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# Sugar Control of Artemisinin Production

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SUGAR CONTROL OF ARTEMISININ PRODUCTION

by

Yi Wang

A Thesis

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

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Degree of Master of Science

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Biotechnology

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APPROVED:

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# Abstract

The role of sugars as regulatory signals has mainly focused on their effects on plant growth, development, gene expression, and metabolism. Little, however, is known about their role in controlling secondary metabolism. Previous work in our lab showed that sugars affect the production of the sesquiterpene antimalarial drug, artemisinin, in hairy roots of *Artemisia annua*. In this study, sugars alone or in combination with their analogues were used to investigate if sugars control artemisinin production in *Artemisia annua* seedlings. Compared to sucrose, a 200% increase in artemisinin by glucose was observed. When the glucose analog, 3-O-methylglucose, which is not phosphorylated effectively by hexokinase, was added with glucose, artemisinin production was dramatically decreased but hexokinase activity was significantly increased compared to glucose. In contrast, neither mannose, which can be phosphorylated by hexokinase, nor mannitol, which can not be transported into cells had any significant effect on artemisinin yield. When different ratios of fructose to glucose were added to seedlings, artemisinin yield was directly proportional to glucose concentration. Although addition of sucrose with glucose gave inconclusive results, sucrose analogues decreased artemisinin production compared to sucrose. These results suggested that both monosaccharide and disaccharide sugars may be acting as signal molecules thereby affecting the downstream production of artemisinin. Taken together, these experiments showed that sugars clearly affect terpenoid production, but that the mechanism of their effects appears to be complex.

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# 1. Introduction

## 1.1 Artemisinin

### 1.1.1 Artemisinin Chemistry and Function

Artemisinin (Figure 1) is the most efficacious antimalarial drug in the world to date (Abdin *et al.*, 2003) and it is only produced in *Artemisia annua* L. plants in very low amounts. Chinese scientists first isolated artemisinin from *A. annua* plants and the structure was later characterized by others as a sesquiterpene lactone with an

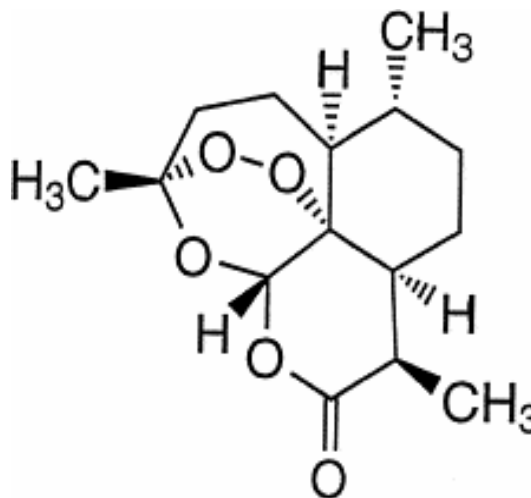


Figure 1 Structure of artemisinin.

endoperoxide linkage (Abdin *et al.*, 2003). This endoperoxide bridge rarely exists in natural products but is essential for the medical function of artemisinin (Balint, 2001; Woerdenbag *et al.*, 1990).

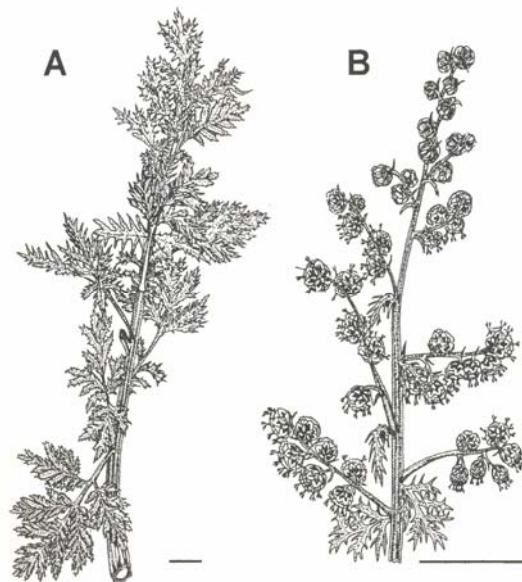
Artemisinin based drugs are the only antimalarials recommended by the World Health Organization (WHO) because of their safety and efficacy against all kind of malaria including cerebral malaria. Antitumor and antimicrobial functions have also been reported (Meshnick *et al.*, 1996; Singh and Lai, 2004; WHO, 2005a; Galal *et al.*, 2005).

### 1.1.2 *Artemisia. annua* L.

*Artemisia. annua*, is also known as sweet wormwood in the United States, and Qing Hao (Chinese: 青蒿) in China. As a Chinese annual herb, the pharmaceutical value of *A. annua* has been recognized since 168 B.C. and it has been used to treat fevers,

hemorrhoids, and malaria in China for centuries (Abdin et al., 2003).

*A. annua* is a qualitative short day plant (Ferreira and Janick, 1995). The mature plant with a single stem can reach about 2m in height. Aromatic leaves are about 2.5-5.0 cm long, deeply dissected and alternately branched around the stem (Figure 2). Two weeks after receiving an inductive stimulus, vegetative shoots develop into inflorescent shoots (Ferreira and Janick, 1995). The 2-3mm yellow nodding capitula are in loose panicles composed of many greenish or yellowish central florets which are bisexual and with little nectar and pistillate marginal florets. The central flowers which can be fertile or sterile are perfect and the anemophilous pollen is trifoliate and smooth, and may or may not have spines (Ferreira and Janick, 1996). Both non-glandular filamentous, 5-celled T-shaped, and biseriate 10-celled glandular trichomes have been found on the surfaces of leaves, stems, and flowers. At least 40 volatile compounds and a lot of nonvolatile compounds have been extracted from *A. annua* and identified (Ferreira and Janick, 1995 and 1996). Artemisinin and other artemisinic compounds are the most important compounds isolated from this plant due to their pharmaceutical value (Ferreira and Janick, 1995 and 1996).



**Figure 2 Vegetative (A) and flowering (B) shoots of *A. annua*.**  
Bar size = 1cm (Taken from Ferreira *et al.*, 1995)

Artemisinin production in whole *A. annua* plant ranges from 0.01 to 0.8% (w/w) (Abdin *et al.*, 2003). In whole plants, the artemisinin level in leaves and inflorescences are much higher than in stems, but in pollen or roots artemisinin is undetectable (Ferreira and

Janick, 1996). Although some have reported that in a single plant, artemisinin production was higher in the upper leaves than the lower leaves (Simon *et al.*, 1990; Duke *et al.*, 1994). Others have found that artemisinin content was evenly distributed (Ferreira and Janick, 1996). At different development stages, artemisinin production in *A. annua* has been reported to be variable but, again, the reports on changes were inconsistent (Woerdenbag *et al.*, 1990; Ferreira and Janick, 1996). Artemisinin is apparently stored in the glandular trichomes of *A. annua* and the glands of old leaves normally rupture open and release their stored materials and, thus, the artemisinin level in older leaves on whole plants is lower (Duke *et al.*, 1994; Ferreira and Janick, 1996).

### **1.1.3 Malaria**

Malaria is characterized by fever, headache, nausea, and muscle pain and caused by the parasite *Plasmodium* (van Agtmael *et al.*, 1999). It is a very serious and even fatal infectious disease and transmitted mainly through female anopheles mosquito. There are four species of *Plasmodium* resulting in malaria including *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae* (WHO, 1998). *P. falciparum* accounts for the most serious version of the disease and death due to its high levels of death rate and spread of antimalarial drug- resistance (Mendis *et al.*, 2001). Every year, about 2 to 3 million deaths (Moorthy *et al.*, 2004) are caused by malaria, of which at least a million deaths are from *P. falciparum* infection (WHO, 2005a); and 40% of the world population is at risk of malaria (Simooya, 2005). *P. falciparum* infects as many as 400 million people a year (WHO, 2005a).

To treat malaria, quinine and quinoline-based drugs such as chloroquine, mefloquine, and primaquine were widely used before the early sixties (Woerdenbag *et al.*,

1990). Some *P. falciparum* strains, however, became resistant to those antimalarial drugs and multiple side effects also exhibited (Abdin *et al.*, 2003). Artemisinin and its derivatives have attracted more and more attention and in 2001, WHO recommended that Artemisinin-based Combination Therapies (ACTs) should be adopted to treat malaria (Mandelbaum-Schmid, 2005) due to little or no cross-resistance with other antimalarial drugs, rapid reduction of the parasite, and efficacious activity against *P. falciparum* stains (Meshnick *et al.*, 1996).

#### **1.1.4 Low production but high demand for artemisinin**

Unfortunately, the level of the production of artemisinin in *A. annua* plants is relatively low, only about 0.01 to 0.8% (DW) (Abdin *et al.*, 2003). A minimum of six months is required for cultivating *A. annua* (WHO, 2004). Due to its unique and complex structure, it is not economically practical to chemically synthesize artemisinin (Abdin *et al.*, 2003). To meet the therapeutic demand, enhanced production of artemisinin is highly desirable. To treat malaria, the treatment courses needed increased dramatically from 2 million treatment courses in 2003 to 30 million courses in 2004 and 70 million treatment courses for 2005. This has, thus, already led to a shortage of artemisinin for ACTs. At least 130 million treatment courses of ACTs in 2006 will be required (WHO, 2005a). Usually, 0.6g artesunate or, for the combination artemether/lumefantrine, 0.48g artemether is needed for one ACT adult treatment course (WHO, 2005b). Because artesunate and artemether are semisynthesized from artemisinin, at least 330 tons artemisinin are needed for just treating malaria infected patients in 2005 and at least 12,000 hectares are required to produce 70 million adult ACT treatments (WHO, 2005b).

### 1.1.5 Approaches for improving artemisinin production

Although artemisinin production can be increased through larger scale field cultivation of *A. annua*, the length of cultivation and manufacturing time, the need for a large amount of land and labor, and the expense of extraction are still problems. Hence, alternative approaches are being studied to enhance artemisinin production using *in vitro* methods.

Transformed hairy roots of *A. annua* have been studied for improving artemisinin production. Compared to suspension cultures, hairy roots are more stable, grow faster, and may be easier to scale-up. Many different culture conditions including light, elicitors, and culture in bioreactors have been investigated (see reviews by Towler *et al.*, 2006; Weathers *et al.*, 2006a, 2006b). However, artemisinin yields in hairy roots are not yet high enough to be economically attractive.

Alternatively, shoot cultures of *A. annua* are also being studied. Different culture conditions such as carbon sources, sugar concentration,  $\text{NH}_4^+/\text{NO}_3^-$  ratio, phosphate concentration, phytohormones (Basile *et al.*, 1993; Woerdenbag *et al.*, 1993; Liu *et al.*, 1998), addition of precursors, such as mevalonic acid (Abdin *et al.*, 2003), elicitors, or addition of metabolic inhibitors have been studied (Abdin *et al.*, 2003). Shoots cultured in bioreactors (Liu *et al.*, 1998) have also been studied. Unfortunately, artemisinin production in shoot cultures, although greater than in hairy roots, is also still much less than in whole plants (Abdin *et al.*, 2003).

More recently, Martin *et al.* (2003) have introduced a portion of the artemisinin pathway into *E. coli*. If this effort succeeds, then *E. coli* may be used to produce high-yield terpenoid-based drugs including artemisinin in large-scale fermentations with

expected costs of extraction also largely decreased (Martin *et al.*, 2003).

## **1.2 Sugars alter *A. annua* hairy root growth and artemisinin production**

Earlier work in this lab was all done using *A. annua* hairy roots; however, the root clone used for all those studies no longer produces adequate amounts of artemisinin even for lab studies. Consequently, studies are now focused on *A. annua* shoot cultures and whole plants. One of the earlier studies with *A. annua* hairy roots, however, showed that compared to sucrose, glucose significantly increased the level of artemisinin (Weathers *et al.*, 2004). Growth, however, was repressed. Fructose, on the other hand, inhibited artemisinin production but did not affect growth. Further, in the presence of sucrose, growth was strikingly increased by glucose but decreased by fructose. No significant difference was observed in artemisinin production between sucrose and sucrose plus glucose. Fructose plus sucrose, however, dramatically inhibited artemisinin production compared to sucrose. These results suggested that sugars may, in addition to being carbon sources, also be acting as regulator or signal molecules affecting the production of artemisinin in *A. annua* hairy roots. The effect of sugars as signal molecules on *A. annua* hairy roots growth and artemisinin production were further investigated by Kast (2005, unpublished results) by using several sugar analogs and his study suggested that sugars may have a regulatory effect on artemisinin production in *A. annua* hairy roots.

It is the focus of this study to determine how sugars affect growth and especially artemisinin production in shoots of *A. annua* seedlings grown *in vitro*.



## **1.3 Sugars**

### **1.3.1 Sugars as carbon and energy source**

Through photosynthesis, plants conserve the energy of light and carbon in the form of sugars. Sugars as carbon and energy entities in plants have long been recognized. The skeletons of nucleic acids, lipids, and proteins, which together with sugars are thought of as the basic building blocks of all cells and organisms, are made up of carbons that are metabolized from sugars through cellular respiration. Through respiration, the energy and reducing potential that are necessary for cellular reactions come from stored sugars. Therefore, the supply and consumption of sugars strongly influence plant growth and development.

### **1.3.2 Sugars as signaling molecules**

Sugars also have recently been recognized as molecules that can be sensed in plants thereby inducing signals that affect metabolism and development. The interaction between sugar molecules and the sensor molecules, usually proteins, is defined as sugar sensing (Smeekens, 2000). Although the concept of sugars as signal molecules is rather new, the validity, the importance, and the complexity of sugars as signal molecules have been supported by a lot of molecular and genetic analyses (see reviews by Smeekens, 2000; Rolland *et al.*, 2002; Frommer *et al.*, 2003; Halford and Paul, 2003).

#### **1.3.2.1 Sugar molecules regulate growth, development, and gene expression**

Sugar signals are involved in almost all physiological activities during the life of a plant including cell cycle, cell differentiation, metabolism, nutrient mobilization, seed germination, hypocotyl elongation, cotyledon development, leaf formation, flowering, adult organ and tissue formation, and leaf senescence (See reviews by Rolland *et al.*,

2002; Gibson, 2005; Table 1). Gene expression also can be regulated by sugar molecules (Rolland *et al.*, 2002; Gibson, 2005; Table 2). Some examples of sugar signals regulating the growth, development, and gene expression are summarized in Table 1 and Table 2.

### **1.3.2.2 Sugar molecules regulate the production of secondary metabolites in plants**

Relatively little is known about sugars acting as signals to control production of plant secondary metabolites. Larronde *et al.* (1998) reported that in cell suspension cultures of *Vitis vinifera*, sucrose dramatically stimulated the production of anthocyanins. Stilbene level, however, was only slightly affected. They further showed that mannose, a glucose analog that can be transported into plants and phosphorylated by hexokinase, can mimic the effect of sucrose on the production of anthocyanins, while another glucose analog, 3-O-methylglucose, which can be taken up into plant cells but very slowly phosphorylated by hexokinase, can not. Also, a specific inhibitor of hexokinase, mannoheptulose, inhibited sucrose stimulation of anthocyanins production. These results suggested that hexokinase appeared to be involved in a sugar signal transduction pathway related to anthocyanin production (Vitrac *et al.*, 2000). In *A. annua* hairy roots, Weathers *et al.* (2004) showed that artemisinin production was stimulated by glucose but inhibited by fructose in comparison to sucrose at the same carbon level. Significant differences were observed in artemisinin production between sucrose and sucrose plus fructose but not between sucrose and sucrose plus glucose although the same carbon amount was supplied in each sugar condition. These results suggested that sugars may also be acting as signal molecules affecting the production of artemisinin. In another study using *Arabidopsis* seedlings, DNA microarray analysis revealed that gene expression related to secondary metabolism was also regulated by glucose, thereby maybe affecting the production of

**Table 1 Selected examples of growth and developmental processes regulated by sugars.**

Sugar	Effects	Related Processes	Reference
Sucrose	Sucrose represses the inhibition of hypocotyl elongation in continuous far-red light in wild-type <i>Arabidopsis</i> seedlings.	Hypocotyl elongation	Dijkwel <i>et al.</i> (1997)
	Sucrose can accelerate flowering in late-flowering <i>Arabidopsis</i> ecotypes and facilitate the leaf morphogenesis and flower in several <i>Arabidopsis</i> late-flowering mutants.	Flowering	Roldan <i>et al.</i> (1999)
	Exogenous sucrose can inhibit sucrose symporter activity in membrane vesicles from sugar beet leaves but cannot affect glucose transporter and alanine symporter. Also equimolar hexose did not elicit the response. This response is reversible.	Nutrient mobilization	Chiou and Bush (1998)
	Sucrose distribution is different in the stage of embryogenesis, growth and starch accumulation in <i>Vicia faba</i> cotyledons. This suggests that sucrose plays an important role in storage cell differentiation.	Cell differentiation	Borisjuk <i>et al.</i> (2002); Gibson (2004)
	Sucrose can mitigate the negative effects of nitrate on the growth rates of soybean nodules.	Nodule growth	Gibson 2005
Glucose	Glucose can delay the rate of germination in wild-type <i>Arabidopsis</i> seeds.	Germination	Price <i>et al.</i> (2003)
	High levels of glucose delay the flowering and increase the rosette leaf number in wild-type <i>Arabidopsis</i> plant.	Leaf formation and flowering	Zhou <i>et al.</i> (1998)
	Glucose stimulates leaf senescence.	Leaf senescence	Gibson 2005
	Glucose can retard seed lipid mobilization in germinating seeds from wild-type <i>Arabidopsis</i> .	Nutrient mobilization	To <i>et al.</i> (2002)
	4-6% glucose represses hypocotyl elongation and suppresses light-inducible cotyledon development in wild-type <i>Arabidopsis</i> seedlings.	Hypocotyl elongation and cotyledon development	Jang <i>et al.</i> (1997)
Sucrose & Glucose	Low concentrations of sucrose and glucose induce CyclinD2 and CyclinD3 expression in <i>Arabidopsis</i> cells and intact seedlings. The induction by sugars is independent of cell cycle progression.	Cell cycle	Riou-Khamlichi <i>et al.</i> (2000)
	Sucrose and glucose adversely regulate source and sink metabolism in photo-autotrophic suspension culture cells of tomato.	Metabolism	Sinha <i>et al.</i> (2002)
	In developing seeds, sucrose regulates differentiation and storage, whereas hexoses control growth and metabolism.	Growth and differentiation	Rolland <i>et al.</i> (2002)

**Table 2 Selected examples of gene expression and protein activity regulated by sugars.**

<b>Sugar</b>	<b>Effects</b>	<b>Related Process</b>	<b>References</b>
Sucrose	Sucrose regulates the <i>Arabidopsis</i> D-type cyclins gene expression.	Cell cycle	Riou-Khamlichi <i>et al.</i> (2000)
	Sucrose induces <i>Arabidopsis</i> nitrate reductase gene transcription.	Nitrate assimilation	Cheng <i>et al.</i> (1992)
Glucose	Glucose represses the expression of photosynthetic genes.	Photosynthesis	Jang and Sheen (1994); Xiao <i>et al.</i> (2000); Price <i>et al.</i> (2004)
	DNA microarray revealed that glucose regulates the expression of a large number of genes related to metabolism of carbohydrate, nitrogen, lipid, inositol, stress response, cell growth, signal transduction, transcription factors, and secondary metabolism in whole <i>Arabidopsis</i> seedlings. Glucose can regulate many related starch biosynthesis genes in dark-adapted <i>Arabidopsis</i> seedlings.	A lot of processes involved Starch metabolism	Price <i>et al.</i> (2004) Price <i>et al.</i> (2004)
Glucose & Sucrose	Glucose significantly represses photosynthetic gene expression in transfected greening maize protoplasts at physiological concentration; while sucrose can inhibit photosynthetic gene expression twofold.	Photosynthesis	Jang and Sheen (1994)
Glucose & Fructose	Glucose and fructose inhibit glyoxylate cycle genes in cucumber cell culture.	Glyoxylate cycle	Graham <i>et al.</i> (1994)
Glucose & Fructose & Sucrose	Glucose, fructose, and sucrose repress $\alpha$ -amylase induction in barley embryos caused by GA3.	Starch hydrolysis	Loreti <i>et al.</i> (2000)

secondary metabolites (Price *et al.*, 2004).

