 medium containing elevated concentrations of some major inorganic nutrients. On the seventh day different concentrations of sucrose were added to the cultures. Although the added sucrose increased the accumulation of anthocyanins 12-fold in the *V. vinifera* cell cultures, accumulation of stilbenes was minimally affected (Larronde et al., 1998). In a later study by Vitrac *et al*. the glucose analog, 3-O-methylglucose, was added to *V. vinifera* cultures, but yielded no difference in anthocyanin production when compared to the negative control. Considering that 3-O-methylglucose is a glucose analog that can be moved into the cells, but is not phosphorylated by hexokinase, these results suggested that hexokinase may be involved. In the same study when another glucose analog, mannose, was added instead of sucrose to *V. vinifera* cells, the accumulation of anthocyanin went up proportionately to the

concentration of added mannose. Although mannose is an analog of glucose, it is phosphorylated by hexokinase, but cannot be further metabolized (Chen and Jones, 2004). These results suggested that hexokinase has a role in regulating anthocyanin production in *V. vinifera* (Vitrac *et al*., 2000). When the hexokinase inhibitor, mannoheptulose, was added, it inhibited the effect of sucrose on the anthocyanin production in the grape cell suspension cultures. These results suggested that hexokinase seemed to be involved with the sugar signal transduction pathway related to anthocyanin production (Vitrac *et al*., 2000).

Artemisinin production was also shown to be stimulated by glucose Weathers *et al*., (2004), and Wang (2006) showed that artemisinin production was significantly decreased when 10% 3-O-methylglucose (3OMG) was added. Wang (2006) also measured hexokinase activity and found that when 10% 3OMG was added along with 90% glucose, activity was decreased compared to controls using 100% glucose. Together these results suggested that sugars are not only carbon sources, but may also regulate artemisinin biosynthesis.

1.4.2 Current signal transduction pathway models

There are several sugar signal transduction pathways working within plants. Sugar signaling molecules can be either monosaccharides or disaccharides and sugar signals can become more complex when both are present in the plant at the same time. Glucose is the main signaling monosaccharide, but its signal can be altered when fructose and other monosaccharides are present (Figure 3). Sucrose is the main signaling disaccharide, but can also be affected when trehalose, maltose, and other dissacharides are present (Figure 4) (Wang, 2006).

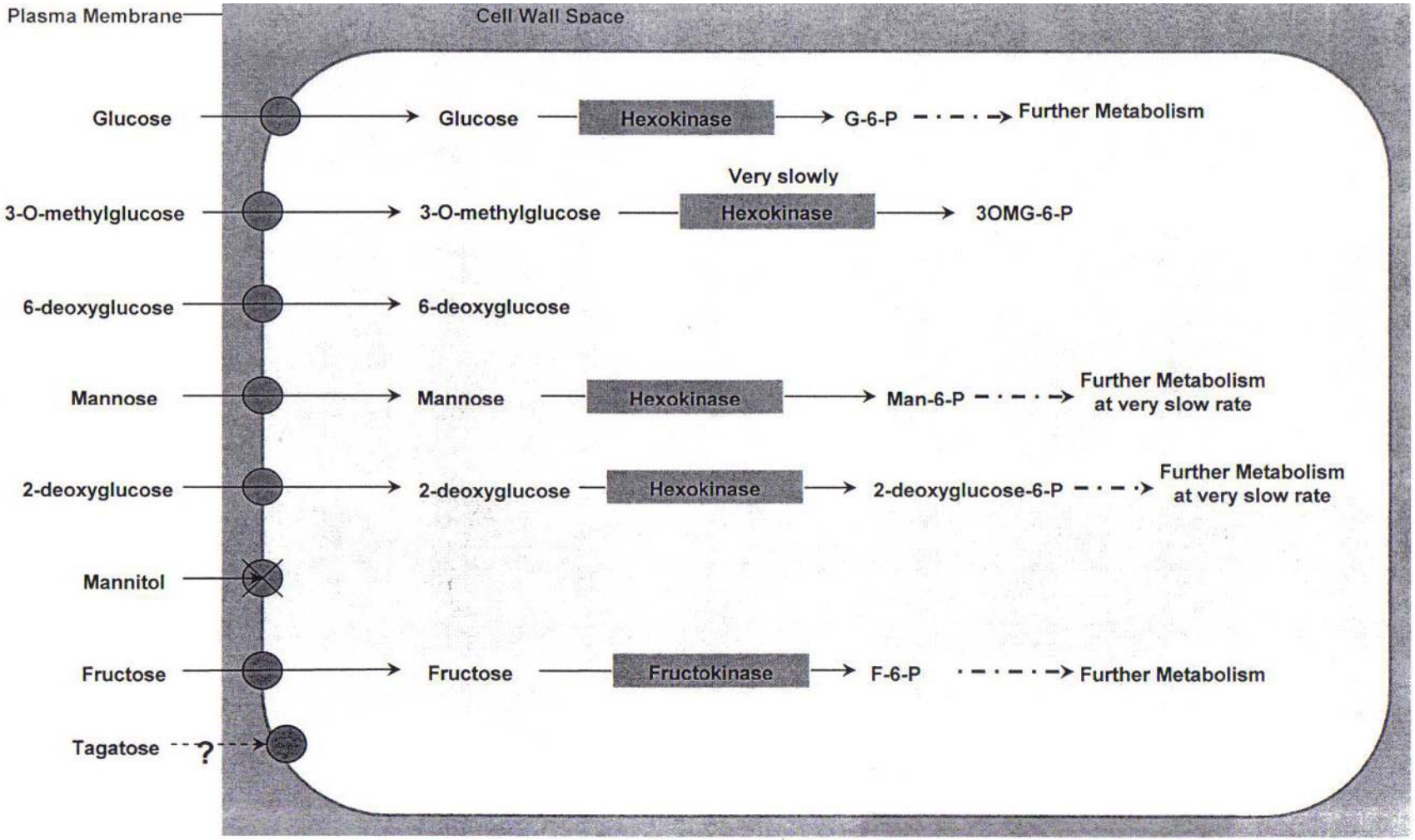

Figure 3 Monosaccharide metabolism and role of monosaccharide analogs in plant cells taken from (Wang, 2006).
Key: ● monosaccharide transporter

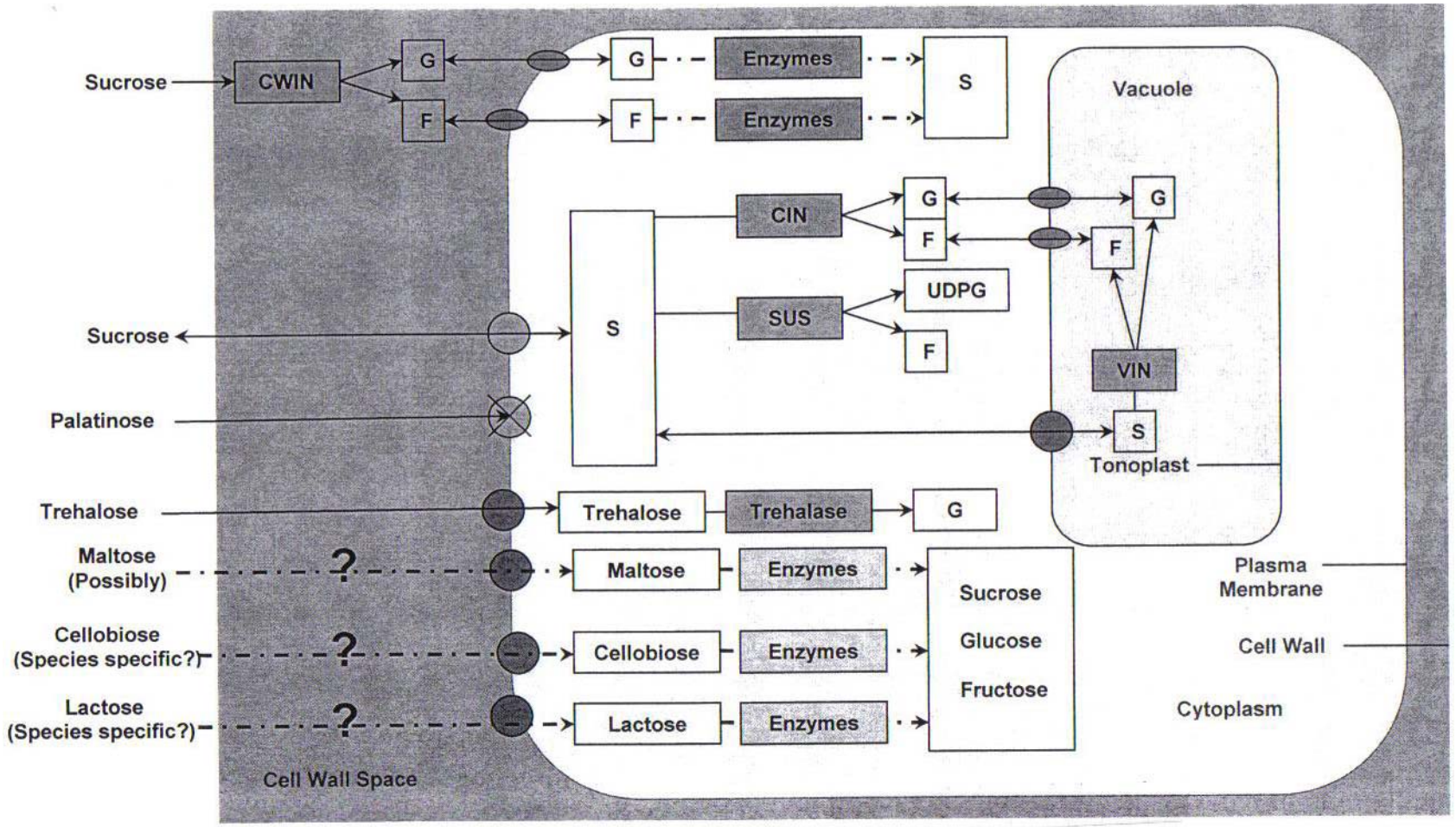

Figure 4 Disaccharide metabolism and role of disaccharide analogs in plant cells taken from (Wang, 2006).
Key: CWIN, cell wall intertase; CIN, cytoplasmic invertase; SUS, sucrose synthase; UDPG, UDP-glucose; VIN, vacuolar invertase; S, sucrose; G, glucose; F, Fructose; ● monosaccharide transporter; ● sucrose transporter; ● unknown transporter

1.4.2.1 Glucose signal transduction pathways

Currently in plants there are three glucose signal transduction pathways proposed. These pathways are based on analyses that have been done on growth and development as well as gene expression and enzyme activity.

1.4.3 Hexokinase as a sugar sensor

Yeast glucose signal transduction has been well studied. Four glucose signaling pathways have been found in yeast, three of which involve hexokinase (Rolland *et al*., 2006). Hexokinase 2 (HXK2) in yeast regulates two different pathways. One of them is the activation of a pathway leading to inactivation of sucrose nonfermenting1 (Snf1) (Moreno *et al*., 2005). Snf1 is similar to a mammalian protein that is responsible for phosphorylation of Mig1, which, when phosphorylated, dissociates from a repressor complex (Rolland et al., 2006). HXK2 can also directly interact with Mig1 to recruit co-repressors (Moreno et al., 2005). There is another regulatory pathway which involves a duel mechanism. This pathway depends on both extracellular glucose or sucrose sensing by a G-protein (Lemaire *et al*., 2004), and uptake of glucose followed by phosphorylation by a hexokinase or glucokinase (Rolland et al., 2006).

There have been many studies focused on the role that hexokinase may play as a sugar sensor in plants. Jang and Sheen (1994) showed that in maize, hexokinase is a sensor in regulation of respiration. They found that 2-deoxyglucose (2-dG), which can be phosphorylated by hexokinase but not further metabolized, when delivering directly into cells causes repression of photosynthetic genes. They also found that 6-deoxyglucose and 3-O-methylglucose (3OMG), which is not efficiently phosphorylated by hexokinase, can not act as a glucose signal.

Using transgenic plants Jang *et al.* (1997) showed that over expression of hexokinase (AtHXK1) in *Arabidopsis* causes hypersensitivity to sugars and that its under expression causes hyposensitivity. They compared the transgenic to wild type plants grown in the same conditions and observed that glucose acts as an inhibitor of greening and growth in seedlings. Seedlings over expressing hexokinase were more inhibited by glucose, while the seedlings under expressing hexokinase grew well, despite glucose. To show that this was due to glucose sensing, Jang *et al*. (1997) also grew the plants on 2-dGlu and there was no difference compared to the glucose-grown seedlings. They also looked directly at the expression level of several genes and found that hexokinase is responsible for sugar repressed genes and sugar induced genes.

1.4.3.1 Localization

Most organisms have multiple isoforms of hexokinase (Olsson *et al*., 2003). For example, yeast has three hexokinases and mammals have four. In *Arabidopsis* there are six hexokinase isoforms (Gonzali, 2002), and rice (*Oryza sativa L*.) has at least ten (Jung-Il *et al.*, 2005). This raises the question: Why do organisms need more than one form of hexokinase? Multiple forms suggest that different hexokinases may have different roles in cells as primary messengers for signal transduction in order to route carbon into diverse locations or pathways within a cell. The localization of different hexokinases is discussed below and summarized in Table 1.

Subcellular locations of the different hexokinases are not yet well known. Locations of hexokinase are of interest because this could provide clues as to the

Table 1. Summery of subcellular localization of hexokinase isoformes.