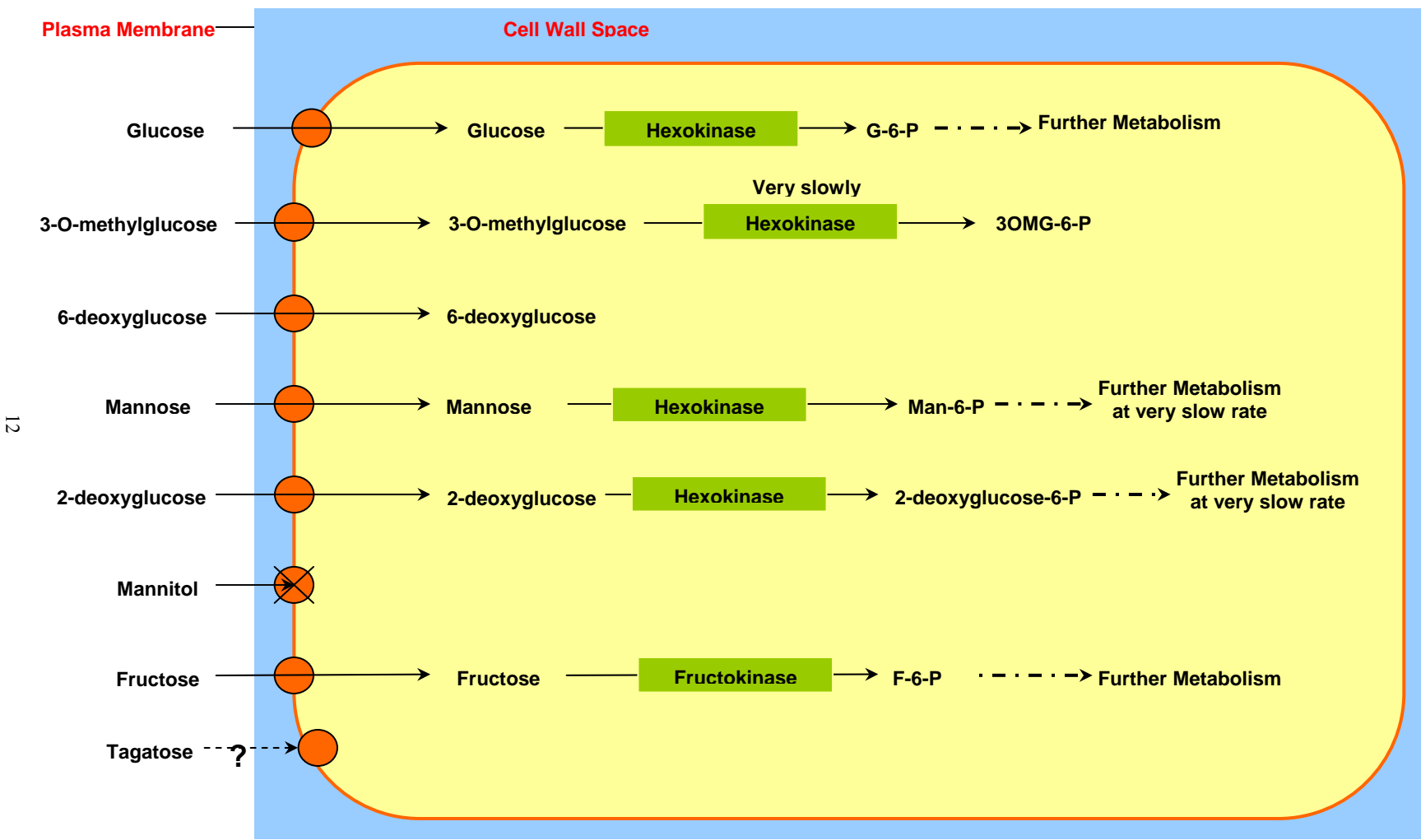
### **1.3.3 Current models for sugar signal transduction pathways**

Both monosaccharide and disaccharide sugars can act as signal molecules in plants. The main monosaccharide that functions as a signal is glucose, but fructose and other monosaccharides can also affect glucose signaling (Figure 3). The main disaccharide signal is sucrose; however, trehalose, maltose, and other disaccharides can affect sucrose signals (Figure 4). Sugar signaling can be further confounded when, as is mainly the case, both monosaccharide and disaccharide sugars are present in plant cells and tissues. What follows is a summary of the known effects.

#### **1.3.3.1 Glucose signal transduction pathways**

Based on analysis of growth and development data, and gene expression and enzyme activity, three possible glucose signal transduction pathways are currently proposed:

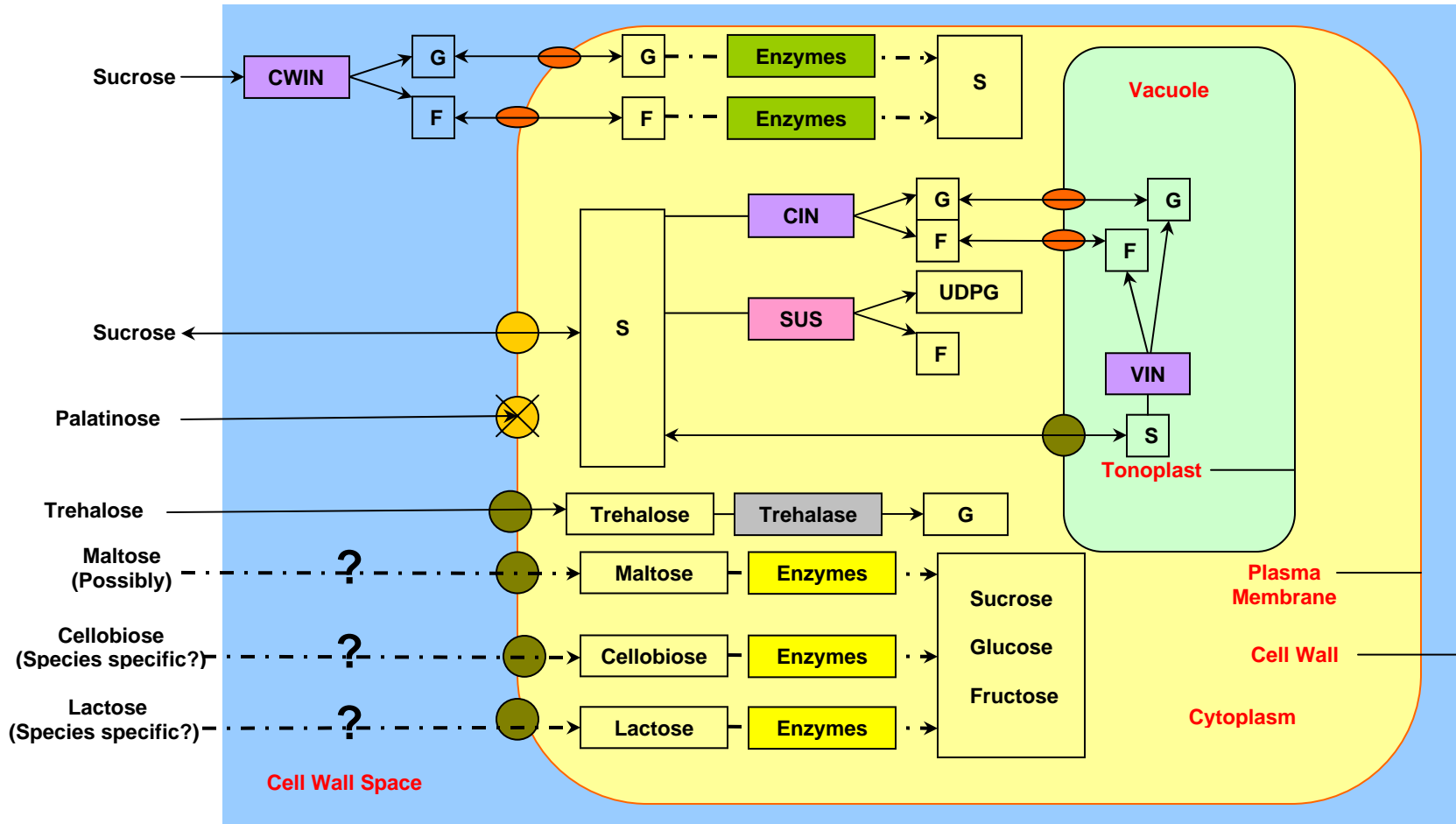
[1] Hexokinase-dependent pathway. In this pathway, a glucose induced response depends on the phosphorylation of glucose by hexokinase (HXK). Jang and Sheen (1994) showed that glucose and 2-deoxyglucose (2DOG), which is an analog of glucose, can be transported into plant cells and phosphorylated by hexokinase into 2-deoxyglucose-6-phosphate which cannot be metabolized further. The 2DOG, however, can cause repression of photosynthetic genes in a maize protoplast transient expression system. This severe repression can be restored by adding mannoheptulose, a specific hexokinase inhibitor. Also using glucose analogs, 6-deoxyglucose and 3-O-methylglucose, which can be efficiently taken up by plant cells but can at best be slowly phosphorylated by hexokinase showed that the glucose transporter located on the plasma membrane cannot



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**Figure 3 Monosaccharide metabolism and role of monosaccharide analogs in plant cells.**

Key: ● monosaccharide transporter



**Figure 4 Disaccharide metabolism and role of disaccharide analogs in plant cells.**

Key: CWIN, cell wall invertase; CIN, cytoplasmic invertase; SUS, sucrose synthase; UDPG, UDP-glucose; VIN, vacuolar invertase; S, sucrose; G, glucose; F, Fructose; ● monosaccharide transporter; ● sucrose transporter; ● unknown transporter

sense the glucose signal (Jang and Sheen, 1994). Taken together, these results suggested that hexokinase is the first and key sensor in the glucose signal transduction pathway. This hexokinase-dependent pathway also exists in other plants (Graham, *et al.*, 1994; Jang, *et al.*, 1997; Umemura, *et al.*, 1998; Pego, *et al.*, 1999; Xiao, *et al.*, 2000).

[2] Glycolysis-dependent pathway. Some metabolite(s) in the glycolytic pathway downstream of glucose-6-phosphate is (are) the sensors via this pathway. Transgenic plants overexpressing *Arabidopsis* hexokinase1 (AtHXK1) gene are glucose hypersensitive, whereas plants overexpressing yeast hexokinase2 (YHXK2) gene are glucose hyposensitive in spite of having an elevated HXK catalytic activity. Expression of two pathogenesis-related (PR) genes by glucose in AtHXK1 plants was higher than in wild-type plants, but less than in YHXK2 plants. This suggested that a glycolysis-dependent sensing pathway post glucose-6-phosphate may exist in plants (Xiao *et al.*, 2000).

[3] Hexokinase-independent pathway. The response induced by glucose in the HXK-independent pathway is not related to glycolysis or hexokinase. Evidence for this mechanism is as follows: Class I patatin genes were induced by 3-O-methylglucose, a glucose analog, in *Arabidopsis* (Martin, *et al.*, 1997). Further, the activities of extracellular sucrose invertase and the corresponding gene expression were enhanced by glucose and another glucose analog, 6-deoxyglucose (Roitsch, *et al.*, 1995). Because both 3-O-methylglucose and 6-deoxyglucose can be transported into plant cells but are not effective substrates for hexokinase, these results suggested that hexokinase may not be a sensor in these cases and the sensor could be upstream of glycolysis.



### 1.3.3.2 Disaccharide signal transduction pathways

Because disaccharides consist of two monosaccharides and there is a shortage of good tools such as mutants and clearly understood disaccharide analogs, the disaccharide signal transduction pathway is not fully understood. Experimental results are, thus, more complicated and difficult to interpret. Based on what is known so far, the disaccharide signal transduction pathway can also be classified into three groups according to specificity:

[1] Sucrose-specific signaling pathway. This pathway is specifically induced by sucrose and supported by the result that equal molar concentrations of hexoses cannot repress the sucrose symporter activity (Chiou and Bush 1998). Since the signal is specifically induced by sucrose, sucrose is probably sensed by specific sensor(s) to sucrose such as sucrose synthase, sucrose invertase, or sucrose transporters located on the plasma membrane (Chiou and Bush 1998; Lalonde, *et al.*, 1999; Sturm, 1999; Stum and Tang, 1999)

[2] Metabolizable disaccharide-specific signaling pathway. The existence of this pathway was verified using non-metabolizable disaccharides. Feeding disaccharides that are not metabolized by plant cells, palatinose, lactulose, or turanose, to barley embryos did not affect the destabilization of  $\alpha$ -amylase mRNA that can be induced by glucose and metabolizable disaccharides (Loreti *et al.*, 2000). These results suggested that in this case, a metabolizable disaccharide is sensed differently from a non-metabolizable disaccharide and that a signaling pathway is present in plants that are sensitive to a specific metabolizable disaccharides (Loreti *et al.*, 2000). Because non-metabolizable disaccharides which cannot be transported into plant cells can not mimic the responses

induced by metabolizable disaccharides, transporters or monosaccharides degraded from metabolizable disaccharides or hydrolytic enzymes may, thus, be the actual sensors.

[3] Trehalose-specific signaling pathway. Exogenous trehalose strongly repressed the elongation of roots and altered the carbon distribution between shoots and roots of *Arabidopsis* seedlings resulting in the accumulation of significant amounts of starch in the shoots (Wingler *et al.*, 2000). Root elongation was restored when sucrose was provided along with trehalose; however starch still accumulated in shoots (Wingler *et al.*, 2000). Compared to sucrose, trehalose induced higher expression of *ApL3*, one of the ADP-glucose-pyrophosphorylase genes, in *Arabidopsis* seedling (Wingler *et al.*, 2000). These results suggested the existence of a trehalose-specific signaling pathway (Wingler *et al.*, 2000).

### **1.3.3.3 Sugar signals can crosstalk with each other**

More than one type of sugar is present in plants. Indeed a diversity of sugars cooperates to modulate plant life. Consequently, it is not surprising that multiple sugar signals coexist in plants. In some cases, each sugar may perform its function independently. For example, in developing *Arabidopsis* seedlings, sucrose is more involved in differentiation and carbon storage than hexoses which are more responsible for growth and metabolism (Rolland *et al.*, 2002). Sucrose alone, or in combination separately with glucose, fructose, maltose, 3OMG, or lactose showed an efficient repression of *ATB2* expression which encodes a light-regulated bZIP transcription factor in *Arabidopsis* seedlings (Rook *et al.*, 1998). Furthermore, trehalose alone or in the presence of sucrose was reported to upregulate *ApL3* expression, which encodes the large subunit of the first enzyme in starch biosynthesis (Wingler *et al.*, 2000). Sugars may also

act antagonistically to each other (Halford and Paul, 2003). For example, sucrose can override the inhibition of root elongation produced by trehalose (Wingler *et al.*, 2000). In *Vicia faba* cotyledons, the increase of sucrose synthase activity and the beginning of starch biosynthesis is accompanied by a rise in the sucrose to glucose ratio (Halford and Paul, 2003). These data also suggested that the plants were able to sense the sucrose to glucose ratio (Halford and Paul, 2003). In this situation, the specific enzymes, genes, or factors appear to be regulated antagonistically by sensors of both sugars.

### **1.3.4 Approaches for discerning between sugars as carbon sources and sugars as signals**

#### **1.3.4.1 Sugar analogs**

Sucrose, glucose, and fructose are the most common sugars present in plants. Since they act not only as carbon sources but also as signals for regulating the growth, development, differentiation, gene expression, and metabolism, it is important to be able to distinguish between these metabolic and signal functions when studying sugar signal transduction pathways. Use of sugar analogs is one approach. Because they are similar to one of the commonly metabolized sugars in structure but seldom used in plant culture or seldom found to exist *in vivo*, these non-metabolizable or partially metabolizable sugars were termed analogs of the corresponding sugar. There are several prominent advantages in using sugar analogs. Sugar analogs do not usually interconvert to other sugars that may also be involved in the signal pathway. In contrast, sucrose, glucose, and fructose can be easily interconverted to each other *in vivo*, making it difficult to discern which sugar is responsible for a specific response. Use of sugar analogs readily overcomes this problem. Furthermore, the metabolism of the analogs may be interrupted at various points

throughout the catabolic pathway which can also aid in identifying regulatory steps. One of the drawbacks of using sugar analogs, however, is that they may inhibit some plant growth or not be sensed as sugars. The uptake and metabolism of sugar analogs may also vary in different plants. For example, the sucrose analog, lactose, can support the growth of calli of *Atropa belladonna* and *Papaver somniferum*, or suspension cells of *Medicago sativa* but cannot be used as a carbon source in suspension cells of *Catharanthus roesus*, calli of *Acer pseudoplatanus*, or *Nicotiana tabacum* calli (Hérouart *et al.*, 1991). Because sugar analogs can be readily obtained and used in sugar studies, they provide a useful tool for doing at least preliminary studies related to sugar signaling. The disadvantages of using analogs can also be avoided by careful design of experiments, thus sugar analogs are still widely used in the investigation of sugar signaling in plants.

#### **1.3.4.2 Transgenic plants**

Transgenic plants are another important tool used in plant signaling studies because specific enzymes or pathways can be targeted and altered. Moore *et al.* (2003) used targeted mutagenesis to obtain HXK1 (hexokinase 1) mutants in which glucose can be sensed but cannot be phosphorylated by hexokinase. Despite lacking catalytic function of an important metabolic enzyme, these mutants still showed various signaling responses in gene expression, cell proliferation, root, and inflorescence growth. These results provided a compelling demonstration that a sugar signaling function can be separated from its metabolism (Moore *et al.*, 2003). As another example, sucrose specific signal regulation of *ATB2* expression was also found through use of a transgenic *Arabidopsis* which encoded a light-regulated bZIP transcription factor in seedlings (Loreti *et al.*, 2001, Rook *et al.*, 1998). Although use of transgenics provides a more powerful approach for

studying sugar signals in plants, it is more difficult and takes more time to develop and validate a desirable transgenic plant model.

#### **1.3.4.3 Genetic screens**

Through the use of genetic screens, some sugar-signaling mutants and corresponding genes involved in sugar signaling have been isolated. For example, a series of *Arabidopsis gin* mutants (glucose-insensitive) have been identified and then were further used to study the physiological function of glucose sensing and signaling (Rolland *et al.*, 2002; Smeekens, 2000). DNA microarray analysis has also been used to study the effects of exogenous sugars on gene expression on a global scale in *Arabidopsis*. Through genetic means, a large number of genes with diverse functions regulated by glucose have, thus, been identified (Price *et al.*, 2004).

Genetic screening is a high throughput approach which can also provide a global profiling of gene expression at a specific developmental stage (Price *et al.*, 2004). Usually genetic screens are used on seedlings but not on mature plants because the latter require more growth space, more processing, and more analytical effort. As a result, mutants and genes that are involved in seedling development will be identified, but genes predominantly related to mature plants will very likely be missed in seedling-based screens (Rook *et al.*, 2003).