Hexokinase Form	Species	Compartment location	Reference
Chloroplast Stroma			
PpHxk1	*Physcomitrella patens*	Chloroplast stroma and maybe thylakoid; has -RR- motif in transit peptide	Olsson *et al.* (2003); Summer *et al.* (2000)
NtHxk2	*Nicotiana tabacum*	Chloroplast stroma	Giese *et al.* (2004)
OsHxk4	*Oryza sativa*	Chloroplast; maybe stroma	Jung-Il *et al.* (2005)
Chloroplast membrane			
PpHxk2	*P. patens*	Chloroplast outer membrane	Olsson *et al.* (2003)
SoHxk1	*Spinacia oleracea*	Chloroplast outer membrane	Weise *et al.* (1999)
AtHxk1	*Arabidopsis thaliana*	Maybe chloroplast outer membrane, because of conserved hydrophobic N-terminal sequence	Olsson *et al.* (2003)
AtHxk2	*A. thaliana*	Maybe chloroplast out membrane, because of conserved hydrophobic N-terminal sequence	Olsson *et al.* (2003)
Cytoplasm			
OsHxk7	*O. sativa*	Cytoplasm	Jung-Il *et al*. (2005)
Mitochondrial Membrane			
PsHxk?[1]	*Pisum sativum*	Mitochondrial membrane	Cosio and Bustamante (1984)

[1] no specific hexokinase was found, only hexokinase activity in mitochondrial membranes fractions.

functional role of the hexokinase isoforms within a cell. The precursors of artemisinin can come from different compartments within the cell. IPP, the major five carbon precursor of all terpenoids, is derived from acetyl-CoA in the cytosol, and from pyruvate in the plastid (Croteau *et al.*, 2000). Hexokinases have been found to be localized not only to the chloroplast outer envelope of spinach leaves (Weise *et al*., 1999), but also bound to the membrane of mitochondria in pea leaves (Cosio and Bustamante, 1984). A recent study has found another novel form of hexokinase (PpHxk1) in the moss, *Physocomitrella patens*. This hexokinase is not bound to the chloroplast outer membrane, but located instead within the chloroplast (Olsson *et al*., 2003). The transit peptide of this internal chloroplast hexokinase contains at least one -RR- motif that further suggests it may be targeted to the thylakoid (Summer *et al.*, 2000). Olsson *et al*. (2003) found another type of hexokinase (PpHxk2) localized to the chloroplast outer membrane. After comparing both hexokinases to those found in a variety of plants, they proposed two general types of hexokinases: a membrane bound hexokinase with highly conserved hydrophobic N-terminal sequences thought to be a membrane anchor, and a hexokinase with a somewhat less conserved N-terminal chloroplast transit peptide acting as a signal for importation into the chloroplast.

In another recent study a functional analysis of *Oryza sativa L*. hexokinases also showed that the two hexokinases, OsHXK4 and OsHXK7 were also located in two different subcellular compartments (Jung-Il *et al*., 2005). OsHXK7 was localized to the cytoplasm and OsHXK4 was localized to the chloroplast. OsHXK4 also was shown to have a predicted chloroplast transit peptide suggesting that it may be imported to the chloroplast stroma (Jung-Il *et al*., 2005). They found that OsHXK4 shares a similar

amino acid sequence to NtHxk2, which has been localized to the chloroplast stroma (Giese *et al*., 2004), further suggesting that OsHXK4 may also be localized to the chloroplast stroma.

Olsson *et al*. (2003) proposed that the PpHxk1 provides a mechanism for bypassing glucose-6-P translocation during times of limited energy supply or when plants were in the dark portions of the photo period. In contrast Wiese *et al*. (1999) proposed that in spinach the membrane bound hexokinase (SoHxk1) could directly phosphorylate glucose as it left the chloroplast. Wiese *et al*. (1999) did not consider SoHxk1 to be a sugar sensor, however, because it was membrane bound; this conclusion was based on the yeast hexokinase sugar sensors which have been characterized as not being membrane bound (Wiese *et al*., 2000). On the other had the *Arabidopsis* hexokinases, AtHxk1 and AtHxk2, both show sugar sensing properties (Jang, *et al*., 1997), yet both also have a conserved hydrophobic sequence suggesting that they are also embedded in a membrane. Olsson *et al*. (2003), thus, predicted that most plants may have similar membrane-bound hexokinases and, if similar to AtHxk1 and AtHxk2, they could also act as sugar sensors. Clearly, identification of which hexokinases are functioning as sugar sensors requires further analysis. In addition, it may be that specific hexokinases act as sensors for very specific metabolic processes, but not for others. Considering that growth and primary metabolites are often not linked to secondary metabolism, it would be reasonable for plant cells to have different sugar regulators, different hexokinases, "in charge" of these different metabolic processes.

2. Objectives

As a first step in determining how broadly sugars affect terpenoid metabolism in *A. annua*, it is important to measure the diversity of terpenoids in shoots and roots in response to different sugars, and to compare these responses to the well studied plant model, *Arabidopsis thaliana*. This project therefore has two main objectives:

1) To compare the terpenoid TLC profiles of plantlets of *A. annua* and *A. thaliana* grown in glucose, sucrose, and fructose.
2) To survey the literature in order to localize in the cell the different hexokinase enzymes found in *A. thaliana* and possibly other plants.

3. Methods

3.1 Sugar Profile Studies

3.1.1 Seed Sterilization

Artemisia annua L. seeds (YU strain, 2005 WPI crop) were imbibed in water for 30 minutes before sterilization. They were then surface sterilized in 10% (v/v) bleach for 12 minutes followed by 70% (v/v) ethanol for 5 minutes. The seeds were then rinsed with 10ml 0.1% sterile PPM (Preservative for Plant Tissue Culture Media, Plant Cell Technology, Inc.), 3 times for 5 minutes each time. This same seed sterilization process was done for the *A. thaliana* seeds, except sterile diH_2O was substituted for the 0.1% PPM. Further changes to the seed sterilization and growth condition processes for A. thaliana are shown in Table 2.

Table 2. Seed Sterilization and Growth Conditions for *A. annua* and *A. thaliana*

A. annua	***A. thaliana***						
1st attempt	1st attempt	2nd attempt[1]	3rd attempt[1]	4th attempt	5th attempt[1]	6th attempt[1]	7th attempt
H_2O; 30 min.	H_2O; 30 min.	H_2O; 30 min.	H_2O; 30 min.	H_2O; 30 min.	H_2O; 30 min.	H_2O; 30 min.	H_2O; 30 min.
10% bleach; 12 min.	10% bleach; 12 min.	10% bleach; 12 min.	10% bleach; 12 min.	10% bleach; 12 min.	10% bleach 12 min.	10% bleach; 12 min.	10% bleach; 5 min.
70% ethanol; 15 min.	70% ethanol; 15 min.	70% ethanol; 15 min.	70% ethanol; 15 min.	70% ethanol; 15 min.	70% ethanol; 15 min.	70% ethanol; 15 min.	70% ethanol; 15 min.
0.1% sterile PPM 3X; 5 min.	0.1% sterile PPM 3X; 5 min.	Sterile H_2O 3X; 5 min.	Sterile H_2O 3X; 5 min.	Sterile H_2O 3X; 5 min.	Sterile H_2O 3X; 5 min.	Sterile H_2O 3X; 5 min.	Sterile H_2O 3X; 5 min.
Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days
Sterile PPM replaced by sterile Gamborg's B5 medium with 3% sucrose	Sterile H_2O replaced by sterile Gamborg's B5 medium with 3% sucrose	Sterile H_2O replaced by sterile Gamborg's B5 medium with 3% sucrose	Sterile H_2O replaced by sterile Gamborg's B5 medium with 3% sucrose	Sterile H_2O replaced by sterile Gamborg's B5 medium with 3% sucrose	Sterile H_2O replaced by sterile Gamborg's B5 medium with 3% sucrose	Sterile H_2O replaced by sterile Gamborg's B5 medium with 3% sucrose	Sterile H_2O replaced by sterile Gamborg's B5 medium with 3% sucrose
Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days	Dark refrigerator for 3 days
Continuous cool white fluorescent light at 24°C at 140 rpm for 5 days	Continuous cool white fluorescent light at 24°C at 100 rpm for 5 days	Continuous cool white fluorescent light at 24°C at 140 rpm for 5 days	Continuous cool white fluorescent light at 24°C at 140 rpm for 5 days	Continuous cool white fluorescent light at 24°C at 140 rpm for 5 days	Continuous cool white fluorescent light at 24°C at 140 rpm for 5 days	Continuous cool white fluorescent light at 24°C at 100 rpm for 5 days	Continuous cool white fluorescent light at 24°C at 100 rpm for 5 days
Rinsed 7 times with sugar-free sterile Gamborg's B5 medium	Rinsed 7 times with sugar-free sterile Gamborg's B5 medium	Contamination; seeds were discarded before reaching final steps	Contamination; seeds were discarded before reaching final steps	No germination after 5 days on shaker	No germination after 5 days on shaker	No germination after 5 days on shaker	Contaminated after 2 days on shaker
Dark refrigerator for 1 day	Dark refrigerator for 1 day	Dark refrigerator for 1 day					
Seeds used in Wang's experiments	Seeds from GH 06	Seeds from GH 06	Seeds purchased from Lehle Seeds	First generation seeds from purchased seeds	Seeds from GH 06	Seeds from GH 06	Seeds from GH 06
Successful sterile germination	Successful sterile germination; all seedlings died after 1 week of growth on plates	Unsuccessful; contamination	Unsuccessful; contamination	Unsuccessful; no germination	Unsuccessful; no germination	Unsuccessful; no germination	Unsuccessful; contaminated before seeds could germinate.

Yellow denotes change in procedure from the previous attempt.

[1]attempted at least twice

3.1.2 Growth Conditions

Sterilized seeds of *A. annua* were imbibed in 30ml 0.1% sterile PPM in a 125ml Erlenmeyer flask in the dark for 3 days in the refrigerator. The 0.1% sterile PPM was then replaced by 30ml sterile Gamborg's B5 medium (Gamborg *et al*., 1968) with 3% (w/v) sucrose at pH 5.7 and seeds were incubated for 3 additional days in the dark in the refrigerator. Seeds were then transferred to continuous cool-white fluorescent light (100 μ E-2s-1) at 24ºC, and at 140rpm (Lab-line Orbit Shaker, Lab-Line Instruments Inc., Melrose Park IL) for 5 days. After 5 days most of the seeds developed to the two-cotyledon stage. The seedlings were then rinsed 7 times by decanting the medium and replacing it with about 50ml of sugar-free B5 medium. The seedlings were left in 50ml of sugar-free B5 medium and placed in the refrigerator for 1 day. Ten seedlings at the two cotyledon stage that looked to be of equal size and health were selected and then inoculated into a 100x25 mm Petri dish containing 20ml autoclaved B5 medium with 0.23% (w/v) Phytagel and 3% (w/v) filter sterilized fructose, sucrose, or glucose. All sugars were filter sterilized using a 0.22μm sterile syringe filter before being added into the B5 medium. Cultures were kept at 24ºC under continuous cool-white fluorescent light (100 μ E-2s-1) for 14 days. This was essentially the same protocol described by Wang (2006).

3.1.3 Growth and Development Analysis

After the two-week growth period, pictures were taken of the seedlings still in their plates, and the dead or contaminated seedlings were discarded. The seedlings were then removed, rinsed with diH_2O, and blotted dry with paper towels. The number of true leaves on each plant was counted and recorded. Using a scalpel each seedling was cut at

the base of the hypocotyl to separate the root from the shoot. Each root or shoot was individually dried at 60ºC for at least 16 hours, and the dry mass of each was measured. The roots and shoots were left in the oven while each one was weighed so the intake of moisture while cooling would be limited.

3.1.4 Extraction of Artemisinin

The dried shoots that were harvested from the same Petri dish were pooled and extracted with 4ml toluene in a chilled water sonicator for 30 minutes. The supernatant was decanted, and the sonication was repeated twice more with 2ml toluene instead of 4ml. The 3 supernatants from this process were pooled and dried under nitrogen at 30ºC. Samples were stored in the -20ºC freezer until TLC analysis. The same procedure was used for extracting and analyzing terpenoids in the root portion of the plants harvested from each sugar condition.

3.1.5 TLC for Terpenoid Analysis

Extracted samples were pooled to give one sample of shoots and roots for each sugar then dried under nitrogen. These samples were then re-suspended in toluene and a volume of the extract equal to 2.5 mg dry weight plant material of roots or shoots from each sugar condition was spotted onto a plastic backed silica gel 60 TLC plate along with 20 μg of the artemisinin standard (AN) according to Table 3.

Table 3. A description of the samples spotted on the TLC plate for terpenoid analysis.

Lane	Sample	Total Amount (mg)	Re-suspension Volume (μL)	Spotted Volume (μL)
1	AN Standard	0.05	200.0	80
2	Glucose Shoots	14.66	211.0	36
3	Sucrose Shoots	23.92	287.0	30
4	Fructose Shoots	20.24	259.0	32
5	Glucose Roots	12.36	178.0	36
6	Sucrose Roots	24.03	221.0	23
7	Fructose Roots	19.52	203.0	26
8	AN Standard	0.05	200.0	80

TLC analysis was performed using benzene: methanol (90:10) mobile phase. The plate was sprayed with an acetic acid: sulfuric acid: p-anisaldehyde (97:2:1) solution, and heated for approximately 5 minutes at 110^{o}C to visualize artemisinic compounds and other terpenes (Driggs, 2006). Artemisinin appeared a fuchsia pink. Other terpenoids were green, brown, and yellow.

3.1.6 Data Analysis

All experiments were done at least in triplicate, and the data were statistically analyzed using the two-tailed t-test function of Microsoft Excel. TLC RF's were calculated based on the center of observed spots and relative to the distance the solvent front traveled from the origin.

4. Results and Discussion

4.1 Sugar Effects on Seedling Growth and Terpenoid Profiles

Our initial objectives included comparative growth and terpenoid profiling of both, *A. annua* and *A. thaliana* seedlings after growth in glucose, fructose, or sucrose.

Unfortunately we were unable to obtain healthy, sterile, seedlings of *A. thaliana* for analysis. Only results from *A. annua* seedlings are, thus, presented here.

Sugars can be transported in and out of plant cells with little effort, and can be transformed into other sugars where they can be metabolized through glycolysis and used as an energy source. To test the effects of glucose, fructose, and sucrose on the growth of *A. annua,* seedlings were grown in Petri dishes containing B5 medium with 3% (w/v) sucrose, glucose, or fructose. The seedlings were grown for 14 days before harvest and analysis. The glucose and fructose results were compared to sucrose because it is the sugar that is normally used for growing seedlings. Only true leaves were counted.

4.1.1 Sugar effects on seedling growth

Seedlings of *A. annua* grown in sucrose had the highest average biomass, followed by seedlings grown in fructose; seedlings grown in glucose had the least biomass (Figure 5). These data suggest that artemisinin plantlets grow best in sucrose, followed by fructose; they grew poorest in glucose. Our sucrose and glucose averages were similar to Wang's findings; however, in contrast to Wang's work we found growth was inhibited by glucose compared to sucrose. Wang found that the average biomass was highest for the seedlings grown in sucrose, with about 6.4 mg. Seedlings grown in glucose had the next highest average of about 6.0 mg, and fructose had the lowest average biomass of about 5.4 mg (Wang, 2006).