### **1.3.5 Interpretation and cautions when using sugar analogs**

#### **1.3.5.1 Sucrose and its analogs**

##### **Sucrose**

There are two ways for exogenous sucrose (Suc; Table 3) to be transported into cells (Figure 4). One is extracellular and occurs after sucrose is hydrolyzed to glucose and

fructose via cell-wall invertase. The two hexoses then are taken up by monosaccharide transporters located on the plasma membrane (Williams *et al.*, 2000). The other transport method is via plasma membrane sucrose transporters. Once in the cytoplasm, sucrose can be inverted into glucose and fructose through the action of cytoplasmic invertase or converted into UDP-glucose and fructose by sucrose synthase in the presence of UDP (Sturm and Tang, 1999). UDP-glucose is involved in the synthesis of starch or sucrose. Cytoplasmic sucrose can also enter the vacuole and be inverted to glucose and fructose via vacuolar invertase (Koch, 2004). Intracellular sucrose also can be metabolized and then eventually stored as starch, triacyl glycerides, polypeptides, or as secondary metabolites in some cells for plant growth, development, and protection (Sturm, 1999).

### Palatinose

Palatinose (Pal; Table 3) is not biosynthesized in higher plants and cannot be recognized and transported by sucrose transporters, so it is used to discern signals that might be perceived by the sucrose transporters (Figure 4; Bouteau *et al.*, 1999; Fernie *et al.*, 2001; Börnke *et al.*, 2002; Sinha *et al.*, 2002). Although some have reported that palatinose does not compete with sucrose for the sucrose transporter (Börnke *et al.*, 2002), a slight inhibition of sucrose uptake rate and an increase in sucrose metabolism rate were

**Table 3 Structures of disaccharides used in this study.**

Name	Structure	Name	Structure	Name	Structure
Sucrose	glc $\alpha$ [1→2] $\beta$ fru	Trehalose	glc $\alpha$ [1→1] $\alpha$ glc	Lactose	gal $\beta$ [1→4] $\alpha$ glc
Palatinose	glc $\alpha$ [1→6] $\alpha$ fru	Maltose	glc $\alpha$ [1→4] $\beta$ glc		
		Cellobiose	glc $\beta$ [1→4] $\beta$ glc		

found when freshly cut discs of growing potato tubers were incubated in the presence of palatinose, which increased the absolute rate of starch synthesis (Ferne *et al.*, 2001). This response suggested at least some interaction probably occurs between palatinose and the sucrose transporter. In contrast, glycolytic and Krebs cycle intermediates were not altered in potato tuber cells when only palatinose was fed (Ferne *et al.*, 2001). Thus, it is possible that some plants or plant organs may respond differently to palatinose.

### **Trehalose**

Trehalose (Tre; Table 3) can be taken up by some plants (Müller, *et al.*, 2001) and hydrolyzed into two glucose monomers via trehalase (Figure 4; Wingler, 2002; Eastmond and Graham, 2003; Eastmond *et al.*, 2003; Schlupepmann *et al.*, 2003). Trehalose appears to be present in all plants (Müller *et al.*, 2001). Induction of sucrose synthase and alkaline invertase activity in soybean roots by trehalose has also been reported (Smeekens, 2000). It is not clear, however, how trehalose effects in analog studies are to be interpreted.

### **Maltose**

Maltose (Mal; Table 3) is the major intermediate degraded at night in the chloroplast from transitory starch. It is then exported across the chloroplast membrane via maltose transporters and into the cytoplasm (Weise *et al.*, 2005). Maltose is metabolized in the cytoplasm via a series of enzymes and possibly converted into sucrose (Figure 4; Lu and Sharkey, 2004; Yan *et al.*, 2005), but the metabolic pathway is not fully understood. Maltose is often used in plant culture media and it has been shown to support plant, plantlet, and plant cell growth (Jang and Sheen, 1994; Rook *et al.*, 1998; Yen *et al.*, 1999). For example, maltose is absorbed by *Vicia faba* L. leaf tissues, developing soybean cotyledons, and laticifer protoplasts of *Hevea* (Bouteau *et al.*, 1999). In spinach

leaves (Bouteau *et al.*, 1999), *Beta vulgaris* leaf tissues (Maynard and Lucas, 1982), and plasma membrane vesicles from sugar beet (*Beta vulgaris* L) (Sakr *et al.*, 1993), however, sucrose uptake was inhibited by maltose. Thus, in some cases maltose may compete with sucrose for the sucrose transporter and, thus, entry into cells.

### **Cellobiose**

Although little is known about the metabolism of cellobiose (Cel; Table 3) in plants, it has been reported to induce depolarization of laticifer protoplast membranes from *Hevea* indicating that laticifers can actively absorb cellobiose (Figure 4; Bouteau *et al.*, 1999). A significant increase in dry weight and in the endogenous glucose, fructose and sucrose content were also observed after feeding cellobiose to barley embryos (Loreti *et al.*, 2000). However, some (Bouteau *et al.*, 1999) also reported that cellobiose cannot be transported across the plasma membrane suggesting again that any cellobiose effects may be species specific.

### **Lactose**

Lactose (Lac; Table 3) can be used as a carbon source in plant cell cultures, but that largely depends on the plant species and type of cultures (H rouart *et al.*, 1991). For example, lactose either does not support or very slowly supports growth of suspension cells of *Catharanthus roesus*, calli of *Acer pseudoplatanus*, and *Nicotiana tabacum* calli. On the other hand, calli of *Atropa belladonna* and *Papaver somniferum*, and suspension cells of *Medicago sativa* readily metabolize and use lactose as a carbon source (H rouart *et al.*, 1991). Lactose has also been reported to support the growth of transgenic *Arabidopsis* seedlings that contain an *ATB2* (*Arabidopsis* bZIP transcription factor gene) promoter –GUS reporter gene construct (Rook *et al.*, 1998).



### **1.3.5.2 Glucose and its analogs**

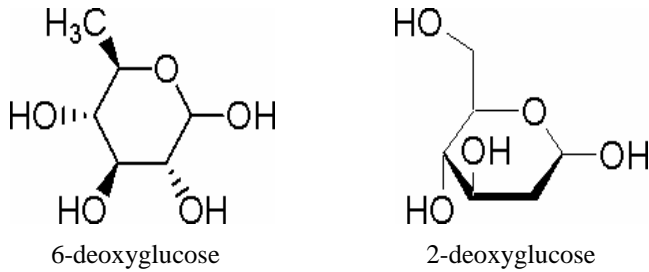
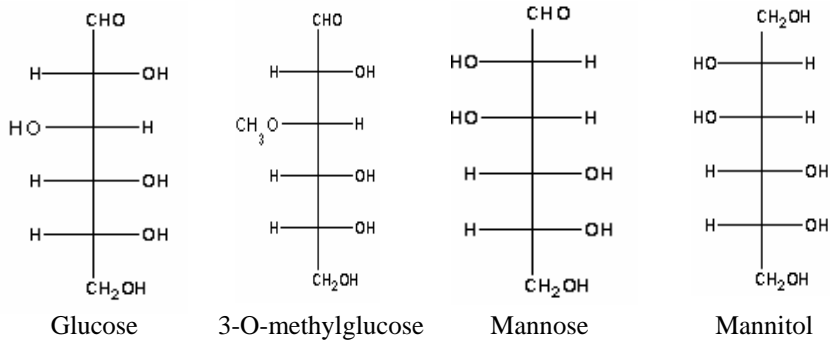
#### **Glucose**

Extracellular glucose (Glc; Figure 5) can be taken up via plasma membrane monosaccharide transporters into the plant cell (Figure 3; Lalonde *et al.*, 1999; Williams *et al.*, 2000; Etxeberria *et al.*, 2005). The intracellular glucose is then phosphorylated into glucose-6-phosphate via a rather non specific hexokinase that has a higher affinity for glucose than for fructose. There are a number of glucose analogs that can be used for studies focused on glucose sensing mechanisms and they are discussed in the following sections.

#### **3-O-methylglucose**

The glucose analog, 3-O-methylglucose (3OMG; Figure 5), can be effectively transported into the cytoplasm via plasma membrane monosaccharide transporters (Figure 3; Lalonde *et al.*, 1999; Gibson, 2000; Ho *et al.*, 2001; Loreti *et al.*, 2001) and then phosphorylated to 3-O-methylglucose-6-phosphate via HXK at a very slow rate about five orders of magnitude lower than for glucose or mannose (Cortès *et al.*, 2003). Because 3OMG also yields a 600 times lower respiration rate than glucose, it is not considered to be a metabolizable sugar (Cortès *et al.*, 2003). Since 3OMG appears to have no influence on hexokinase activity (Gonzali *et al.*, 2002), it has often been used to investigate whether hexokinase is a sensor in sugar signaling. Inhibition by 3OMG of a metabolic process would suggest that hexokinase is involved in regulation of that process. Glucose and 3OMG can, however, reciprocally inhibit the uptake of each other (Gogarten and Bentrup F-W., 1983; Getz *et al.*, 1987), thus, demonstrating that 3OMG also acts as a competitive inhibitor of the glucose transporter.

A. Glucose and its analogs.



B. Fructose and its analog.

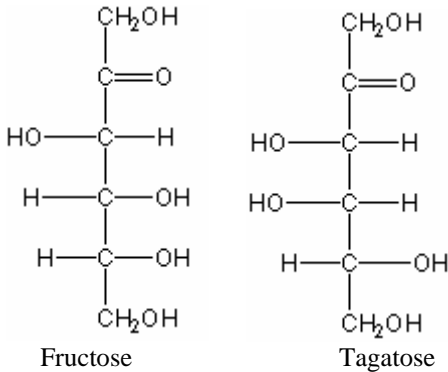


Figure 5 Structures of glucose and fructose and their analogs.

## Mannose

Mannose (Man; Figure 5) usually does not exist in plants (Pego *et al.*, 1999). It can be effectively transported into the plant cell via the plasma membrane monosaccharide transporters (Figure 3; Lalonde *et al.*, 1999; Gibson 2000; Ho *et al.*, 2001; Loreti *et al.*, 2001) and then phosphorylated by HXK to mannose-6-phosphate (Man-6-P) (Baskin *et al.*, 2001) at the expense of ATP (Pego *et al.*, 1999). Mannose is then very slowly

metabolized further (Pego *et al.*, 1999; Baskin *et al.*, 2001) due to the absence or very low concentrations of mannose phosphate isomerase that is necessary for subsequent metabolic steps (Pego *et al.*, 1999; Brouquisse *et al.*, 2001). Because of the accumulation of Man-6-P, there is a shortage of Pi in the cytoplasm, thereby causing a decreased synthesis of ATP and an imbalance in metabolism (Brouquisse *et al.*, 2001), which can be seen as reduced growth. Mannose alteration in specific metabolic responses would suggest that some step downstream of hexokinase might be a regulator.

### **6-deoxyglucose and 2-deoxyglucose**

Two other glucose analogs are 6-deoxyglucose (6DOG; Figure 5) and 2-deoxyglucose (2DOG; Figure 5). Like 3OMG, 6-deoxyglucose can be transported into plant cells but can not act as a substrate for hexokinase (Figure 3; Loreti *et al.*, 2001; Gibson, 2000). On the other hand, 2-deoxyglucose, like mannose, can enter plant cells and be phosphorylated by hexokinase into 2-deoxyglucose-6-P which is subsequently poorly metabolized (Loreti *et al.*, 2001; Gibson, 2000). Responses to 6DOG would indicate a mechanism similar to that when 3OMG is present, while 2DOG response indicates a mechanism similar to that of mannose.

### **Mannitol**

Mannitol (Mtl; Figure 5) is not metabolized by most plants, and can not enter plant cells (Figure 4; Gibson, 2000). It is usually used as an agent to alter osmotic pressure and is not usually used in sugar analog studies. If Mtl affected some metabolic response, this would suggest either an osmotic effect, or the participation of a monosaccharide transporter in the process.

### **1.3.5.3 Fructose and its analog**

#### **Fructose**

Extracellular fructose (Fru; Figure 5) can be taken up via plasma membrane monosaccharide transporters into the plant cell (Figure 4; Lalonde *et al.*, 1999; Williams *et al.*, 2000; Etxeberria *et al.*, 2005). Fructose is more specifically phosphorylated by fructokinase into fructose-6-phosphate which is further metabolized by the cell for energy and carbon skeletons. In most plant cells, cytoplasmic glucose and fructose can also easily be converted to sucrose via a series of enzymes in the cytoplasm (Loreti *et al.*, 2000).

#### **Tagatose**

The only really good analog for fructose is tagatose (Tag, Figure 5). Unfortunately, little is known about how tagatose may work in plants or even if it enters plant cells. Any alteration in metabolism in the presence of tagatose would merely indicate that fructose is somehow involved in the response.

### **1.3.6 Crosstalk between sugar signaling and other signalings**

In addition to sugar signaling pathways, other signaling pathways including those involving phytohormones, nitrogen, light, and stress also exist in plants. Sugar signaling interacts with all of these. For example, using *Arabidopsis* glucose insensitive 2 (*gin2*) mutants that lose the function of specific hexokinase1 (HXK1) revealed that the HXK1 is a central link between light, sugar, and hormone signaling because these mutants also displayed different reactions to phytohormones and light compared to wild type plants (Moore *et al.*, 2003). Other *Arabidopsis* sugar-signaling mutants also indicated that the sugar and hormone signals are connected in plants. For example, the *gin1* mutant is

insensitive to glucose and also involved in abscisic acid biosynthesis. The glucose hypersensitive mutants, *ein2*, showed insensitivity to ethylene, jasmonate, and paraquat (León and Sheen, 2003). Further, Ho *et al.* (2001) found that stress-related genes such as alcohol dehydrogenase (ADH2), heat shock protein 86 (HSP86), and ubiquitin precursor (Ubi) were negatively regulated by both sucrose and glucose in rice cell cultures. Use of DNA microarray analysis revealed that in whole *Arabidopsis* seedlings (Price *et al.*, 2004), some genes can be regulated by both glucose and nitrogen, and genes related to abscisic acid biosynthesis or response, or ethylene perception are also involved in sugar signaling. The genetic screen also showed that many stress-related genes are induced by sugars (Price *et al.*, 2004).

#### **1.4 Summary**

Sugars as an important nutrient play vital roles in plant life. The importance of the signaling role of sugars, rather recently recognized, has revealed some possible signal transduction pathways, and also possible mechanisms about connections between sugars, and other signaling pathways. Although significant progress has been made, a lot of questions remain unanswered. For example, how do sugars participate into specific function? What are the molecules that participate as sensors in specific signaling pathway? Are sugar signaling pathways also involved in the regulation of secondary metabolite production? Finally how do sugar signaling pathways interact with other signaling pathways within the cell regulatory network and cooperate to modulate plant growth and development?

## 2 Objectives

Little is known about the role of sugars as signal molecules in controlling terpenoid production in plants. In this project, several questions were posed:

1. Do sugars alter the production of the terpenoid, artemisinin, differently?
2. If they do, what are the possible mechanisms of their signal effects on the production of artemisinin?
3. Is there crosstalk among these sugar signaling pathways related to artemisinin production?

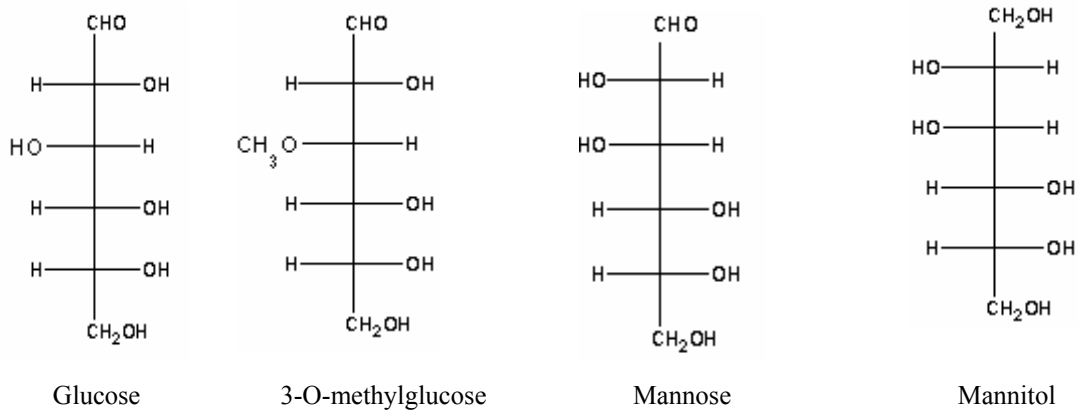
## 3 Materials and Methods

### 3.1 Growth of plant materials in sugars

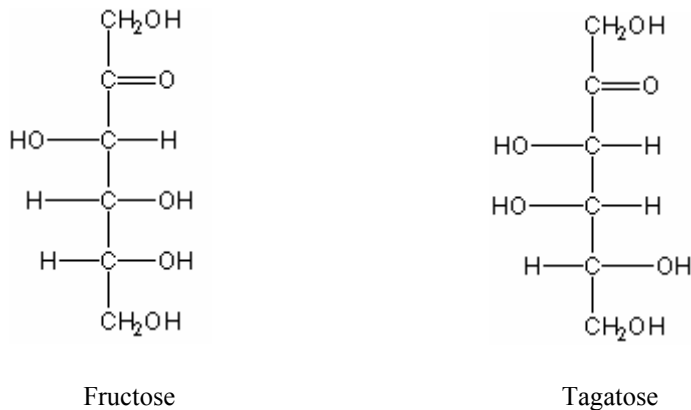
*Artemisia annua* L. seeds (YU strain, 2004 WPI crop) were surface sterilized in 10% (v/v) bleach for 12 minutes followed by 70% (v/v) ethanol for 5 minutes. Then seeds were washed with 10ml 0.1% (v/v) sterile PPM (Preservative for Plant Tissue Culture Media, Plant Cell Technology, Inc.), 3 times, 5 minutes each time. The sterilized seeds were imbibed in 30ml 0.1% sterile PPM in a 125ml Erlenmeyer flask in the dark for 3 days in the refrigerator. After that, seeds were incubated in 30ml Gamborg's B5 medium (Gamborg *et al.*, 1968) with 3% (w/v) sucrose at pH5.7 in the dark in the refrigerator for 3 days, then transferred to continuous cool-white fluorescent light ( $100\mu\text{Em}^{-2}\text{s}^{-1}$ ) at 24°C, and at 140rpm (Lab-line Orbit Shaker, Lab-Line Instruments Inc., Melrose Park IL). After 5 days, most seeds developed to the two-cotyledon stage. To eliminate exogenous sugar effects, the seedlings were washed by pipetting out the medium and replacing the liquid with about 50ml of sugar-free B5 medium. This was done 7 times, after which the seedlings were then maintained in the dark for 1 day. Ten seedlings at the two-cotyledon stage were manually selected and then inoculated into a Petri dish containing 20ml autoclaved B5 medium with 0.23% (w/v) Phytigel to which filter-sterilized sugars were added. Cultures were maintained at 24°C under continuous cool-white fluorescent light ( $100\mu\text{Em}^{-2}\text{s}^{-1}$ ) for 14 days. For liquid culture, ten seedlings at the two-cotyledon stage were inoculated into 125mL Erlenmeyer flasks containing autoclaved 20mL B5 medium with filter-sterilized sugars.

All sugars and their respective analogs used in this project are shown in Figure 6 and Table 3 and were filter sterilized using a separate 0.22 $\mu$ m sterile syringe filter before addition into B5 medium. Except lactose, which was from Malinkrodt, all other sugars and analogs were bought from Sigma Aldrich (St. Louis, Mo). All sugars were added to either Petri dishes or flasks at a constant carbon level equivalent to 3% (w/v) sucrose.

A. Glucose and its analogs



B. Fructose and its analog



**Figure 6 Structures of monosaccharides used in this study.**

### 3.2 Analysis of growth and development

After two-week growth on Petri dishes, seedlings were removed, washed with diH<sub>2</sub>O and blotted dry with paper towels. The leaves of each seedling were counted. Each



seedling was cut at the base of the hypocotyl yielding the shoot and root portions. Individual shoots and roots were dried at 60°C for at least 16 hours and the dry mass of each was measured. All dead or contaminated seedlings were discarded.

### **3.3 Extraction and analysis of artemisinin**

The dried shoots of all seedlings harvested from the same Petri dish or Erlenmeyer flask were pooled together, weighed, and extracted with 1mL toluene in a chilled water sonicator for 30 minutes. The supernatant was decanted and the sonication was repeated twice. The 3 supernatants from shoots harvested from the same Petri dish or Erlenmeyer flask were pooled and dried under nitrogen at 30°C. Samples were stored in the freezer at -20°C until analysis by high performance liquid chromatography (HPLC). Artemisinin in each sample was analyzed as the Q260 method by HPLC according to Smith *et al.* (1997).

### **3.4 Assay of hexokinase activity**

Seedlings grown in Petri dishes for 14 days were harvested, and shoots from 2-3 plates containing same medium were pooled together and frozen using liquid nitrogen, and then ground using mortar and pestle. The powder was halved. One half of frozen shoots was dried, extracted and analyzed by HPLC for artemisinin as described above, and the other half was stored in the freezer at -80°C until hexokinase activity was assayed.

About 0.1g of frozen fresh plant material and liquid nitrogen were added to the mortar and pestle. After the liquid nitrogen was evaporated, 10% insoluble Polyvinylpyrrolidone (PVPP) was added and homogenized to powder. Then 1ml ice-cold extraction buffer containing 50mM HEPES-KOH (pH7.5), 5mM MgCl<sub>2</sub>, 1mM EDTA (pH7.0), 15mM KCl, 2.5mM dithiothreitol, 0.1% Triton X-100, 10% glycerol, 1X protease inhibitor (Roche Diagnostics #1836145) was added and ground thoroughly. The

extract was centrifuged at 17,400 x g for 3min at 4°C and the crude supernatant was directly used for assay of hexokinase activity and total protein. The SOP for this assay is in the Appendix.

Hexokinase activity was measured using a coupled assay with glucose-6-phosphate dehydrogenase and NAD reduction. The assay mixture contained 50mM Bicine-KOH pH 8.5, 15mM KCl, 5mM MgCl<sub>2</sub>, 2.5mM ATP, 1mM NAD, and 2 units glucose 6-P dehydrogenase in 450µl at room temperature. For one assay, 450µl of the assay mixture, 400µl H<sub>2</sub>O, and 100µl plant extract were added in 1 ml cuvette, mixed, and the spectrophotometer (Hitachi U2800) was zeroed at 340nm. Then, 50µl of 0.1 M glucose was added to initiate the reaction and the absorbance at 340nm was monitored for 30min using the time scan method on the instrument. The protein concentration was determined according to the method of Bradford (Bradford, 1976) using BSA as standard.

### **3.5 Statistical analysis**

Each sugar experiment was repeated 2-6 times and the results pooled and averaged. Data were analyzed using SPSS 14.0 for MS Windows (SPSS Inc). Growth data were analyzed using ANOVA, which tests the hypothesis that three or more group means are not different based on the assumption that data are from a normally distributed population (Glantz, 2001). ANOVA was followed by Dunnett's *post hoc* test, which tests the difference among all other groups against a single control group (Glantz, 2001). Alternatively, the Student-Newman-Keuls *post hoc* test was used, which tests the difference among all groups to each other pairwise (Glantz, 2001). An independent-samples T-Test was used in conjunction with different experimental conditions; this tests the difference between two group means based on the assumption that data are from a

normally distributed population (Glantz, 2001). When there are multiple groups in an experiment, ANOVA should be used (Glantz, 2001). Artemisinin production data were analyzed using Kruskal-Wallis test; this tests the hypothesis that three or more group means are not different without the assumption that data are from a normally distributed population (Glantz, 2001). It was followed by Dunn's *post hoc* test, when the sample sizes are different; Dunn's *post hoc* test is used to test the difference among pairwise groups or all other groups against one control group (Glantz, 2001). Alternatively, the Mann-Whitney U-test was used, which tests the difference between two group means without assuming that data are from a normally distributed population (Glantz, 2001).

## 4 Results

### 4.1 Effects of single, common sugar metabolites on growth and artemisinin

#### production

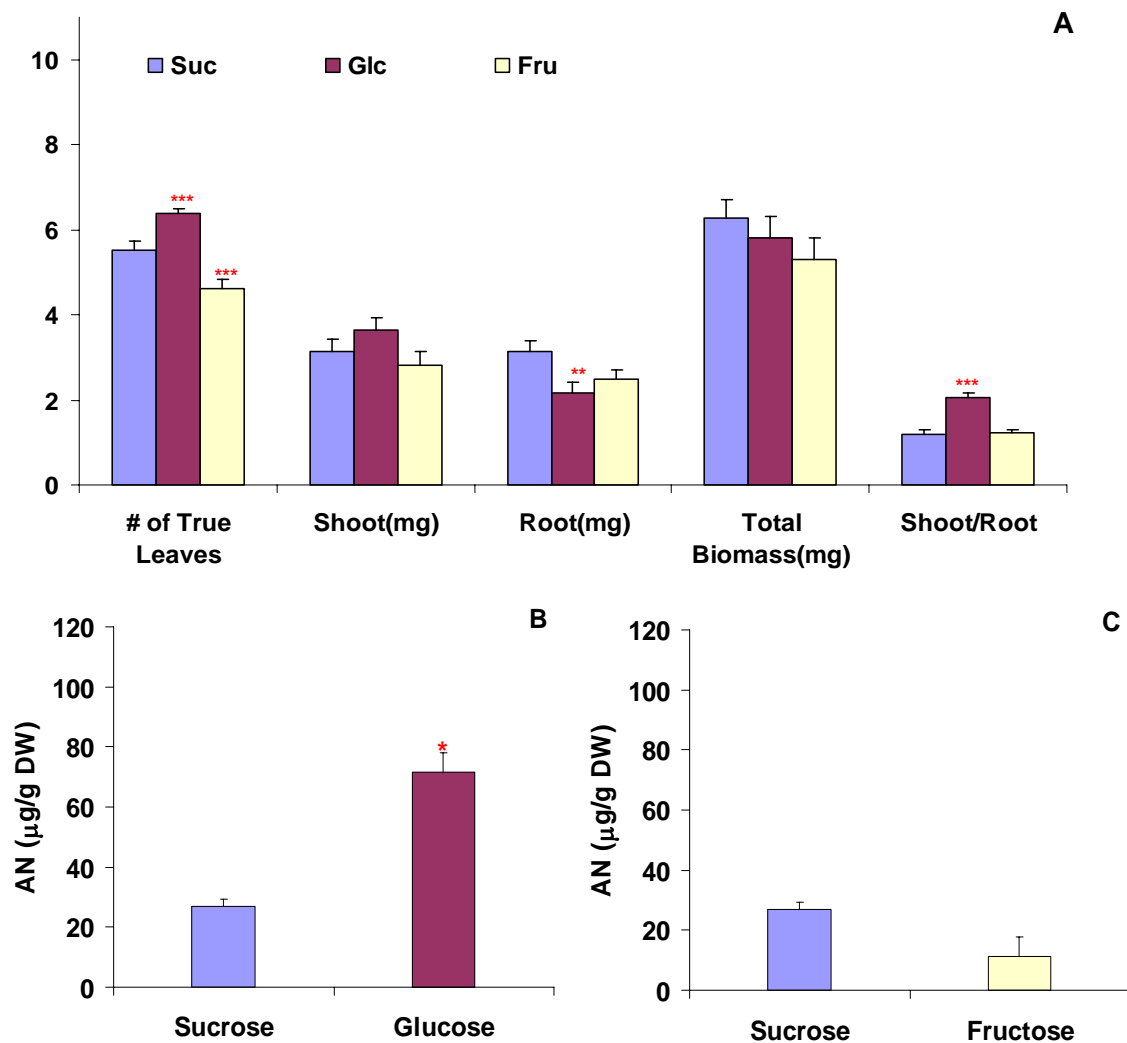
Sucrose, glucose, and fructose are the most common sugars existing in all plants. They can be easily transported into plant cells, readily converted to each other, and normally metabolized through glycolysis. Thus, it is necessary to first understand the effects of these common sugars on *A. annua* growth and artemisinin production. Equimolar carbon (equivalent to the amount of carbon in 3% (w/v) sucrose) of sucrose, glucose, or fructose was added into B5 medium and seedlings were cultured in Petri dishes for 14 days. The effects of glucose or fructose were compared with sucrose because sucrose is the common carbon source used for seedling culture. The number of true leaves was significantly increased by glucose but decreased by fructose; glucose also inhibited root growth (Figure 7A and Table 4). Furthermore, the shoot to root biomass ratio of seedlings grown in glucose was statistically higher than in sucrose. Importantly, seedlings grown in medium with 100% Glc produced nearly two times more artemisinin

**Table 4 Summary of growth and artemisinin production responses of glucose and fructose compared to sucrose.**

Compare to Suc	Glc	Fru
# of True Leaves	↑	↓
Shoot Biomass (mg)	nc	nc
Root Biomass (mg)	↓	nc
Total Biomass (mg)	nc	nc
Shoot/Root	↑	nc
AN (µg/g DW)	↑	nc

↑ or ↓ indicates statistically significant increase or decrease; nc indicates no statistically significant change.

than those grown in sucrose (Figure 7B) while artemisinin production from fructose is about half of that in sucrose (Figure 7C). A significant difference was only detected, however, between the artemisinin yields in glucose and sucrose ( $P = 0.035$ ). These results showed that glucose stimulated artemisinin production, while fructose inhibited it compared to sucrose.



**Figure 7 Effects of sucrose, glucose, and fructose on growth and artemisinin production.**

Data are mean of total replicates + SE. A. Effect on growth. Data were analyzed using ANOVA followed by Dunnett's *post hoc* test comparing each group to the control (sucrose). The total replicates for sucrose: 74; glucose: 73; fructose: 64. B. Effect of sucrose and glucose on artemisinin production. Data were analyzed using the Mann-Whitney U test. There were 7 replicates for each condition. C. Effect of sucrose and fructose on artemisinin production. Data were analyzed using the Mann-Whitney U test. The total replicates for sucrose: 7; fructose: 6. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

## 4.2 Effects of glucose and its analogs on growth and artemisinin production

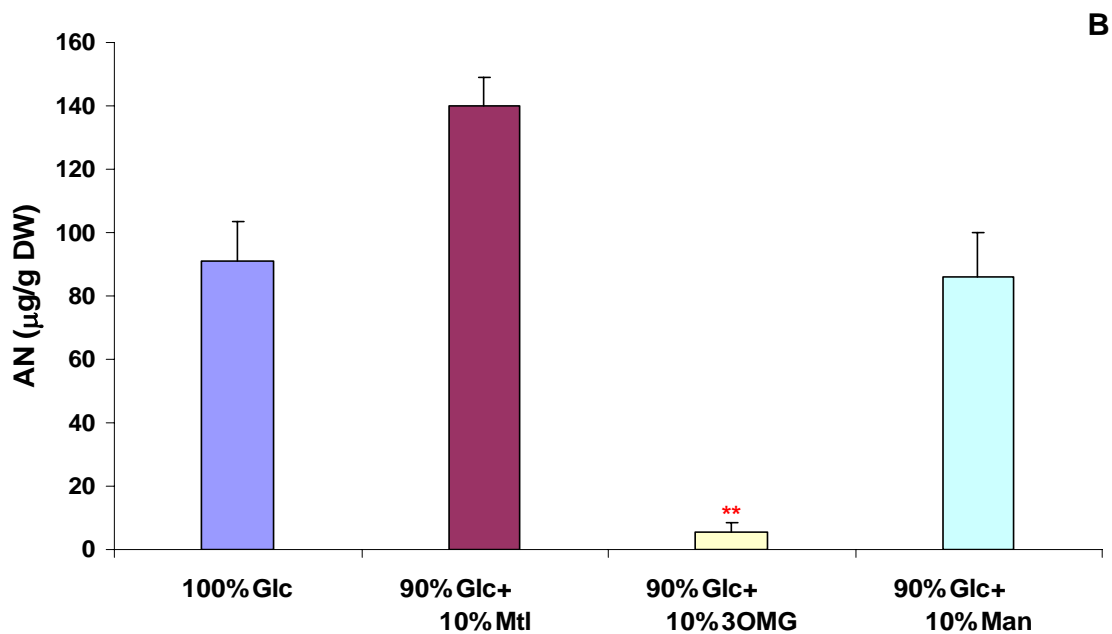
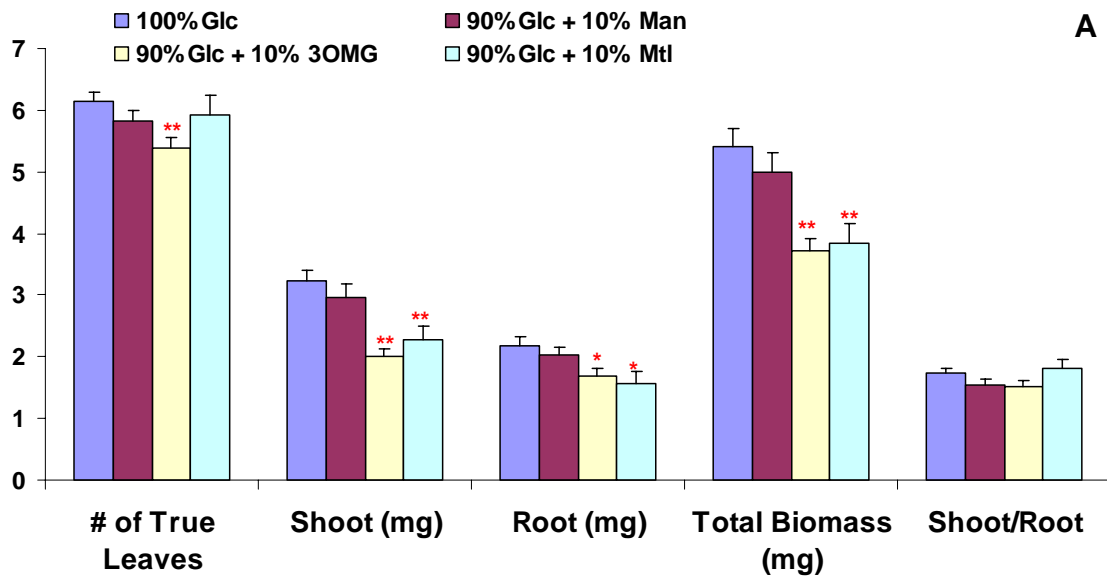
Three glucose analogs, 3OMG, Man, or Mtl were used to investigate how *A. annua* growth and artemisinin production are affected by glucose. To eliminate its possible “toxicity” to seedlings, each glucose analog accounted for only 10% total carbon in the medium with glucose comprising the remaining 90% of carbon. The total carbon was always equivalent to 3% (w/v) sucrose. Glucose at 100% was used as control. In the presence of 10% 3OMG, the numbers of true leaves, shoot mass, root mass and total biomass were significantly decreased compare to seedlings grown in 100% Glc (Figure 8A and Table 5). Shoot mass, root mass, and total biomass of seedlings were also remarkably inhibited by the addition of 10% Mtl (Figure 8A and Table 5). No significant differences in growth were observed, however, between addition of 10% Man and the 100% Glc control (Figure 8A and Table 5).

Artemisinin production decreased by about 95% compared to the glucose control, when 10% glucose analog, 3OMG, was added to medium (Figure 8B and Table 5). Although 3OMG appeared to have inhibited artemisinin production compared to glucose, neither Man nor Mtl had any significant effect on artemisinin production (Figure 8B and

**Table 5 Summary of growth and artemisinin production responses of glucose analogs compared to 100% Glc.**

Compare to 100% Glc	90% Glc + 10% Man	90% Glc + 10% 3OMG	90% Glc + 10% Mtl
<b># of True Leaves</b>	nc	↓	nc
<b>Shoot Biomass (mg)</b>	nc	↓	↓
<b>Root Biomass (mg)</b>	nc	↓	↓
<b>Total Biomass (mg)</b>	nc	↓	↓
<b>Shoot/Root</b>	nc	nc	nc
<b>AN (µg/g DW)</b>	nc	↓	nc

↑ or ↓ indicates statistically significant increase or decrease; nc indicates no statistically significant change.



**Figure 8 Effects of glucose and its analogs on growth and artemisinin production.**

Data are mean of total replicates + SE. A. Effect on growth. Data were analyzed using ANOVA followed by Dunnett's *post hoc* test comparing each group to the control (100% Glc). The total replicates for 100% Glc: 66; for 90% Glc + 10% 3OMG: 55; for 90% Glc + 10% Man: 63; for 90% Glc + 10% Mtl: 26. B. Effect on artemisinin production. Data were analyzed using Kruskal-Wallis test followed by Dunn's *post-hoc*. There were 8 replicates for each condition except for 90% Glc + 10% Mtl, which only contained 3 replicates. \*P<0.05; \*\* P<0.01.

Table 5). These results suggested that artemisinin production may be regulated by glucose at hexokinase.

Although these results with 3OMG appeared to distinguish the metabolic and signal functions of glucose, it remained to be determined if signaling of glucose could still be sensed at very low 3OMG concentration. To investigate this, 0%, 1% and 10% 3OMG were independently added to seedlings with the added remaining carbon provided by glucose. No significant difference in biomass and shoot to root biomass ratios was observed between the addition of 1% 3OMG and 100% Glc. On the other hand, 10% 3OMG significantly inhibited shoot mass, root mass, and total biomass, but stimulated the ratio of shoots to roots (Figure 9A and Table 6). Artemisinin production was also significantly inhibited by the addition of 10% 3OMG ( $P = 0.037$ ; Figure 9B and Table 6), but not by 1% 3OMG ( $P = 0.310$ ; Figure 9C and Table 6) compared to 100% Glc.