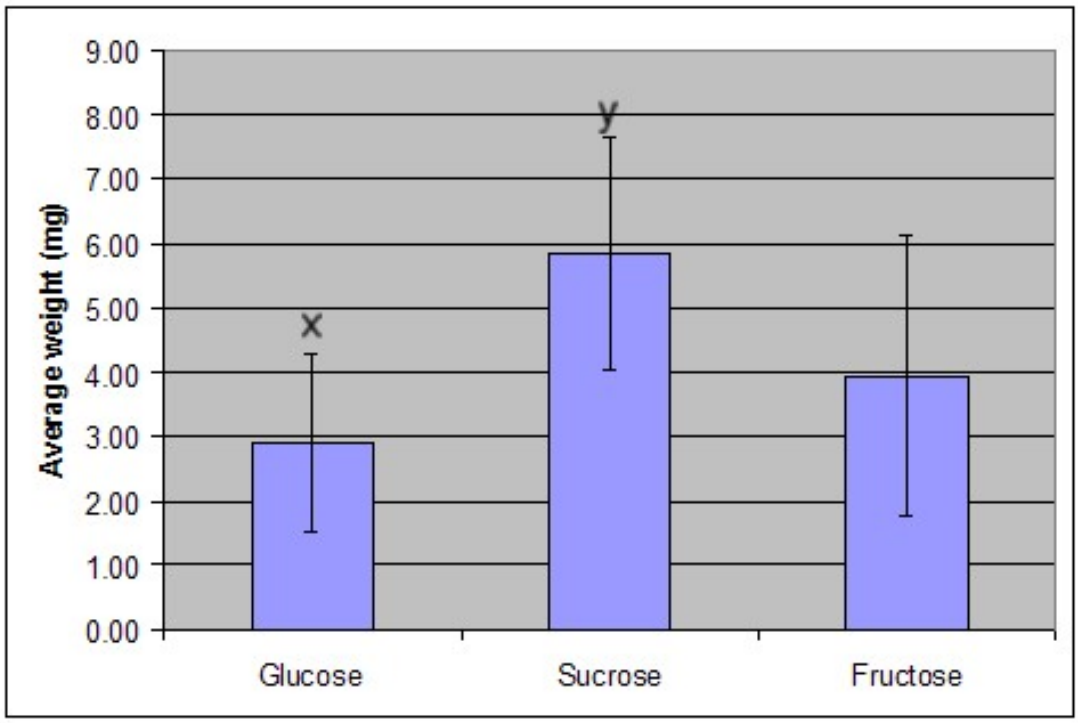

Figure 5. Effects of sucrose, glucose, and fructose on total biomass of *A. annua*. Average weight of pooled seedlings after 14 days of growth in glucose, fructose or sucrose at 30 g/L. Each group had 24 to 22 seedlings; outliers were eliminated from data. Letters above bars indicate statistical significance: x,y, sucrose vs. glucose in roots, $p<0.01$. A t-test was done using Microsoft Excel comparing glucose to sucrose and fructose to sucrose. The test did not show fructose to be significantly different from sucrose. Error bars represent plus or minus one standard deviation.

The average weight of the seedlings grown in sucrose was the highest of all these sugars for both roots and shoots (Figure 6). In contrast lowest seedling weight for both roots and shoots was observed when glucose was the sole carbon source (Figure 6). These data are similar to the growth responses shown in Figure 5. Wang, on the other hand, found that in comparison to sucrose the shoot biomass of seedlings grown in fructose or glucose was not significantly different, but the root biomass of glucose, about 2.3 mg, was significantly lower than the biomass of seedlings grown in sucrose, about 3.1 mg (Wang, 2006). Weathers *et al.* (2004) found similarly that growth of hairy root cultures of *A. annua* was inhibited when grown in glucose compared to sucrose; growth

in fructose was not significantly different from sucrose. When roots were grown in sucrose in combination with glucose or fructose, results varied even more. While sucrose and glucose stimulated root growth, sucrose and fructose inhibited it (Weathers *et al.*, 2004). In contrast equivalent molar amounts of all these sugars gave the best growth overall. Clearly sugars have an intriguing and complex effect on growth.

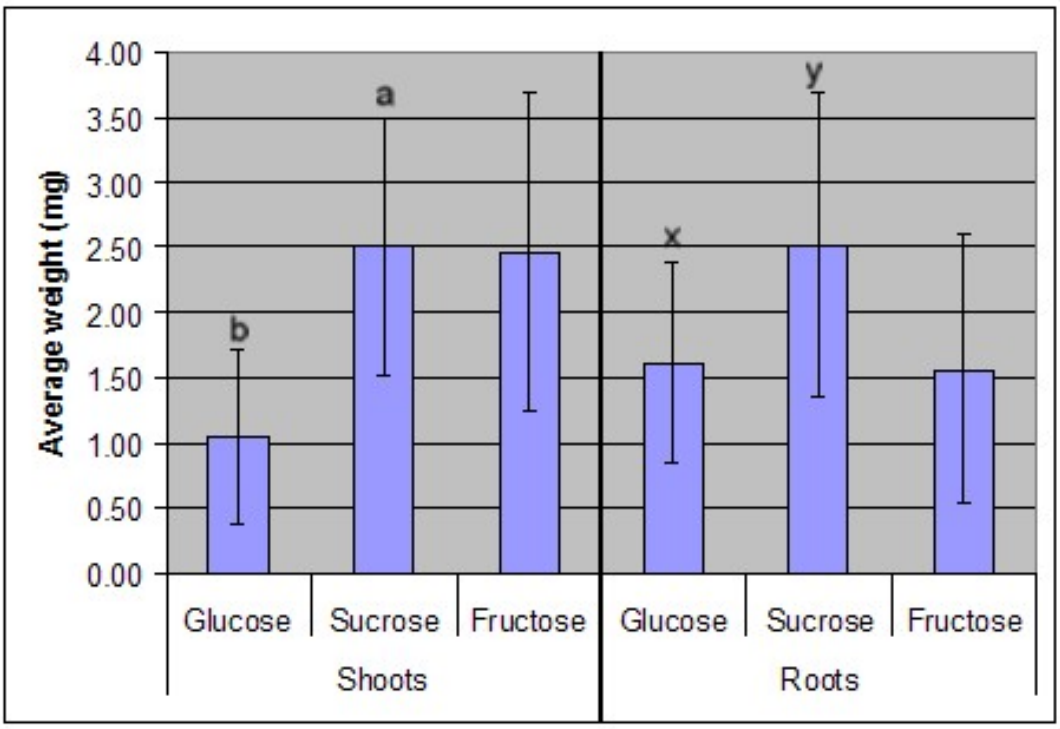

Figure 6. Effects of sucrose, glucose, and fructose on root and shoot biomass of A. annua. Average weight of pooled shoots and pooled roots after 14 days growth in glucose, fructose or sucrose at 30 g/L. Each group had 24-25 seedlings; outliers were eliminated from data. Each seedling shoot or root was weighed individually. Letters above bars indicate statistical significance: a,b, sucrose vs. glucose in roots; x,y, sucrose vs. glucose in shoots, $p<0.01$. A t-test was done using Microsoft Excel comparing glucose to sucrose and fructose to sucrose. Error bars represent plus or minus one standard deviation. glucose to sucrose and fructose to sucrose. Error bars represent plus or minus one standard deviation.

When shoot to root ratios were calculated for seedlings grown in each sugar, fructose had the highest shoot to root ratio followed by sucrose, and then glucose (Figure 7). This shows that, compared to glucose or sucrose, seedlings grown in fructose grew larger shoots. Wang's results showed that glucose yielded the highest shoot to root ratio of about 2.1. Sucrose and fructose had equally lower shoot to root ratios of about 1.2 (Wang, 2006). Although our results contradict Wang's study, they are consistent with the results of Weathers *et al*. (2004) which showed that glucose inhibited hairy root growth.

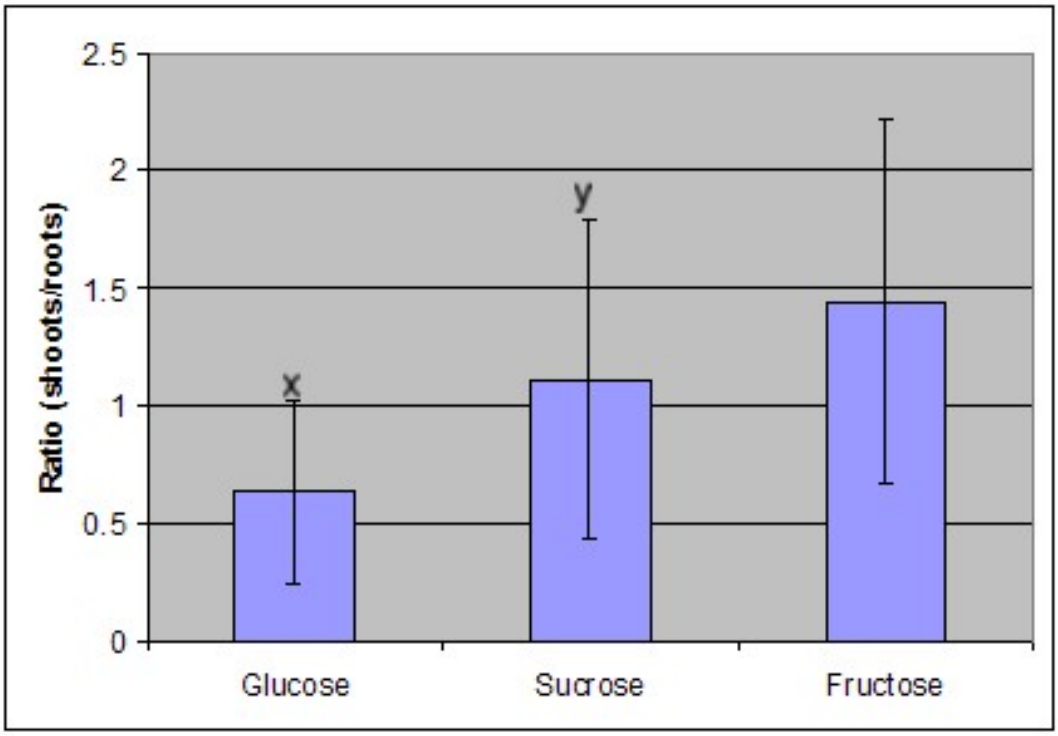

Figure 7. Shoot to root ratio of seedlings grown in glucose, sucrose and fructose. Shoot verse root ratios for seedlings grown for 14 days in 30g/L of sucrose, glucose or fructose. Each group had 24 seedling; outliers were eliminated from data. Letters above indicate statistical significance: x,y, sucrose vs. glucose; a,b, sucrose vs. fructose, $p<0.01$. A t-test was done using Microsoft Excel comparing glucose to sucrose and fructose to sucrose.

Seedlings of *A. annua* grown in glucose and fructose have fewer leaves than seedlings grown in sucrose (Figure 8). These results contradict Wang's findings, which showed more leaf production in glucose than in sucrose. Wang reported that in comparison to sucrose the number of leaves on seedlings grown in glucose was greater, about 6.5 average leaves, and the number of leaves on seedlings grown in fructose was lower, about 4.6 leaves (Wang, 2006).

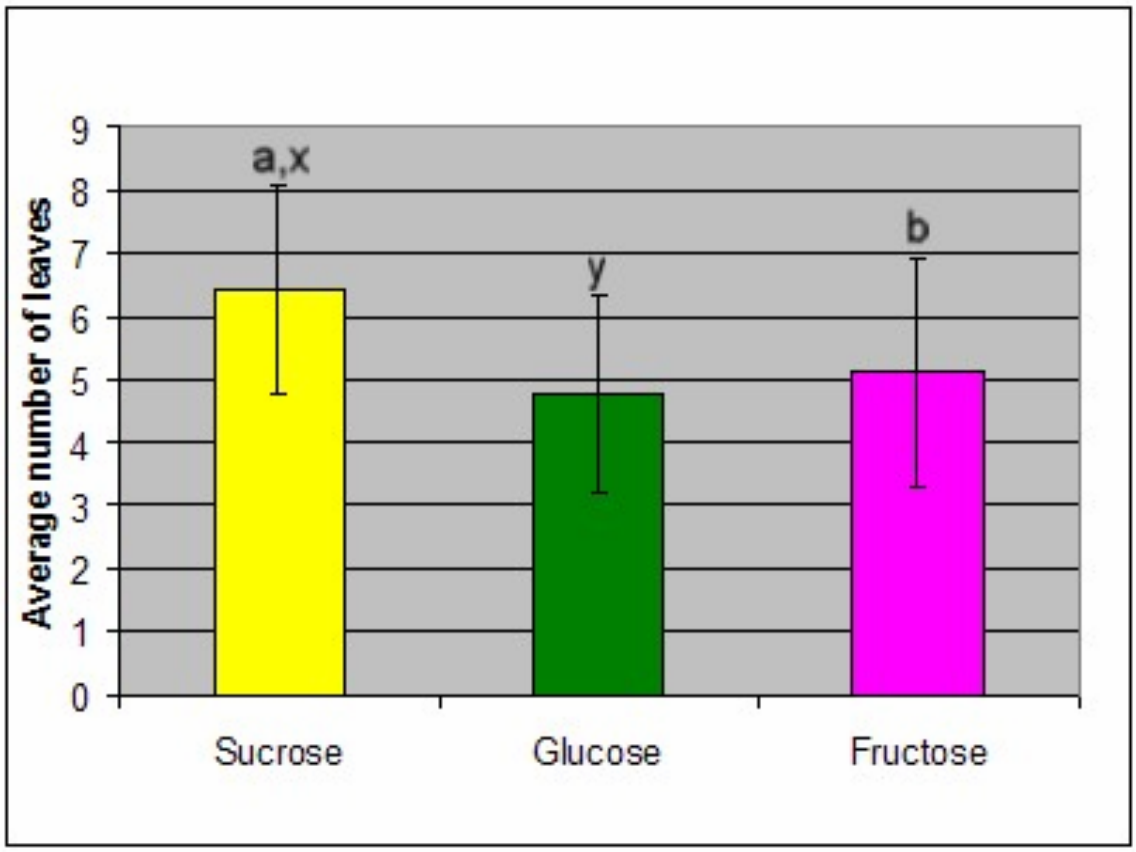

Figure 8. Effects of sucrose, glucose, and fructose on leaf count.
Average number of leaves for seedlings grown for 14 days in 30g/L of sucrose, glucose or fructose. Each group had at least 28 plants. Letters above indicate statistical significance: x,y, sucrose vs. glucose; a,b, sucrose vs. fructose, $p<0.01$. A t-test was done using Microsoft Excel comparing glucose to sucrose and fructose to sucrose.