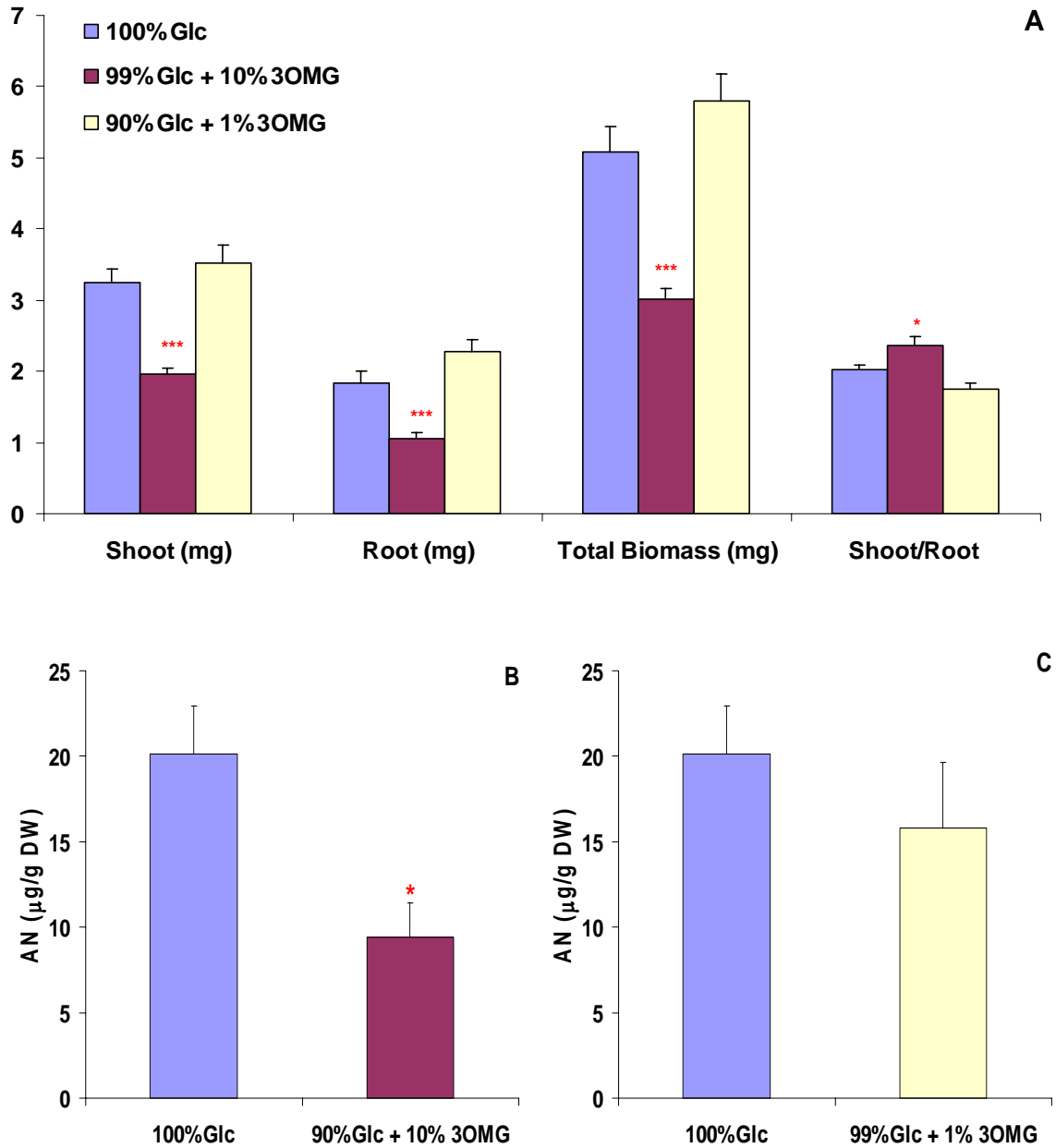
The 3OMG analog cannot be phosphorylated effectively by hexokinase (Cortès *et al.*, 2003), and artemisinin production was significantly decreased compared to the 100% Glc control when 10% of it was added (Figure 8B, 9B, and 10). Considering that the glucose analog, Man, an effective substrate of hexokinase produced a level of artemisinin

**Table 6 Summary of growth and artemisinin production responses of combination of 10% or 1% 3OMG compared to 100% Glc.**

Compared to 100% Glc	90% Glc + 10% 3OMG	90% Glc + 1% 3OMG
Shoot Biomass (mg)	↓	nc
Root Biomass (mg)	↓	nc
Total Biomass (mg)	↓	nc
Shoot/Root	↑	nc
AN (µg/g DW)	↓	nc

↑ or ↓ indicates statistically significant increase or decrease; nc indicates no statistically significant change.

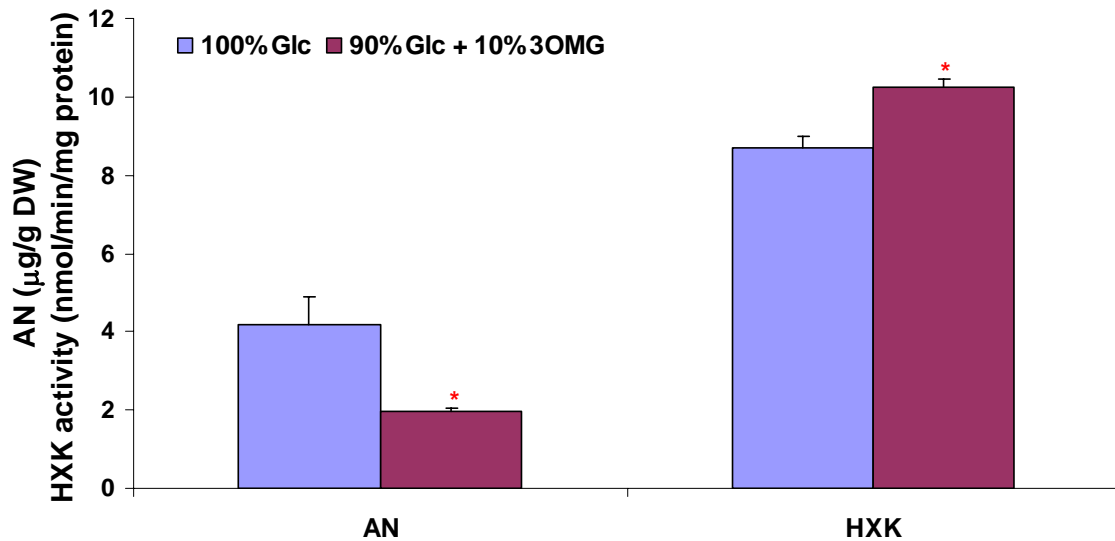




**Figure 9** Effects of glucose and combination of 10% or 1% 3OMG on growth and artemisinin production.

Data are mean of total replicates + SE. A. Effect on growth. Data were analyzed using ANOVA followed by Dunnett's *post hoc* test comparing each group to the control (100% Glc). The total replicates for 100% Glc: 60; for 99% Glc + 1% 3OMG: 58; for 90% Glc + 10% 3OMG: 59. B. Effect of glucose and combination of 10% 3OMG on artemisinin production. Data were analyzed using the Mann-Whitney U test. There were 6 replicates for each condition. C. Effect of glucose and combination of 1% 3OMG on artemisinin production. Data were analyzed using the Mann-Whitney U test. There were 6 replicates for each condition. \*P<0.05; \*\*\* P<0.001.

equivalent to the 100% Glc control, this suggested that hexokinase may play a role in the control of artemisinin accumulation through a glucose signal transduction pathway. Thus hexokinase activity was measured in seedlings grown on 100% Glc and on 10% 3OMG medium. Compared to the control, 100% Glc, hexokinase activity was significantly increased ( $P=0.05$ ) when 10% 3OMG was added (Figure 10). Taken together, these results indicate that glucose may indeed be affecting a downstream control on artemisinin production possibly through a hexokinase sensor.



**Figure 10 Effects of glucose or combination of 10% 3OMG on artemisinin production and hexokinase activity.**

Data are the mean of three replicates + SE. Data were analyzed using the Mann-Whitney U-test. \* $P=0.05$ .

### **4.3 Effects of fructose and its analog on growth and artemisinin production.**

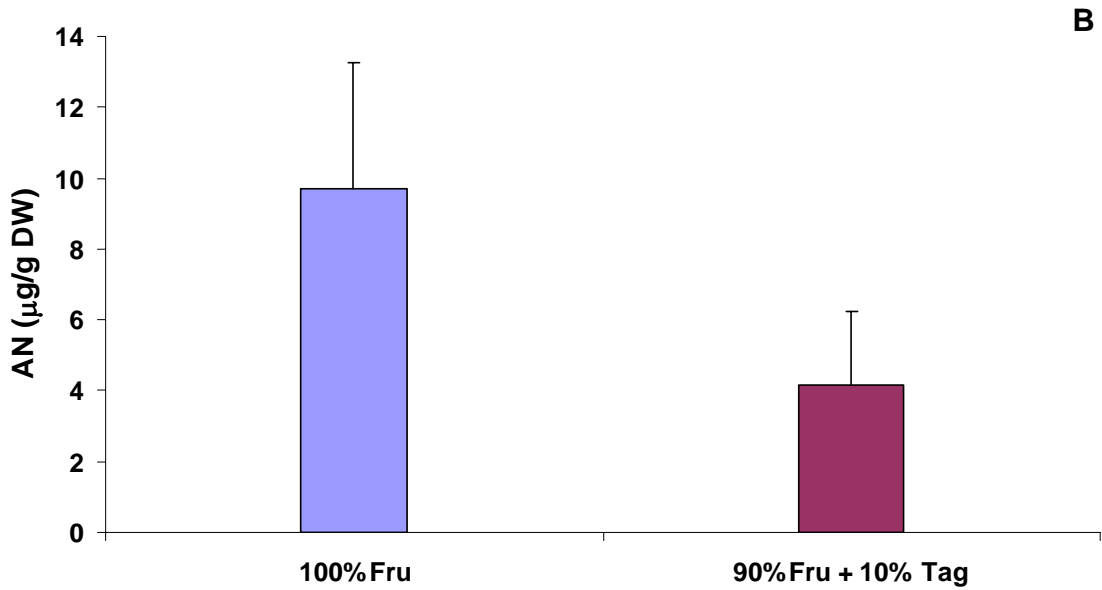
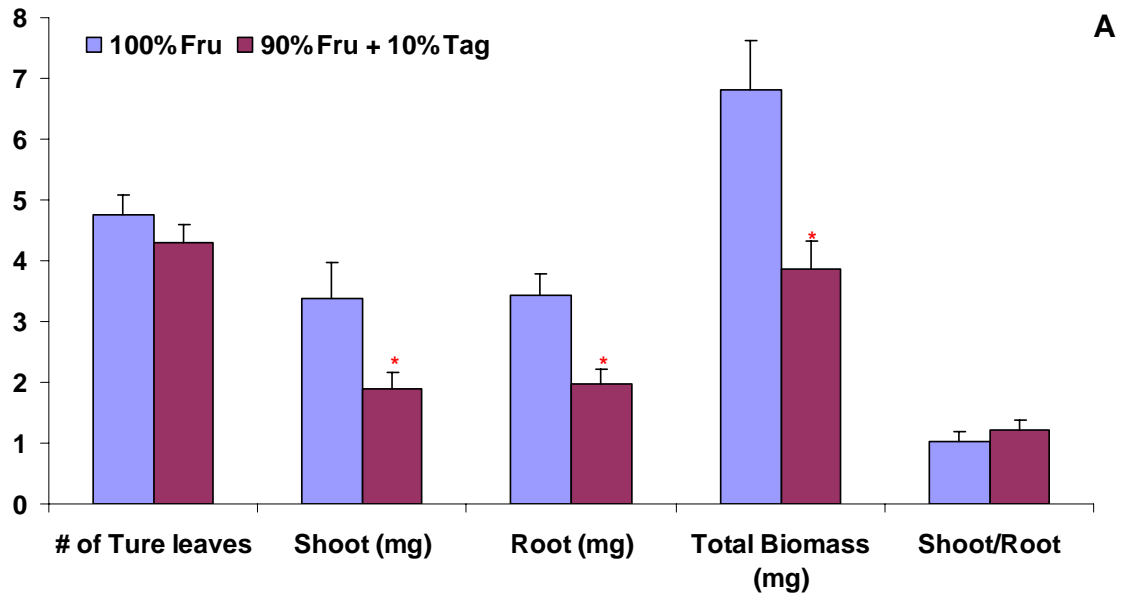
To investigate the effect of fructose as a signal molecule, 10% Tag, the analog of fructose was added to 90% Fru (the final carbon amount was equivalent to the amount of carbon in 3% (w/v) sucrose), and compared to seedlings grown in 100% Fru, the control. After two weeks growth in Petri dish, the shoots, roots, and total biomass of seedlings grown on the 10% Tag were about half of those grown on the medium with fructose alone (Figure 11A), but the ratios of shoot to root biomass were similar between the two sugar conditions.

Although artemisinin production by seedlings grown on the medium with fructose alone was about double that by seedlings grown in the presence of 10% Tag (Figure 11B), it was not statistically different. Consequently, no conclusion can be made about the effect of Tag and fructose on artemisinin production.

### **4.4 Effects of sucrose and its analogs on growth and artemisinin production**

Although monosaccharides are the main components involved in plant cell metabolism, disaccharides also have crucial roles. The effect of sucrose and some its analogs on growth and artemisinin production was also measured.

Sucrose alone or combined with its analogs was added to Petri dishes using the same method as described for the monosaccharide experiments. In contrast to the shoots of seedlings, all of the sucrose analogs inhibited root growth (Figure 12A and Table 7). Total biomass was also decreased by the addition of 10% Pal or 10% Tre when compared to 100% Suc (Figure 12A and Table 7). Similarly, seedlings grown in sucrose with 10% Tre or 10% Lac had greatly stimulated shoot to root biomass ratios compared to 100% Suc (Figure 12A and Table 7).



**Figure 11 Effects of fructose and its analog on growth and artemisinin production.**

Data are mean of the total replicates + SE. A. Effect on growth. Data were analyzed using independent-samples T-Test. The total replicates for 100% Fru: 21; for 90% Fru + 10% Tag: 28. B. Effect on artemisinin production. Data were analyzed using Mann-Whitney U-test. There were three replicates for each condition. \*P<0.05.

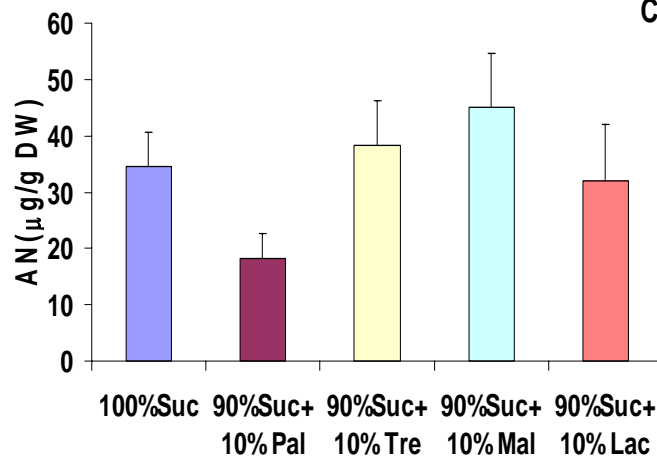
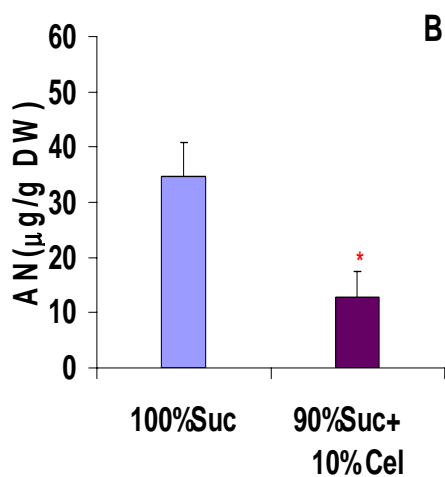
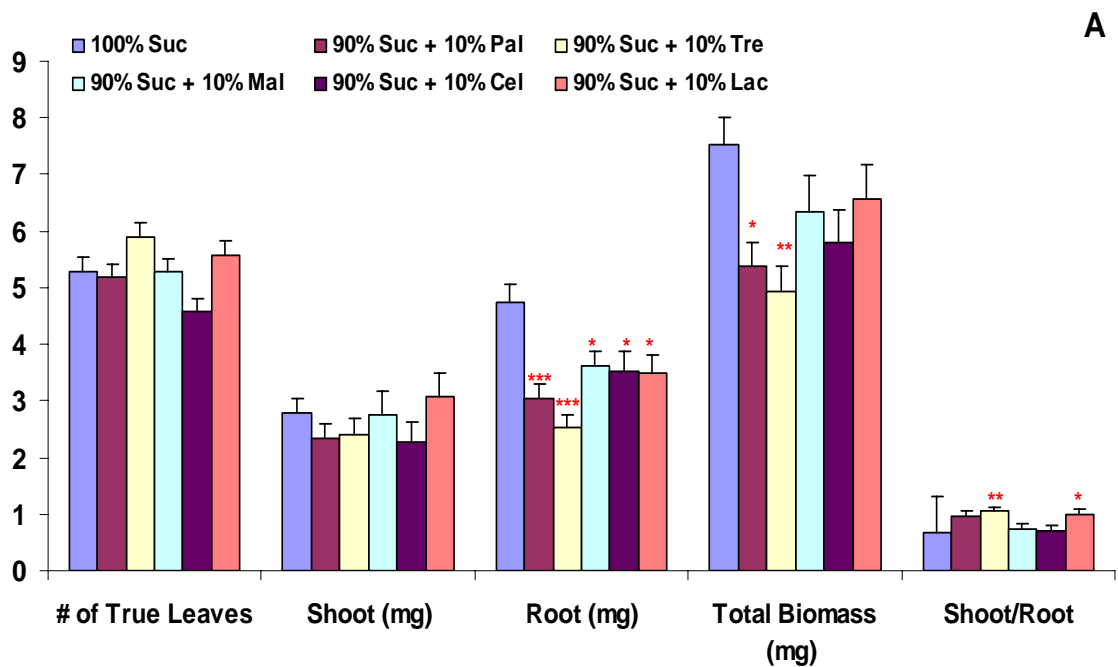
Artemisinin production, however, responded to these five sucrose analogs differently (Figure 12B, C, and Table 7). Artemisinin production was decreased in presence of Pal, Cel, and Lac. In contrast, when Tre or Mal, were present, artemisinin production increased. A statistically significant decrease in artemisinin production was only found between addition of 10% Cel and 100% Suc control (Figure 12B and Table 7;  $P = 0.047$ ).

Although the addition of 10% Pal did not statistically decrease the artemisinin production ( $P=0.068$ , Mann-Whitney U-test) when the seedlings were cultured in Petri dishes, conducting the experiment in shake flasks did give a significant result. There was significantly more total biomass produced by seedlings exposed to 10% Pal plus 90% Suc than those grown in 100% Suc (Figure 13A). Artemisinin levels were significantly reduced by 80% in the presence of 10% Pal (Figure 13B). Both results were significant at the  $P= 0.05$  level. These results showed that sucrose may also be providing some control over artemisinin production and possibly at the transporter stage because Pal can not be transported into cell and some interaction probably occurs between Pal and the sucrose transporter (Bouteau *et al.*, 1999; Fernie *et al.*, 2001; Börnke *et al.*, 2002; Sinha *et al.*, 2002).