4.1.2 Terpenoid profiles for A. annua seedlings grown in sucrose, glucose, and fructose

The TLC results showed that considerably more terpenes were produced in *A. annua* shoots than in roots (Figure 9). Relative R_f values are shown in Table 4. The diversity of terpenes in roots is different from that in shoots; some terpenes present in one organ are absent in the other. Although it appeared that more terpenes were produced in glucose-fed seedlings than in seedlings fed sucrose or fructose, results were not definitive. Wang (2006) reported that in *A. annua* seedlings shoots grown in glucose increased artemisinin production compared to seedlings grown in sucrose or fructose. This suggested there was a link between glucose and the terpenoid pathways. Weathers *et al.* (2004) also saw an increase in artemisinin levels when roots were grown in glucose alone. They did not, however, see any significant difference in artemisinin production when glucose was combined with sucrose. Compared to fructose alone, Weathers *et al*. (2004) observed an inhibition in artemisinin production when fructose and sucrose were combined and fed to roots. Because glucose is a more preferred substrate for phosphorylation by hexokinase compared to fructose (Olsson *et al*., 2003), these data also suggested that glucose was acting as a signal that was possibly being sensed by hexokinase to affect terpenoid metabolism. Wang's data also suggest that glucose was acting as a signal that was possibly being sensed by hexokinase to affect terpenoid metabolism.

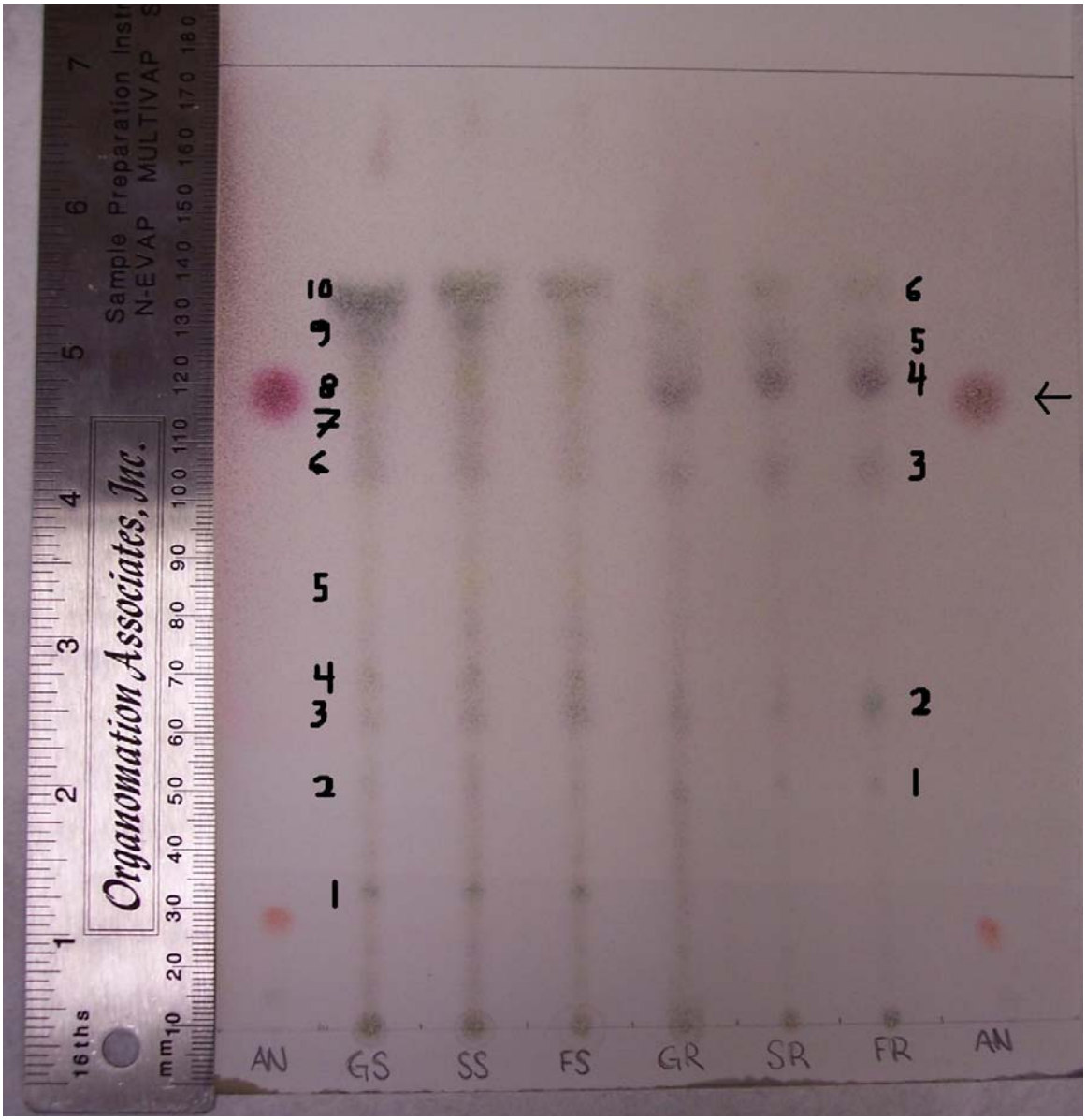

Figure 9. TLC of seedlings extracted after 14 days grown on sucrose, glucose or fructose. AN, artemisinin marker, indicated by arrow. GS, Glucose shoots; SS, sucrose shoots; FS, fructose shoots; GR, glucose roots; SR, sucrose roots; FR, fructose roots. Each lane was extracted from a pool of at least 28 plants.

Table 4. R_f values calculated for the TLC plate shown in Figure 7.

	Distance traveled (cm)		
Solvent	16.7	R_f	Color
AN	11.1	0.66	Pink/Brown
Shoot Spots			
1	2.5	0.15	Green
2	4.1	0.24	Green
3	5.4	0.32	Green
4	6.2	0.37	Brown
5	7.7	0.46	Yellow
6	9.8	0.59	Brown
7	10.5	0.63	Brown
8	11.0	0.66	Yellow
9	12.0	0.72	Green
10	12.6	0.75	Green
Root Spots			
1	4.1	0.24	Green
2	5.4	0.32	Green
3	9.8	0.59	Brown
4	11.2	0.67	Brown
5	11.6	0.69	Brown
6	12.6	0.75	Yellow

5. Conclusions

At the same carbon level *A. annua* grew better in sucrose than glucose, and there was no significant change in growth between sucrose and fructose. Collectively these data do not match those found by Wang (2006). There was a significant difference in terpenoids produced in the shoots verses the roots in all three sugars. Jang *et al.* (1997) transformed *Arabidopsis thaliana* with binary vectors with fusions of the cauliflower mosaic virus 35S RNA promoter and AtHXK1 and AtHXK2 in sense and anti-sense orientations. They used a constitutive promoter to bypass transcriptional regulation. Hypersensitivity to glucose was seen in the plants over expressing hexokinase in the form of stunted growth and lack of greening in seedlings. It may be possible that a hypersensitivity to hexokinase could cause over production of artemisinin and other terpenoids in *A. annua*. If *A. thaliana* shows a similar terpenoid profile it would suggest that terpenoids are regulated similarly by glucose in both *A. thaliana* and *A. annua*. However, specific hexokinase enzymes and their corresponding genses would have to be isolated and located to over express hexokinase in *Artemisia*. Hexokinase located in the cytoplasm would be a likely target since the sesquiterpene pathway is located in the cytoplasm (Rodríguez-Concepción et al., 2004). The compilation of hexokinase localizations in Table 1 provided information such as the specific hexokinase number, plant species and location within the cell. This table will aid future research because it may be used conveniently to design experiments to answer basic questions on hexokinase location and function in plant terpenoid biochemistry. It would be interesting to learn more about where hexokinases are located in *A. annua* and whether any specific

hexokinase might be responsible for the signal transduction pathway that leads to the increased production of artemisinin.