**Table 7 Summary of growth and artemisinin production responses of sucrose analogs compared to 100% Suc.**

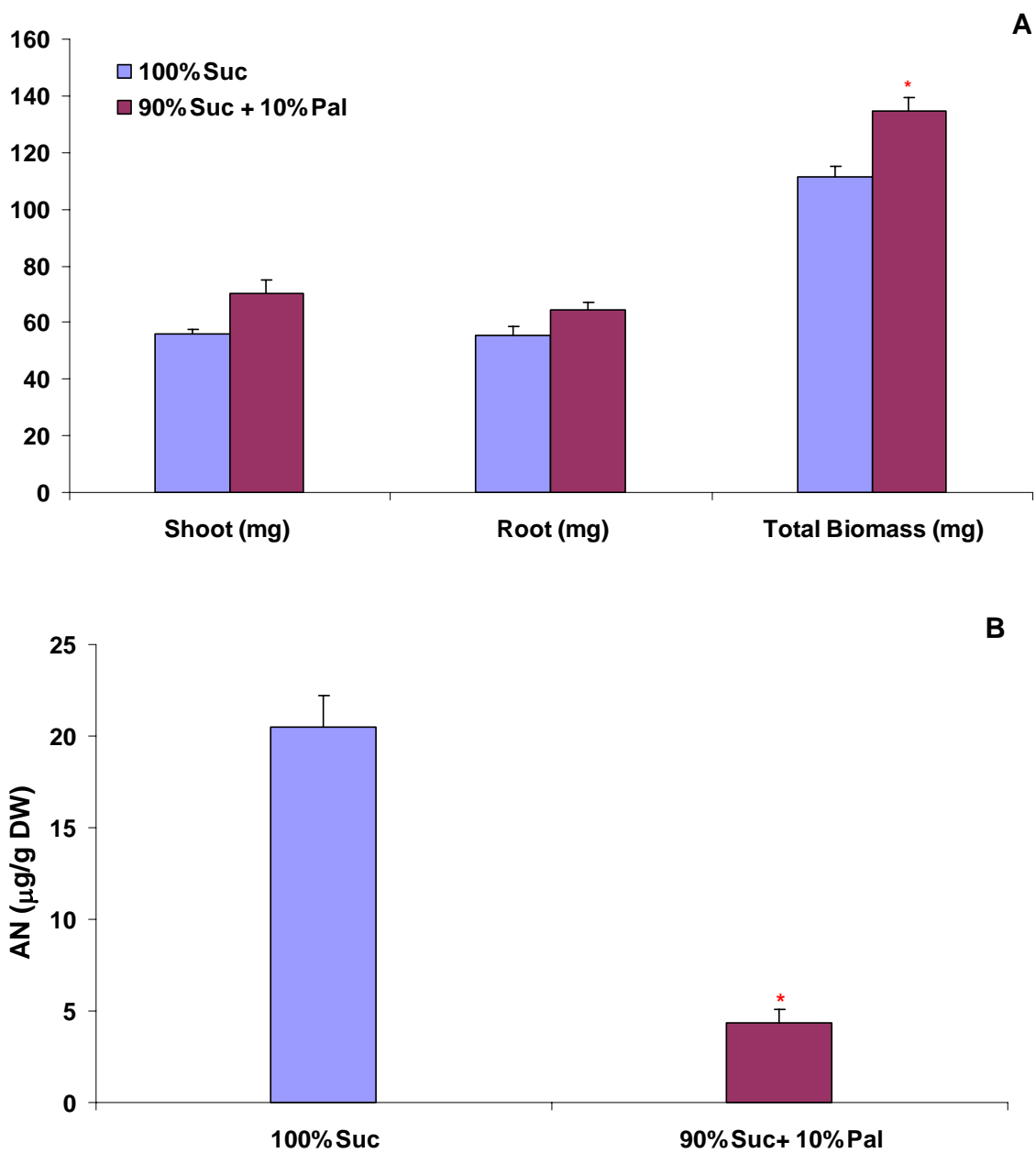
Compared to 100% Suc	90% Suc + 10% Pal	90% Suc + 10% Tre	90% Suc + 10% Mal	90% Suc + 10% Cel	90% Suc + 10% Lac
# of True Leaves	nc	nc	nc	nc	nc
Shoot Biomass (mg)	nc	nc	nc	nc	nc
Root Biomass (mg)	↓	↓	↓	↓	↓
Total Biomass (mg)	↓	↓	nc	nc	nc
Shoot/Root	nc	↑	nc	nc	↑
AN ( $\mu\text{g/g DW}$ )	nc	nc	nc	↓	nc

↑ or ↓ indicates statistically significant increase or decrease; nc indicates no statistically significant change.



**Figure 12 Effects of sucrose and its analogs on growth and artemisinin production.**

Data are the mean of total replicates + SE. A. Effect on growth. Data were analyzed using ANOVA followed by Dunnett's *post hoc* test comparing each group to the control (100% Suc). The total replicates for 100% Suc: 47; for 90% Suc + 10% Pal: 47; for 90% Suc + 10% Tre: 50; for 90% Suc + 10% Mal: 51; 90% Suc + 10% Cel: 46; 90% Suc + 10% Lac: 43. B. Effect of sucrose and its analog, Cel, on artemisinin production. Data were analyzed using the Mann-Whitney U test. There were 5 replicates for each condition. C. Effect of sucrose and its analogs on artemisinin production. Data were analyzed using the Kruskal-Wallis test. The total replicates for 100% Suc was 5; for other conditions were 6. \*P<0.05; \*\* P<0.01; \*\*\* P<0.001.



**Figure 13 Effects of sucrose and combination of 10% palatinose on growth and artemisinin production in liquid medium.**

Data are mean of the three replicates + SE. A. Effect on growth. Data were analyzed using independent-samples T-Test. There were 3 replicates for each condition. B. Effect on artemisinin production. Data were analyzed using Mann-Whitney U-test. There were 3 replicates for each condition. \*P<0.05.

#### **4.5 Effects of sugar combinations on growth and artemisinin production**

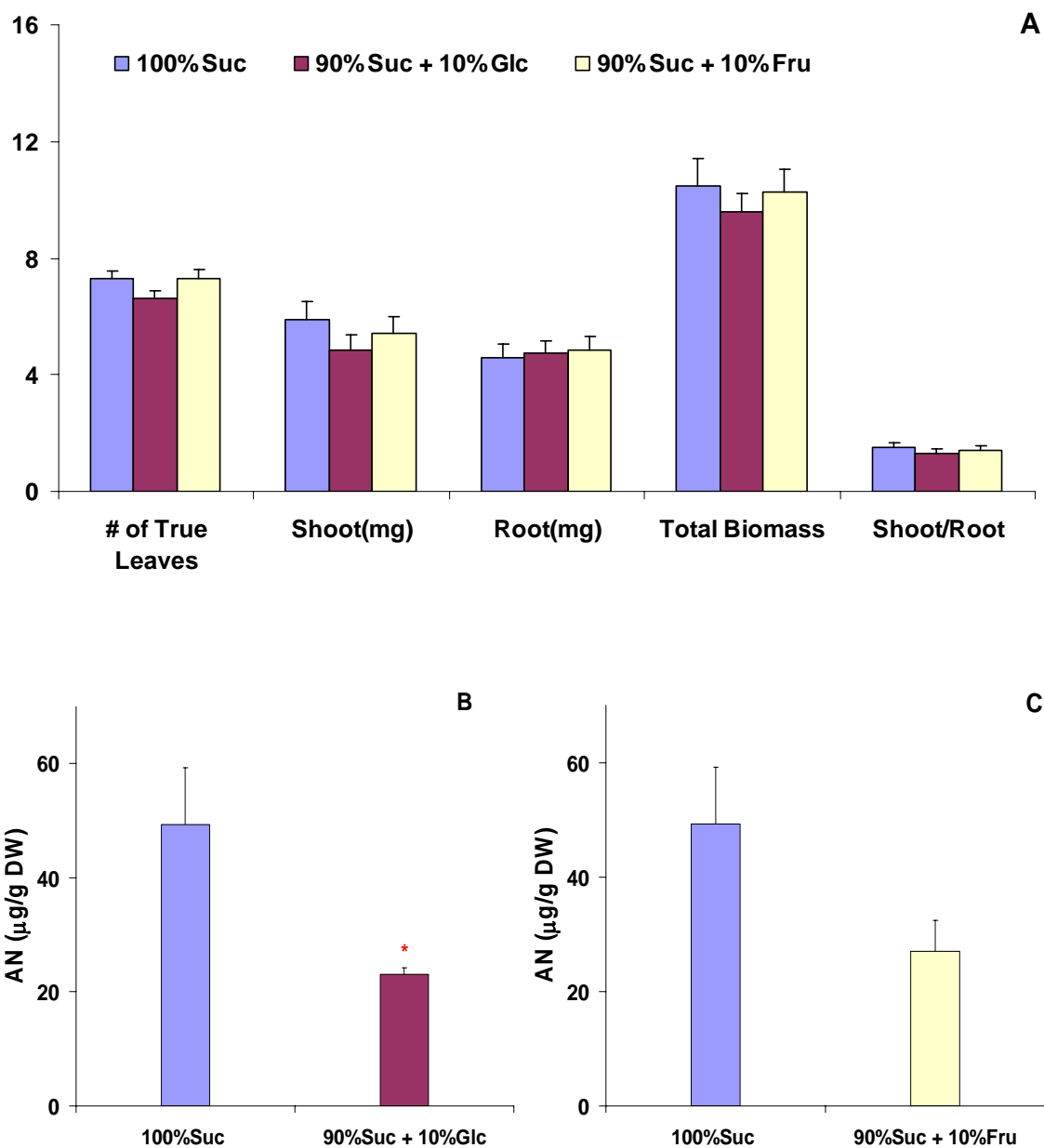
Because sucrose can be hydrolyzed rather rapidly (Kim *et al.*, 2003) into glucose and fructose via plant cell-wall invertase or cytoplasmic invertase (Williams *et al.*, 2000), addition of extracellular glucose or fructose in the presence of sucrose will inevitably change the ratio of these three sugars. When a small amount of glucose is added with sucrose, the relative glucose concentration is increased relative to cultures provided only sucrose. In the same way, when a little fructose and sucrose are provided exogenously, the relative fructose concentration is increased. A mixture of sugars should, thus, alter artemisinin production. Considering that normal plant cells would always have dynamic sugar concentrations, experiments were done using sucrose supplemented with 10% of either glucose or fructose following the previously described methods. Addition of 10% Glc or 10% Fru to 90% Suc did not affect seedling growth significantly compared to 100% Suc (Figure 14 A), despite a rather large decrease in artemisinin levels (Figure 14 B, C). A significant difference, however, was only observed between addition of 10% Glc and the 100% Suc control (Figure 14B;  $P=0.05$ ), but not between the 10% Fru and the 100% Suc control (Figure 14C). This surprising decrease in artemisinin was in contrast to that in 100% Glc (Figure 7B) and clearly showed that the sugar response is not simple.

#### **4.6 Effects of sugar ratios on growth and artemisinin production.**

The previous experiments suggested that sucrose, glucose, and fructose may be signal molecules affecting the production of artemisinin in *A. annua* seedlings, and that they interact with each other.

To compare how fructose and glucose might work coordinately in altering growth and





**Figure 14 Effects of sucrose and combination of either 10% Glc or 10% Fru on growth and artemisinin production.**

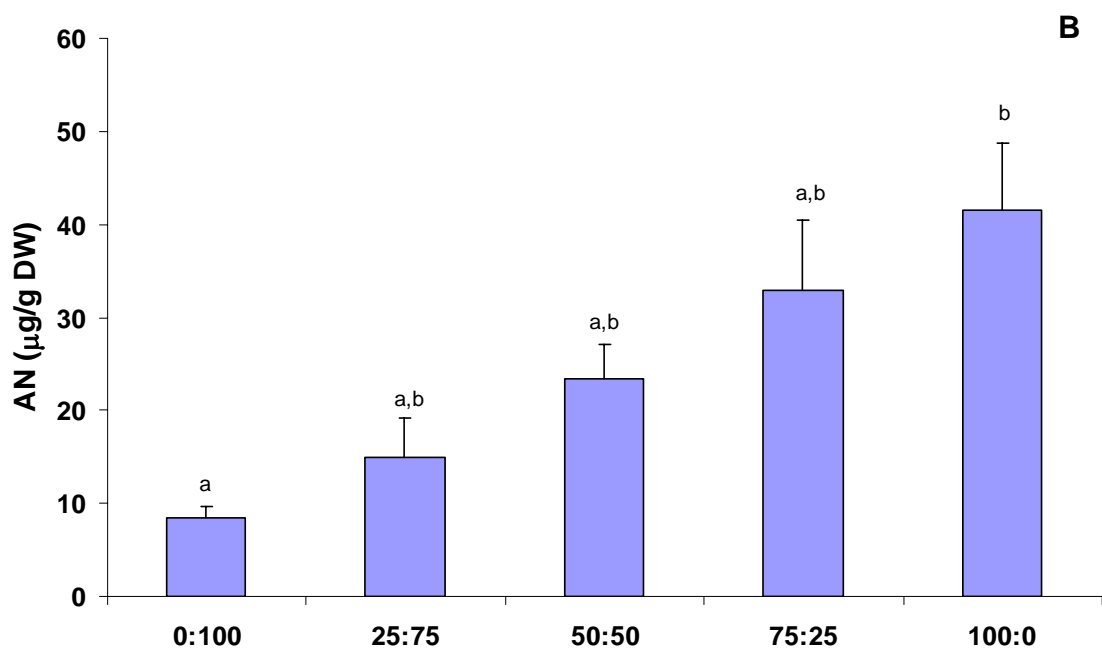
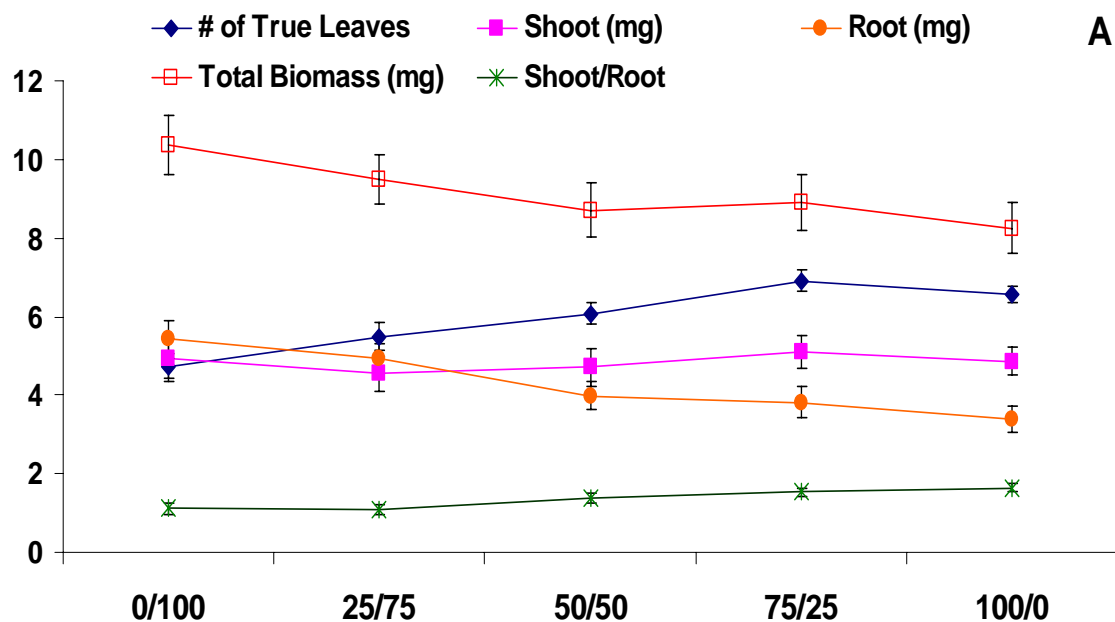
Data are mean of total replicates + SE. A. Effect on growth. Data were analyzed using ANOVA followed by Dunnett's *post hoc* test comparing each group to the control (100% Suc). The total replicates for 100% Suc: 29; for 90% Suc + 10% Glc: 30; for 90% Suc + 10% Fru: 28. B. Effect of 100% Suc and combination of 10% Glc on artemisinin production. Data were analyzed using Mann-Whitney U-test. There were 3 replicates for each condition. B. Effect of 100% Suc and combination of 10% Fru on artemisinin production. Data were analyzed using Mann-Whitney U-test. There were 3 replicates for each condition. \*P=0.05.

especially artemisinin production, five different ratios of these two sugars were tested on *A. annua* seedlings. As the concentration of glucose was increased relative to fructose, the number of true leaves first increased until the 75/25 (Glc/fru) ratio was reached (Figure 15A). In contrast, root mass decreased with increasing glucose level (Figure 15A). Overall, these data resulted in a slightly increasing shoot/root biomass ratio as the ratio of glucose increased compared to fructose (Figure 15A).

The artemisinin production steadily increased as the glucose to fructose ratio increased (Figure 15B). However, no statistically significant differences were found among these conditions except when fructose and glucose were added alone.

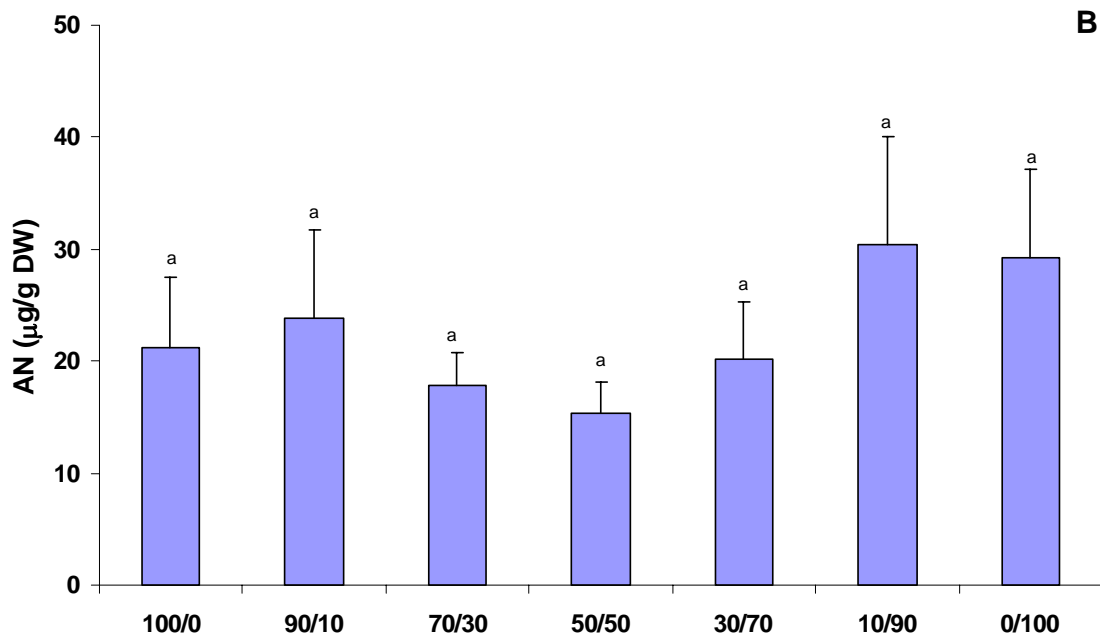
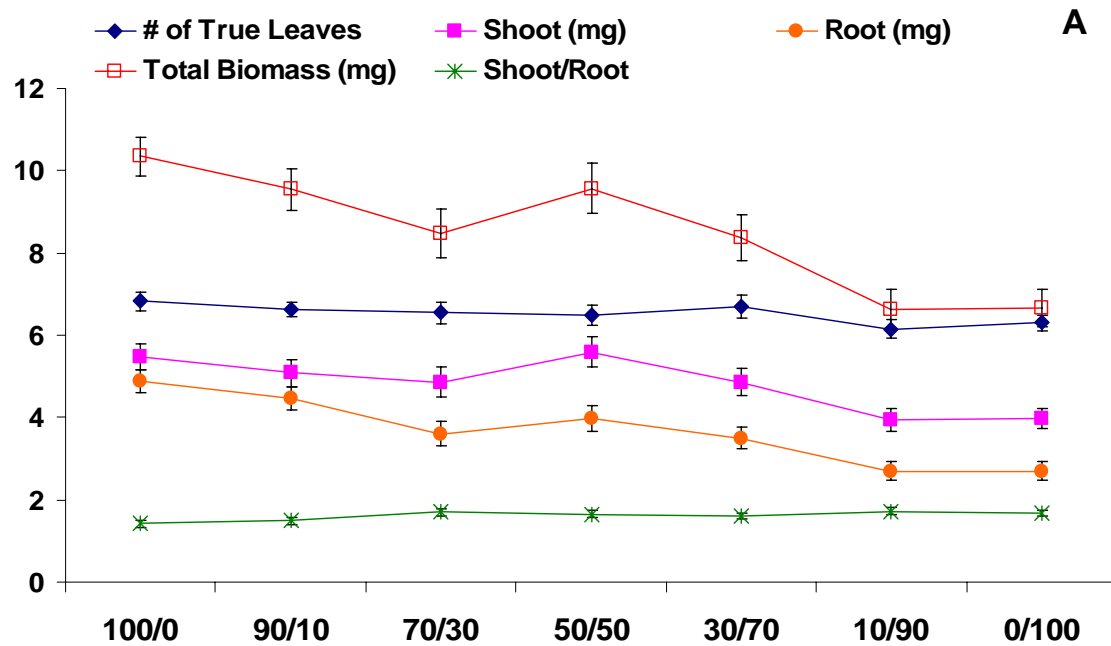
Because sucrose is also usually present in cells along with glucose and fructose, growth and artemisinin production were measured in seedlings grown in seven different concentrations of glucose plus sucrose using the previously described approach. Sucrose clearly stimulated *A. annua* plant growth compared to glucose (Figure 16A). This was reflected by the higher shoot mass, root mass, and total biomass of seedlings grown on 100% Suc compared to 100% Glc medium (Figure 16A). For the most part these growth responses were the same as shown in Figure 7.

Although artemisinin production (Figure 16B) increased a little in seedlings when glucose was provided in Petri dishes as 10% carbon source, artemisinin levels decreased as the concentration of glucose increased until the 50/50 concentration was reached. Afterwards, artemisinin production began to increase as glucose levels approached 90% (Figure 16B). Unfortunately, artemisinin production was not significantly different among these different Suc/Glc ratios.



**Figure 15 Effects of % Glc/% Fru ratio on growth and artemisinin production.**

Data are mean of total replicates  $\pm$  SE. A. Effect on growth. Data were analyzed using ANOVA followed by Student-Newman-Keuls *post hoc* test comparing all sugar groups to each other pairwise. The total replicates for 100/0: 25; 75/25: 29; 50/50: 39; 25/75: 35; 0/100: 43. B. Effect on artemisinin production. Data were analyzed using Kruskal-Wallis Test followed by Dunn's *post hoc* test to compare each other pairwise. There were 5 replicates for each ratio condition except 75/25, which contained 4. Groups that are statistically similar ( $P > 0.05$ ) are labeled with the same letter.



**Figure 16 Effects of % Suc/% Glc ratio on growth and artemisinin production.**

Data are mean of total replicates  $\pm$  SE. A. Effect on growth. Data were analyzed using ANOVA followed by Student-Newman-Keuls *post hoc* test comparing all sugar groups to each other pairwise. The total replicates for 100/0: 106; 90/10: 108; 70/30: 75; 50/50: 76; 30/70: 75; 10/90: 76; 0/100: 69. B. Effect on artemisinin production. Data were analyzed using Kruskal-Wallis Test to compare each other pairwise. The total replicates are 11 for 100/0 and 90/10; 7 for 50/50; 8 for other conditions. Groups that are statistically similar ( $P > 0.05$ ) are labeled with the same letter.

Taken together, the results of the mixed sugar experiments indicated that glucose clearly has a stimulatory effect on growth and artemisinin production, especially in combination with fructose. When all three sugars are present, however, the glucose stimulation effect is less clear and the sugar effect on artemisinin regulation appears complex.

## 5 Discussion

Sugars are the major carbon source for *in vitro* cultured plants. While the effects of different types of sugars and their concentrations on plant growth have long been recognized and used to optimize *in vitro* culture, very little is known about their effects on the control of secondary metabolite production. Three common sugars, sucrose, glucose, and fructose, were studied for their impact not only on plant growth and development, but particularly on artemisinin production. Artemisinin production was significantly increased compared to sucrose when equimolar carbon of glucose was fed to seedlings of *A. annua* (Figure 7B), while fructose appeared to have an inhibitory effect (Figure 7C). These results suggested that besides acting as growth nutrients, sugars were also affecting artemisinin production and possibly acting as signal molecules to regulate artemisinin biosynthesis. The major focus of this discussion, therefore, is on the evidence from this study on the effect of sugars acting as signal molecules on artemisinin production.

### **Glucose regulates artemisinin production possibly through a hexokinase sensor.**

Using different glucose analogs in combination with glucose, this study showed that glucose appears to be acting as a signal that is possibly perceived through hexokinase for controlling artemisinin production. When 3OMG comprised 10% of the total carbon was provided to seedlings, artemisinin production was significantly inhibited compared to the 100% Glc control (Figure 8B), while neither Man nor Mtl had this effect. Like Man, however, 3OMG can enter the cell through a transporter (Lalonde *et al.*, 1999; Gibson, 2000; Ho *et al.*, 2001; Loreti *et al.*, 2001), but it is not effectively phosphorylated by HXK (Cortès *et al.*, 2003) thereby suggesting that HXK may be acting as a sensor that

can control artemisinin production further downstream. These results are consistent with the hypothesis of HXK acting as a putative sugar sensor. This conclusion is similar to what has been observed in other studies. For example, Jang and Sheen (1994) showed that the expression of photosynthetic genes was inhibited by glucose and the glucose analogs that can be phosphorylated by HXK. In contrast, 3OMG and 6DOG, which can be taken up by cells but are not an effective substrate of HXK, did not inhibit the expression of those genes. Furthermore, the repression of photosynthetic genes also did not occur when G-6-P was directly delivered into the cells. Based on these results, Jang and Sheen (1994) proposed that HXK is the sensor in glucose signaling. Later Moore *et al.*, (2003) showed that an *Arabidopsis* mutant lacking HXK catalytic activity still showed glucose signaling functions like wild-type plants. Taken together, those studies provided compelling evidence that HXK can both act as a catalyst and sense a glucose signal. The apparent stimulation by Mtl of artemisinin production above the 100% Glc control (Figure 8B) also suggests that a monosaccharide transporter is probably not involved in the sensing process because Mtl does not enter the cell (Gibson, 2000). These results are consistent with the study of Jang and Sheen (1994). In their study, neither L-glucose, which cannot be efficiently transported by plant cell, nor 3OMG, which can be transported into plant cell but cannot be effectively phosphorylated by HXK, repressed the expression of photosynthetic genes compared to glucose.

There is also the possibility that the inhibition of artemisinin production induced by the addition of 10% 3OMG may be caused by 3OMG possibly acting as a toxin because growth was also significantly inhibited (Figure 8A). This is unlikely, however, because while artemisinin production was 95% inhibited, growth was only inhibited 30% (Figure

8). Furthermore, in the presence of 10% Mtl, which also inhibited growth (Figure 8A), artemisinin production was actually stimulated when compared to 100% Glc (Figure 8B). Moreover, addition of only 1% 3OMG, while stimulating growth beyond the 10% 3OMG, still inhibited artemisinin production (Figure 9). These results together, suggest that the reduced growth observed in the presence of 10% 3OMG was, thus, not necessarily the cause of the decreased artemisinin production (Figure 8B).

Considering that artemisinin production was significantly inhibited by 10% 3OMG but not by 1% 3OMG (Figure 9B and C), also suggested that the signal effect of glucose on artemisinin production may be dependent on glucose concentration. Seedlings were subsequently fed different ratios of Glc/Fru and both growth and artemisinin were measured. As the proportion of glucose increased relative to fructose, the level of artemisinin also increased (Figure 15B), suggesting that the concentration of these two monosaccharides is sensed and that their ratio affects the yield of artemisinin, a distant downstream product. This is further supported by the data that show inhibition of artemisinin production by 100% Fru compared to sucrose or glucose and by further inhibition if 10% Tag, a fructose analog, is added to fructose (Figure 11B). Inhibition of artemisinin production by fructose is in contrast to the results of Jung *et al.* (1992) who reported the stimulation by fructose of catharanthine yield in hairy roots of *C. roseus*. These results do not, however, exclude crosstalk between the different sugars in regulating artemisinin production.

When the activity of HXK was measured in seedlings grown in 10% 3OMG, HXK specific activity increased compared to that of seedlings grown in 100% Glc (Figure 10). These results have several possible interpretations. First, hexokinase, a known glucose



signal sensor, may affect artemisinin production through its catalytic activity. The addition of 10% 3OMG actually decreased by 10% the total carbon that was metabolically available and since hexokinase activity increased concomitantly by 10% (Figure 10), it is possible that the increase in HXK activity may be in response to the total carbon available to the seedlings after 14 days in culture. Several studies, however, have indicated that glucose signaling is uncoupled from glucose metabolism (Jang and Sheen *et al.*, 1994; Jang *et al.*, 1997). Further, HXK1 mutants lacking catalytic activity still showed various signaling functions (Moore *et al.*, 2003). All of the signaling functions previously studied, however, have been related to gene expression or related to plant development, not secondary metabolism, so separation of glucose signaling from glucose metabolism can not necessarily be assumed with respect to artemisinin production. Second, some unknown metabolite(s) downstream of glucose phosphorylation may be involved in artemisinin production with or without glucose signaling and this metabolite might be what is being sensed by HXK (Xiao *et al.*, 2000). Third, increased hexokinase activity could also be due to some stress produced by the presence of 3OMG. For example, it was reported by Fox *et al.* (1998) that hexokinase activity is stimulated in shoots of *Echinochloa phyllopogon* by anaerobic stress. Further, 3OMG also can act as a competitive inhibitor of the glucose transporter, therefore inhibiting glucose entrance to plant cells which could subsequently induce an increase in HXK activity (Gogarten and Bentrup F-W, 1983; Getz *et al.*, 1987). Clearly additional studies of hexokinase activity in seedlings grown in sucrose, fructose, and glucose in combination with its other analogs and in the presence of an HXK inhibitor like N-acetyl glucosamine should be undertaken.

Disaccharide signal transduction pathways are not as clearly understood as the

glucose signaling pathways. Sucrose analogs were used to learn more about this pathway and how it might regulate artemisinin production. Loreti *et al.* (2000) had previously shown that similar to our results, disaccharides containing a fructose moiety had an inhibitory effect on  $\alpha$ -amylase in barley embryos. Even the non metabolizable fructose moiety-containing-disaccharides, palatinose, turanose, and lactulose repressed the enzyme indicating that the fructose moiety was necessary for sensing the disaccharide and that it was independent of the glucose sensing system (Loreti *et al.*, 2000). In *A. annua*, however, only Pal was tested for its effects on artemisinin production and compared to 100% Suc, addition of 10% Pal significantly inhibited artemisinin production (Figure 13). This suggested that instead of only specific glucose sensitivity, there may also be a disaccharide transporter that is involved in a signaling effect on artemisinin production because Pal is not transported into the cell. Similar to the conclusions reached by Loreti *et al.* (2000), these results suggest that besides a HXK, glucose sensor, there may also be a sucrose transporter sensor that responds to sucrose to induce a signal to produce artemisinin.

When other disaccharide analogs were fed to seedlings, some interesting results were observed. Addition of 10% Cel, for example, significantly decreased artemisinin production compared to 100% Suc (Figure 12B). Mal, on the other hand, did not significantly alter artemisinin compared to the 100% Suc control (Figure 12C). Cel (Table 1) consists of two  $\beta$  glucose units with a 1 $\rightarrow$ 4 linkage, while Mal (Table 1), a stereoisomer of Cel, consists of an  $\alpha$ -glucose and a  $\beta$ -glucose also with a 1 $\rightarrow$ 4 linkage. Mal is known to be transported by a membrane transporter across the chloroplast membrane and into the cytosol (Weise *et al.*, 2005) where it is potentially converted into

sucrose via a series of steps (Figure 3; Lu and Sharkey, 2004; Yan *et al.*, 2005). Less is known about cellobiose. The difference in the stereo structures of these two analogs, however, may be the feature sensed by plant cells through plasma membrane transporter or some intracellular enzyme, thereby differently affecting artemisinin production.

Crosstalk is known to exist between sugar signaling systems (Rolland *et al.*, 2002, Rook *et al.*, 1998, Wingler *et al.*, 2000, Halford and Paul, 2003), and was also observed in artemisinin production. With the exception of the Glc/Fru ratio results, it is difficult to interpret sugar combination experiments. In an experiment where either 10% Glc or 10% Fru was added to 90% Suc, it appeared that addition of a small amount of glucose to sucrose fed seedlings of *A. annua* significantly decreased artemisinin production compared to the 100% Suc control (Figure 14B). This result was unexpected considering the Glc/Fru results (Figure 15B) and the significant stimulation of artemisinin production in seedlings that were fed only glucose (Figure 7B), but may be explained as an apparently antagonistic action between signals perceived from sucrose and from glucose (Halford and Paul, 2003). Such an antagonistic effect and the complexity of the sugar signals that govern artemisinin production are even more pronounced when seedling are fed different ratios of Glc/Suc (Figure 16B).

## 6 Conclusion

At the same carbon level and compared to sucrose, the stimulation of artemisinin production by glucose, and inhibition by fructose clearly showed that these sugars can control artemisinin production. By feeding small amounts of different sugar analogs to seedlings, results further suggested that there may be at least two possible sugar sensing mechanisms that are involved in controlling artemisinin production in *A. annua*. The first, in response to addition of 10% 3OMG, HXK appears to be a sensor that can detect differential concentrations of glucose and fructose thereby altering artemisinin production further downstream. The second mechanism, in response to addition of 10% Pal, appears to involve a sucrose transporter that senses a sucrose specific signal. When both monosaccharide and disaccharide sugars are present, there appears to be crosstalk between the putative sugar signals, but the mechanism is complex, further studies to elucidate complete understanding of the mechanism of action are warranted.

## 7 References

- Abdin MZ, Israr M, Rehman RU, Jain SK 2003 Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. *Planta Medica* 69: 289-299.
- Balint GA 2001 Artemisinin and its derivatives: An important new class of antimalarial agents. *Pharmacology & Therapeutics* 90: 261-265.
- Basile DV, Akhtari N, Durand Y, Nair MSR 1993 Toward the production of artemisinin through tissue culture: determining nutrient-hormone combinations suitable for cell suspension cultures. *In Vitro Cellular & Developmental Biology* 29P: 143-147.
- Baskin TI, Remillong EL, Wilson JE 2001 The impact of mannose and other carbon sources on the elongation and diameter of the primary root of *Arabidopsis thaliana*. *Australian Journal of Plant Physiology* 28: 481-488.
- Borisjuk L, Walenta S, Rolletschek H, Mueller-Klieser W, Wobus U, Weber H 2002 Spatial analysis of plant metabolism: sucrose imaging within *Vicia faba* cotyledons reveals specific developmental patterns. *The Plant Journal* 29(4): 521-530.
- Börnke F, Hajirezaei M, Heineke D, Melzer M, Herbers K, Sonnewald U 2002 High-level production of the non-cariogenic sucrose isomer palatinose in transgenic tobacco plants strongly impairs development. *Planta* 214(3): 356 – 364.
- Bouteau F, Dellis O, Bousquet U, Rona JP 1999 Evidence of multiple sugar uptake across the plasma membrane of laticifer protoplasts from *Hevea*. *Bioelectrochemistry and Bioenergetics* 48: 135-139.
- Bradford M, 1976 A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72:248-254.
- Brouquisse R, Evrard A, Rolin D, Raymond P, Roby C 2001 Regulation of protein degradation and protease expression by mannose in maize root tips. Pi sequestration by mannose may hinder the study of its signaling properties. *Plant Physiology* 125: 1485-1498.
- Cheng CL, Acedo GN, Cristinsin M, Conkling MA 1992 Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription. *Proceedings of the National Academy of Science of USA* 89: 1861-1864.
- Chiou TJ and Bush DR 1998 Sucrose is a signal molecule in assimilate partitioning. *Proceedings of the National Academy of Science USA* 95: 4784-4788.
- Cortès S, Gromova M, Evrard A, Roby C, Heyraud A, Rolin DB, Raymond P, Brouquisse RM 2003 In plants, 3-O-methylglucose is phosphorylated by hexokinase but not perceived as a sugar. *Plant Physiology* 131: 824-837.
- Dijkwel PP, Huijser C, Weisbeek PJ, Chua NN, Smeeckens SCM 1997 Sucrose control of phytochrome A signaling in *Arabidopsis*. *The Plant Cell* 9: 583-595.
- Duke, MV, Paul, RN, Elsohly, HN, Sturtz, G, Duke, S 1994 Localization of artemisinin in foliar tissues of gland and glandless biotypes of *Artemisia annua* L. *International Journal of Plant Sciences* 155(3):

365-372.

- Eastmond PJ and Graham LA 2003 Trehalose metabolism: A regulatory role for trehalose-6-phosphate? *Current Opinion in Plant Biology* 6: 231-235.
- Eastmond PJ, Li Y, Graham IA 2003 Is trehalose-6-phosphate a regulator of sugar metabolism in plants? *Journal of Experimental Botany* 54(382): 533-537.
- Etxeberria E, González P, Tomlinson P, Pozueta-Romero J 2005 Existence of two parallel mechanisms for glucose uptake in heterotrophic plant cells. *Journal of Experimental Botany* 56(417): 1905-1912.
- Fernie AR, Roessner U, Geigenberger P 2001 The sucrose analog palatinose leads to a stimulation of sucrose degradation and starch synthesis when supplied to discs of growing potato tubers. *Plant Physiology* 125: 1967-1977.
- Ferreira JFS and Janick J 1995 Floral morphology of *Artemisia annua* with special reference to trichomes. *International Journal of Plant Sciences* 156(6): 807-815.
- Ferreira JFS and Janick J 1996 Distribution of artemisinin in *Artemisia annua*. J. Janick (ed.), *Progress in new crops*. ASHS Press, Arlington, VA. P. 579-584.
- Fox TC, Green BJ, Kennedy RA, and Rumpho ME 1998 Changes in hexokinase activity in *Echinochloa phyllopogon* and *Echinochloa crus-galli* in response to abiotic stress. *Plant Physiology* 118: 1403-1409.
- Frommer WB, Schulze WX, Lalonde S 2003 Hexokinase, jack-of-all-trades. *Science* 300: 261-263.
- Gamborg OL, Miller RA, Ojima K 1968 Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50:151-158.
- Galal AM, Ross SA, Jacob M, ELSohly MA 2005 Antifungal activity of artemisinin derivatives. *Journal of Natural Products* 68(8):1274-6.
- Getz HP, Knauer D, Willenbrink J 1987 Transport of sugars across the plasma membrane of beetroot protoplasts. *Planta* 171: 185-196.
- Glantz SA 2001 *Primer of biostatistics*. Fifth edition New York: McGraw-Hill. Pp. 89, 95, 103, 339, 343, 362, 366.
- Gibson SI 2000 Plant sugar-response pathways: part of a complex regulatory web. *Plant Physiology* 124: 1532-1539.
- Gibson SI 2004 Sugar and phytohormone response pathways: navigating a signaling network. *Journal of Experimental Botany* 55(395): 253-264.
- Gibson SI 2005 Control of plant development and gene expression by sugar signaling. *Current Opinion in Plant Biology* 8(3): 93-102.
- Gonzali S, Alpi A, Blando F, Bellis LD 2002 *Arabidopsis* (HXK1 and HXK2) and yeast (HXK2) hexokinases overexpressed in transgenic lines are characterized by different catalytic properties. *Plant Science* 163: 943-954.
- Gogarten JP and Bentrup F-W 1983 Fluxes and compartmentation of 3-O-methyl-D-glucose in *Riccia fluitans*. *Planta* 159: 423-431.