6. References

Abdin, M. Z., Israr, M., Rehman, R. U., Jain, S. K. (2003). Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. *Planta Med.* 69: 289-299.

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch J., Springer J. (1980). Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol- lowering agent. *Proc. Natl. Acad. Sci*. USA 77: 3957–3961.

Balint, G. (2001). Artemisinin and its derivatives an important new class of antimalarial agents. *Pharmacology & Therapeutics.* 90: 261-265.

Chen, J., Jones, A. M. (2004). AtRGS1 function in *Arabidopsis thaliana*. *Methods in Enzymology.* 389: 338-350.

Cosio, E., Bustamante, E. (1984). Subcellular localization of hexokinase in pea leaves. *J. Bio. Chem*. 259(12): 7688-7692.

Croteau, R., Kutchan, T., Lewis, N. (2000). Natural products (secondary metabolites). In Buchanan, B., Gruissem, W., Jones, R. (Eds.) *Biochemistry & Molecular Biology of Plants*. Rockville, Maryland: American Society of Plant Physiologts. Pp. 1250-1317.

Driggs, M. D. (2006). Is a precursor to the anti-malarial drug, artemisinin, glycosylated *in planta*? Major Qualifying Project. Biology and Biotechnology. Worcester Polytechnic Institute.

Gamborg O.L., Miller R.A., Ojima K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research.* 50: 151-158.

Gonzali, S., Amedeo, A., Blando, F., Bellis, L. (2002). *Arabidopsis* and yeast hexokinase overexpressed in transgenic lines are characterized by different catalytic properties. *Plant Science*. 163: 943-954.

Jang, J., Sheen, J. (1994). Sugar sensing in higher plants. *The Plant Cell*. 6: 1665-1679.

Jang, J., Leon, P., Zhou, L., Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. *The Plant Cell*. 9: 5-19.

Jung-Il C., Nayeon R., Seho K., Sang-Kyu L., Junok L., Ki-Hong J., Youn-Hyung L. Seong H. B., Joris W., Gynheung A., Tae-Ryong H., Jong-Seong J. (2005) Structure, expression, and functional analysis of the hexokinase gene family in rice (*Oryza sativa L.*) *Planta*. 224: 598–611.

Koch, K.E. (1996). Carbohydrate modulated gene expression in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 509–540.

Larronde, F., Krisa, S., Decendit, A., Chèze, C., Deffieux, G., Mérillon, J. (1998) Regulation of polyphenol production in *Vitis vinifera* cell suspension cultures by sugars. *Plant Cell Reports*. 17: 946–950.

Lemaire, K., Velde, S., Dijck, P., Thevelein, J. (2004) Glucose and sucrose act as agonist and mannose as antagonist ligands of the G Protein-coupled receptor gpr1 in the yeast *Saccharomyces cerevisiae*. *Molecular Cell*. 16: 293–299.

Meshnick, S., Taylor, T., Kamchonwongpaisian, S. (1996). Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbio. Rev.* 60(2): 301-315.

Morenol, F., Ahuatzi, D., Riera, A., Palomino, C., Herrero, P. (2005) Glucose sensing through the Hxk2-dependent signaling pathway. *Biochemical Society Transactions.* 33: 265-268.

Olsson, T., Thelander, M., Ronne, H. (2003). A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylation enzyme in the moss *Physcomitrella patens*. *J. Bio. Chem*. 278(45): 44439-44447.

Pandey, A., Tekwani, B., Singh, R., Chauhan, V. (1999). Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification system in the malarial parasite. *J. Bio. Chem.*, 274(27): 19383-19388.

Rodríguez-Concepción, M., Forés, O., Martínez-García, J.F., González, V., Phillips, M.A., Ferrer, A., Boronat, A. (2004). Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during *Arabidopsis* seedling development. *The Plant Cell*. 16: 144-156.

Rolland F., Baena-Gonzalez E., Sheen J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* 57: 675-709.

Sheen, J., Zhou, L., Jang, J.C. (1999). Sugars as signaling molecules. *Curr. Opin. Plant Biol.* 2: 410–418.

Smeekens, S. (2000). Sugar-induced signal transduction in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 51: 49–81.

Steliopoulos, P., Wüst, M., Adam, K.P., Mosandl, A. (2002). Biosynthesis of the sesquiterpene germacrene D in *Solidago canadensis*: 13C and 2H labeling studies. *Phytochemistry.* 60:13–20.

Summer E., Mori H., Settles A., Cline K. (2000) The thylakoid delta pH-dependent pathway machinery facilitates RR-independent N-tail protein integration. *J. Biol. Chem*. 275(31): 23483-23490.

Vitrac, X., Larronde, F., Krisa, S., Decendit, A., Deffieux, G., Merillon, J. (2000). Sugar sensing and Ca2+-calmodulin requirement in *Vitis vinifera* cells producing anthocyanins. *Phytochemistry.* 53(6): 659-65.

Wang, Y. (2006). Sugar control of artemisinin production. MS Graduate Thesis, Biology and Biotechnology. Worcester Polytechnic Institute.

Weathers, P. J., Cheetham, R. D., Teoh, K. (1994). Artemisinin production by transformed roots of *Artemisia annua*. *Biotech. Letters.* 16(12): 1281–1286.

Weathers, P. J., Hemmavanh, D. D., Walcerz, D. B., Cheetham, R. D., Smith, T. C. (1997). Interactive effects of nitrate and phosphate salts, sucrose, and inoculum culture age on growth and sesquiterpene production in *Artemisia annua* hairy root cultures. *In Vitro Cell. & Devel. Biol. – Plant.* 33: 306–312.

Weathers, P. J., DeJesus-Gonzalez L., Kim, Y. J., Souret, F. F., Towler, M. J. (2004). Alteration of biomass and artemisinin production in *Artemisia annua* hairy roots by media sterilization method and sugars. *Plant Cell Reports*. 23: 414–418.

Wiese, A., Groner, F., Sonnewald, U., Deppner, H., Lerchl, J., Hebbeker, U., Flugge, U., Weber, A. (1999). Spinach hexokinase I is located in outer envelope membrane of plastids. *FEBS lett.*, 461: 13-18.

Woerdenbag, H., Luers, J., Uden, W., Pras, N., Malingre, T., Alferman, W. (1993). Production of new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. *Plant Cell, Tissue and Organ Culture*. 32(2): 247-257.

World Health Organization (WHO). (2004). *Malaria Medicines and Supplies Service (MMSS)*. Retrieved September 21, 2006, from http://www.rollbackmalaria.org.