- Graham IA, Denby KJ, Leaver CJ 1994 Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. *The Plant Cell* 6: 761-772.
- Halford NG and Paul MJ 2003 Carbon metabolite sensing and signaling. *Plant Biotechnology* 1: 381-398.
- Hérouart D, Sangwan RS, Sanwan-Norreel BS 1991 Selection and characteristics of a lactose-adapted *Datura innoxia* cell line grown in suspension culture. *The Plant Cell* 21: 97-104.
- Ho SL, Chao YC, Tong WF, Yu SM 2001 Sugar coordinately and differentially regulates growth- and stress-related gene expression via a complex signal transduction network and multiple control mechanisms. *Plant Physiology* 125: 877-890.
- Jang JC and Sheen J 1994 Sugar sensing in higher plants. *The Plant Cell* 6: 1665-1679.
- Jang JC, León P, Zhou L, Sheen J 1997 Hexokinase as a sugar sensor in higher plants. *The Plant Cell* 9: 5-19.
- Jung KH, Kwak SS, Kim SW, Lee H, Choi CY, Lin JR 1992 Improvement of the catharanthine productivity in hairy root cultures of *Catharanthus roseus* by using monosaccharides as a carbon source. *Biotechnology Letters* 14:695-700.
- Kim YJ, Weathers PJ, Wyslouzil BE 2003 Growth dynamics of *Artemisinin annua* hairy roots in three culture systems. *Biotechnology and Bioengineering* 83: 428-443.
- Koch, K 2004 Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology* 7: 235-246.
- Lalonde S, Boles E, Hellmann H, Barker L, Patrick JW, Frommer WB, Ward JM 1999 The dual function of sugar carriers: Transport and sugar sensing. *The Plant Cell* 11: 707-726.
- Larronde F, Krisa S, Decendit A, Cheze C, Deffieux G, Merillon JM 1998 Regulation of polyphenol production in *Vitis vinifera* cell suspension cultures by sugars. *Plant Cell Reports* 17(12): 946 – 950.
- León P and Sheen J 2003 Sugar and hormone connections. *Trends in Plant Science* 8(3): 110-116.
- Liu CZ, Wang YC, Guo C, Ouyang F, Ye HC, Li GF 1998 Production of artemisinin by shoot cultures of *Artemisia annua* L. in a modified inner-loop mist bioreactor. *Plant Science* 135: 211-217.
- Loreti E, Alpi A, Perata P 2000 Glucose and disaccharide-sensing mechanisms modulate the expression of  $\alpha$ -amylase in barley embryos. *Plant Physiology* 123: 939-948.
- Loreti E, Bellis LD, Alpi A, Perata P 2001 Why and how do plant cells sense sugars? *Annals of Botany* 88: 803-812.
- Lu Y, Sharkey TD 2004 The role of amylomaltase in maltose metabolism in the cytosol of photosynthetic cells. *Planta* 214: 466-473.
- Mandelbaum-Schmid J 2005 World Health Organization drives efforts to boost antimalarial drug supply. News release 6 June 2005. Communications Officer, Roll Back Malaria WHO/Geneva. <http://www.who.int/mediacentre/news/releases/2005/pr24/en/index.html>
- Martin T, Hellmann H, Schmidt R, Willmitzer L, Frommer WB 1997 Identification of mutants in metabolically regulated gene expression. *The Plant Journal* 11(1): 53-62.
- Martin VJJ, Pitera DJ, Withers ST, Newman JD, Keasling JD 2003 Engineering a mevalonate pathway in

- Escherichia coli* for production of terpenoids. *Nature Biotechnology* 21(7):796-802.
- Maynard JW, Lucas WJ 1982 Sucrose and glucose uptake into *Beta vulgaris* leaf tissues. *Plant Physiology* 70: 1436-1433.
- Mendis K, Sina BJ, Marchesini P, Carter R 2001 The neglected burden of *Plasmodium vivax* malaria. *American Journal of Tropical Medicine and Hygiene* 64 (Suppl. 1-2): 97-105.
- Meshnick SR, Taylor TE, Kamchonwongpaisan S 1996 Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological Reviews* 60(2): 301–315.
- Moore B, Zhou L, Rolland F, Hall Q, Cheng WH, Liu YX, Hwang I, Jones T, Sheen J 2003 Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* 300:332-336.
- Moorthy VS, Good MF, Hil AVS 2004 Malaria vaccine developments. *The Lancet* 363:150-156.
- Müller J, Aeschbacher RA, Winkler A, Boller T, Wiemken, A 2001 Trehalose and trehalase in *Arabidopsis*. *Plant Physiology* 125: 1086-1093.
- Pego JV, Weisbeek PJ, Smeekens SCM 1999 Mannose inhibits *Arabidopsis* germination via a hexokinase-mediated step. *Plant Physiology* 119: 1017-1023.
- Price J, Li TC, Kang SG, Na JK, Jang JC 2003 Mechanisms of glucose signaling during germination of *Arabidopsis*. *Plant Physiology* 132: 1424-1438.
- Price, J., Laxmi, A., Martin, S.K.S., Jang, J.C. 2004 Global transcription profiling reveals multiple sugar signal transduction mechanisms in *Arabidopsis*. *The Plant Cell* 16:2128-2150.
- Riou-Khamlichi C, Menges M, Healy JMS, Murray JAH 2000 Sugar control of the plant cell cycle: differential regulation of *Arabidopsis* D-type cyclin gene expression. *Molecular and Cellular Biology* 20(13): 4513-4521.
- Roitsch T, Bittner M, Godt DE 1995 Induction of apoplasmic invertase of *Chenopodium rubrum* by D-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. *Plant Physiology* 108: (285-294).
- Roldan M, Gomez-Mena C, Ruiz-Garcia L, Salinas J, Martinez-Zapater JM 1999 Sucrose availability on the aerial part of the plant promotes morphogenesis and flowering of *Arabidopsis* in the dark. *The Plant Journal* 2(5): 581-590.
- Rolland F, Moore B, Sheen J 2002 Sugar sensing and signaling in plants. *The Plant Cell* 14: S185-S205.
- Rook F, Gerrits N, Kortstee A, Kampen MV, Borrias M, Weisbeek P, Smeekens S 1998 Sucrose-specific signaling represses translation of the *Arabidopsis* ATB2 bZIP transcription factor gene. *The Plant Journal* 15(2): 253-263
- Rook F, Bevan MW 2003 Genetic approaches to understanding sugar-response pathways. *Journal of Experimental Botany* 54 (382): 495-501.
- Sakr S, Lemoine R, Gaillard C, Delrot S 1993 Effect of cutting on solute uptake by plasma membrane vesicles from sugar beet (*Beta vulgaris* L.) leaves. *Plant Physiology* 103(1): 49-58.
- Schlupmann H, Pellny T, Dijken AV, Smeekens S, Paul M 2003 Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. *Plant Biology* 100(11): 6849-6854.



- Smeekens S 2000 Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 51:49-81.
- Simooya O. 2005 The WHO 'Roll Back Malaria Project': planning for adverse event monitoring in Africa. *Drug Safety* 28(4):277-86.
- Simon JE, Charles D, Ceibert E, Grant L, Janick J, Whipkey, A 1990 *Artemisia annua* L.: A promising aromatic and medicinal. In: J. Janick and J.E. Simon (eds.), *Advances in new crops*. Timber Press, Portland, OR p. 522-526.
- Smith T, Weathers PJ, Cheetham RD 1997 Effects of gibberellic acid on hairy root cultures of *Artemisia annua*: growth and artemisinin production. *In Vitro Cellular and Developmental Biology-Plant* 33:75-79.
- Singh NP and Lai HC 2004 Artemisinin induces apoptosis in human cancer cells. *Anticancer Research* 24(4):2277-80.
- Sinha AK, Hofmann MG, Römer U, Köckenberger W, Elling L, Roitsch T 2002 Metabolizable and non-metabolizable sugars activate different signal transduction pathways in tomato. *Plant Physiology* 128: 1480-1489.
- Sturm A 1999 Invertase. Primary structures, functions, and roles in plant development and sucrose partitioning. *Plant Physiology* 121: 1-7.
- Sturm A, Tang GQ 1999 The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science* 4(10): 401-407.
- To JPC, Reiter WD, Gibson SI 2002 Mobilization of seed storage lipid by *Arabidopsis* seedlings is retarded in the presence of exogenous sugars. *BioMed Central Plant Biology* 2: 4.
- Towler M, Kim YJ, Correll M, Wyslouzil B, Weathers, PJ. 2006 Design, development, and applications of mist bioreactors for micropropagation and hairy root culture. In: *Plant Tissue Culture Engineering*. Eds. Gupta, SD, Ibaraki, Y. Springer, The Netherlands. Pp. 119-134. Invited.
- Umemura T, Perata P, Futsuhara Y, Yamaguchi J 1998 Sugar sensing and  $\alpha$ -amylase gene repression in rice embryos. *Planta* 204: 420-428.
- van Agtmael MA, Eggelte TA, and van Boxtel CJ 1999 Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *Trends in Pharmacological Sciences* 20:199-205.
- Vitrac X, Larronde F, Krisa S, Decendit A, Deffieux G, Mérillon JM 2000 Sugar sensing and  $Ca^{2+}$ -calmodulin requirement in *Vitis vinifera* cells producing anthocyanins. *Phytochemistry* 53: 659-665.
- Weathers PJ, DeJesus-Gonzalez L, Kim YJ, Souret FF, Towler MJ 2004 Alteration of biomass and artemisinin production in *Artemisia annua* hairy roots by media sterilization method and sugars. *Plant Cell Report* 23:414-418.
- Weathers PJ, Wobbe KK, Wyslouzil BE 2006a In vitro production of artemisinin: biosynthesis, regulation, and bioprocess development. In: *Molecular Plant Cell-Based Bioprocessing* (ed. Zhang, W, Springer, Dordrecht, The Netherlands. Submitted December 2005.
- Weathers PJ, Elkholy S, Wobbe KK 2006b Artemisinin: the biosynthetic pathway and its regulation in

- Artemisia annua*, a terpenoid-rich species. In Vitro Cellular and Developmental Biology-Plant, accepted for publication.
- Weise SE, Kim KS, Stewart RP, Sharkey TD 2005  $\beta$ -Maltose is the metabolically active anomer of maltose during transitory starch degradation. *Plant Physiology* 137: 756-761.
- WHO Roll Back Malaria Partnership 1998 What is malaria?  
[http://www.rbm.who.int/cmc\\_upload/0/000/015/372/RBMInfosheet\\_1.htm](http://www.rbm.who.int/cmc_upload/0/000/015/372/RBMInfosheet_1.htm)
- WHO Roll Back Malaria Partnership 2004 Surge in demand leads to shortage of artemisinin-based combination therapy for malaria.  
[http://www.who.int/malaria/cmc\\_upload/0/000/015/364/RBMInfosheet\\_9.pdf](http://www.who.int/malaria/cmc_upload/0/000/015/364/RBMInfosheet_9.pdf)
- WHO Roll Back Malaria Department 2005a World Health Organization drives efforts to boost antimalarial drug supply.  
<http://www.who.int/mediacentre/news/releases/2005/pr24/en/>
- WHO Roll Back Malaria Department 2005b Roll Back Malaria Department Meeting on the production of artemisinin and ACTs. Second Draft Report.  
<http://www.who.int/malaria/docs/arusha-artemisinin-meeting.pdf>
- Williams LE, Lemoine R, Sauer N 2000 Sugar transporters in higher plants - a diversity of roles and complex regulation. *Trends in Plant Science* 5(7): 283-290.
- Wingler A, Fritzius T, Wiemken A, Boller T, Aeschbacher RA 2000 Trehalose induces the ADP-glucose pyrophosphorylase gene, *ApL3*, and starch synthesis in *Arabidopsis*. *Plant Physiology* 124: 105-114.
- Wingler A 2002 The function of trehalose biosynthesis in plants. *Phytochemistry* 60: 437-440.
- Woerdenbag HJ, Lugt CB, Pras N 1990 *Artemisia annua* L.: a source of novel antimalarial drugs. *Pharmaceutisch Weekblad. Scientific edition* 12(5): 169-81.
- Woerdenbag HJ, Lüers, JFJ, Uden WV, Pras N, Malingré TM, Alfermann AW 1993 Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. *Plant Cell, Tissue and Organ Culture* 32:247-257.
- Xiao W Y, Sheen J, Jang JC 2000 The role of hexokinase in plant sugar signal transduction and growth and development. *Plant Molecular Biology* 44: 451-461.
- Yan L, Jackson PG, Thomas, D S 2005 Daylength and circadian effects on starch degradation and maltose metabolism. *Plant Physiology* 138: 2280-2291.
- Yen HE, Chen YC, Yen SK, Lin JH 1999 Sugar uptake by photomixotrophic soybean suspension cultures. *Botanical Bulletin of Academia Sinica* 40: 147-152.
- Zhou L, Jang JC, Jones TL, Sheen J 1998 Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. *Proceedings of the National Academy of Science USA* 95(17): 10294-10299.

# 8 Appendix

## 8.1 Hexokinase assay protocols

### 8.1.1 Shoot Extraction

#### Final Concentration of Extraction buffer

50mM Hepes-KOH, pH 7.5  
5mM MgCl<sub>2</sub>·6H<sub>2</sub>O  
1mM EDTA, pH 7.0  
15mM KCl  
10 % Glycerol  
0.1% Triton X-100  
1X protease inhibitor

#### Procedure of Extract Buffer Preparation

1. Add to beaker:

5ml	0.5M	Hepes-KOH pH 7.5	FW:238.8
1.25ml	0.2M	MgCl <sub>2</sub> ·6H <sub>2</sub> O	Sigma M2393
1ml	0.05M	EDTA pH 7.0	Sigma E9884; pH7.0 adjusted using KOH
0.75ml	1.0M	KCl	Sigma P4504
5ml		Glycerol	Sigma G5150
1 tablet		Protease inhibitor	Roche Diagnostics #1836145
30ml		H <sub>2</sub> O	

2. Mix using stir bar until tablet is dissolved.

3. Add 0.5 ml 10% Triton (Sigma T6878), mix gently then adjust the volume to 50ml.

4. Store at -20°C. The buffer can be thawed and refrozen a number of times.

#### Experimental Use

1. Thaw the frozen extract buffer.

2. Add 25.6µl, 0.1M DTT (Acros Organics, #16568-0250) in 1 ml extract buffer.

#### Extraction Procedure

1. Weigh 0.1g frozen plant material.

2. Add 10 % (w/w) polyvinylpyrrolidone (PVPP; Sigma P6755).

3. Add tissue and liquid N<sub>2</sub> to mortar with pestle, after the liquid N<sub>2</sub> evaporates, add PVPP and 1ml extraction buffer, grind thoroughly.

4. Pour directly into centrifuge tube and balance

5. Centrifuge for 3 min at 17,400 x g at 4°C.

6. The clear supernatant was collected and assayed for hexokinase activity.

### 8.1.2 Hexokinase Activity Assay Procedures

#### Final concentration of Assay Buffer

50mM Bicine-KOH, pH 8.5  
15mM KCl  
5mM MgCl<sub>2</sub>·6H<sub>2</sub>O  
2.5mM ATP, pH6.8-7  
1mM NAD  
2units glucose 6-P dehydrogenase (G6PDH)  
5 mM glucose

#### Assay mixture for 5 assays

0.475ml	0.5263M	Bicine-KOH pH8.5	Sigma B3876
0.125ml	0.2M	MgCl <sub>2</sub> ·6H <sub>2</sub> O	Sigma M2393
0.075ml	1M	KCl	Sigma P4504
1ml	0.0124M	ATP pH6.8-7	Sigma A-2383, pH adjusted using KOH
0.375ml	0.0133M	NAD	Sigma 43410
0.2ml	50units/ml	G6PDH	

#### 50units/ml G6PDH

944µl	1M	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	Sigma A4915
5µl	2M	Tris pH7.5	Sigma T1503
1µl	0.2M	MgCl <sub>2</sub> ·6H <sub>2</sub> O	Sigma M2393
9µl		G6PDH	Sigma G8404-200Unit
41µl		H <sub>2</sub> O	

#### Assay procedure

1. Add to a 1-ml cuvette:  
450µl assay mixture  
100µl plant crude extract  
400µl H<sub>2</sub>O
2. Cover and invert the cuvette several times, zero spectrophotometer at 340nm.
3. Add 50µl 2mM glucose to cuvette, cover it with cap and invert 2-3 times.
4. Time scan the  $\Delta$ Abs change at 340nm.
5. Assay mixture is checked by 2 units yeast hexokinase, Sigma H5625, before assay samples. At 340nm,  $\Delta$ Abs change  $\geq 1$  by 2 units yeast hexokinase within several minutes indicates the validity of the assay mixture.

#### Reaction Rate Calculations

The mM extinction coefficient of NADH = 6.22

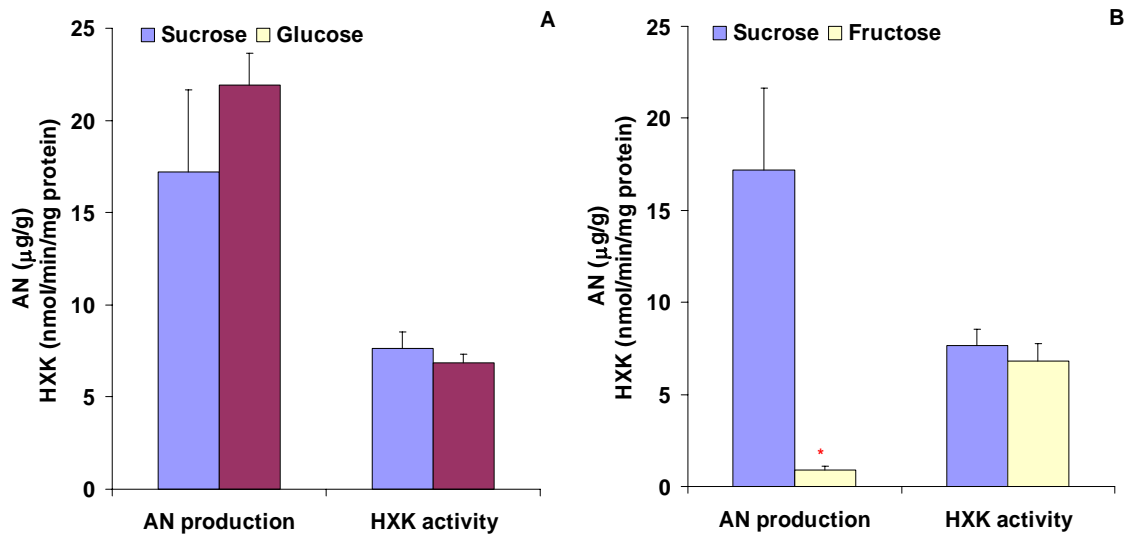
Total activity (nmol/min) =

$$(\Delta A_{340}/\text{min}) \times (\text{total assay volume}/ \text{added extract volume}) \times (1/6.22) \times 1000$$

Specific activity (nmol/min/mg protein) = Total activity (nmol/min)/total protein (mg)

## 8.2 Effects of sucrose, glucose, or fructose on artemisinin production and hexokinase activity.

To compare the HXK activity in seedlings grown on medium with sucrose, glucose, or fructose, equimolar carbon (carbon molar equivalent to 3% (w/v) sucrose) of sucrose, glucose, or fructose was added into B5 medium, seedlings were cultured for 14 days, and then artemisinin and hexokinase was extracted and assayed using the methods described in Material and Method section except 1 ml ice-cold extraction buffer was used to extract 0.05g frozen shoots grown on medium with fructose.



**Figure 17 Effects of sucrose, glucose or fructose on artemisinin production and hexokinase activity.** Data are mean of three replicates + SE. A. Effect of sucrose and glucose on artemisinin production and hexokinase activity. Data were analyzed using Mann-Whitney U-test. B. Effect of sucrose and fructose on artemisinin production and hexokinase activity. Data were analyzed using Mann-Whitney U-test. \*  $P > 0.05$ .