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Steviol glycoside biosynthesis

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Abstract

Steviol glycosides are found in high concentrations in the leaves of the Paraguayan perennial herb *Stevia rebaudiana* and their intense sweetness, as well as high concentration in *Stevia* leaf tissue, has made them the subject of research interest for over 100 years. Steviol glycosides are diterpenoids whose biosynthetic pathways share four steps in common with gibberellic acid formation. The convergence of genomics and plant biochemistry has led to the rapid elucidation of the genes coding for the various enzymes in the biosynthetic pathway. Functional characterization of the enzymes coded for by those genes is on-going. The first committed step in the pathway is the synthesis of the aglycone steviol and the various glycosides found in the leaf tissue result from the elaboration of steviol by a number of glucosyltransferases.

Keywords: *Stevia rebaudiana*; Asteraceae; Stevia; Biosynthesis; Diterpene glycoside; Terpene cyclase; P450; Glucosyltransferase

1. Steviol glycosides

The elucidation of biochemical pathways has been a longstanding goal of scientists studying plant secondary metabolism. The emergence of genomic resources like whole genome sequences and tools like high throughput expressed sequence tags (ESTs) have accelerated our understanding of these pathways (Rodríguez-Concepción and Boronat, 2004). One example of this acceleration is in the characterization of the genes and enzymes involved in the biosynthesis of steviol glycosides. Steviol glycosides are tetracyclic diterpenes derived from the same kaurenoid precursor as gibberellic acid (Fig. 1). Their intense sweetness and use as high potency sweeteners has made them the subject of significant scientific and commercial interest since they were first brought to the attention of Europeans in 1899 (Soejarto, 2002). The leaves of *Stevia rebaudiana* (Bertoni) Bertoni accumulate at least eight steviol glycosides (SGs), the concentrations of which vary quite widely depending on the genotype and production environment (Kinghorn and Soejarto, 1985; Phillips, 1987; Brandle and Rosa, 1992; Brandle et al., 1998; Starratt et al., 2002). The diversity of SGs results from elaboration of the aglycone steviol by various glycosyltransferases (Shibata et al., 1991, 1995; Richman et al., 2005). As a result of differential glycosylation, each SG has distinctive organoleptic properties. For example, stevioside (**18**) is reported to be 143 times sweeter than sucrose on a weight basis, but rebaudioside A (**19**) is 242 times sweeter (Kasai et al., 1981). The taste quality of rebaudioside A is better than stevioside, because it is more sweet and less bitter (DuBois and Stephenson, 1985). Either a sugar unit or a carboxyl at the C19 and either a sugar or a hydroxyl at the C-13 are essential for sweetness (Kasai et al., 1981). Rhamnosylation of the C13-glucose at the C2′ position instead of glycosylation results in dulcoside A, and when that compound is glucosylated at the C3′ position the result is rebaudioside C. The sweetness and the taste quality of the two rhamnosylated glycosides is inferior to their glucosylated counterparts (Tanaka, 1997).

Although SGs are widely used in food products and as dietary supplements in many countries around the world, reports of anti-fertility effects and mutagenicity have left

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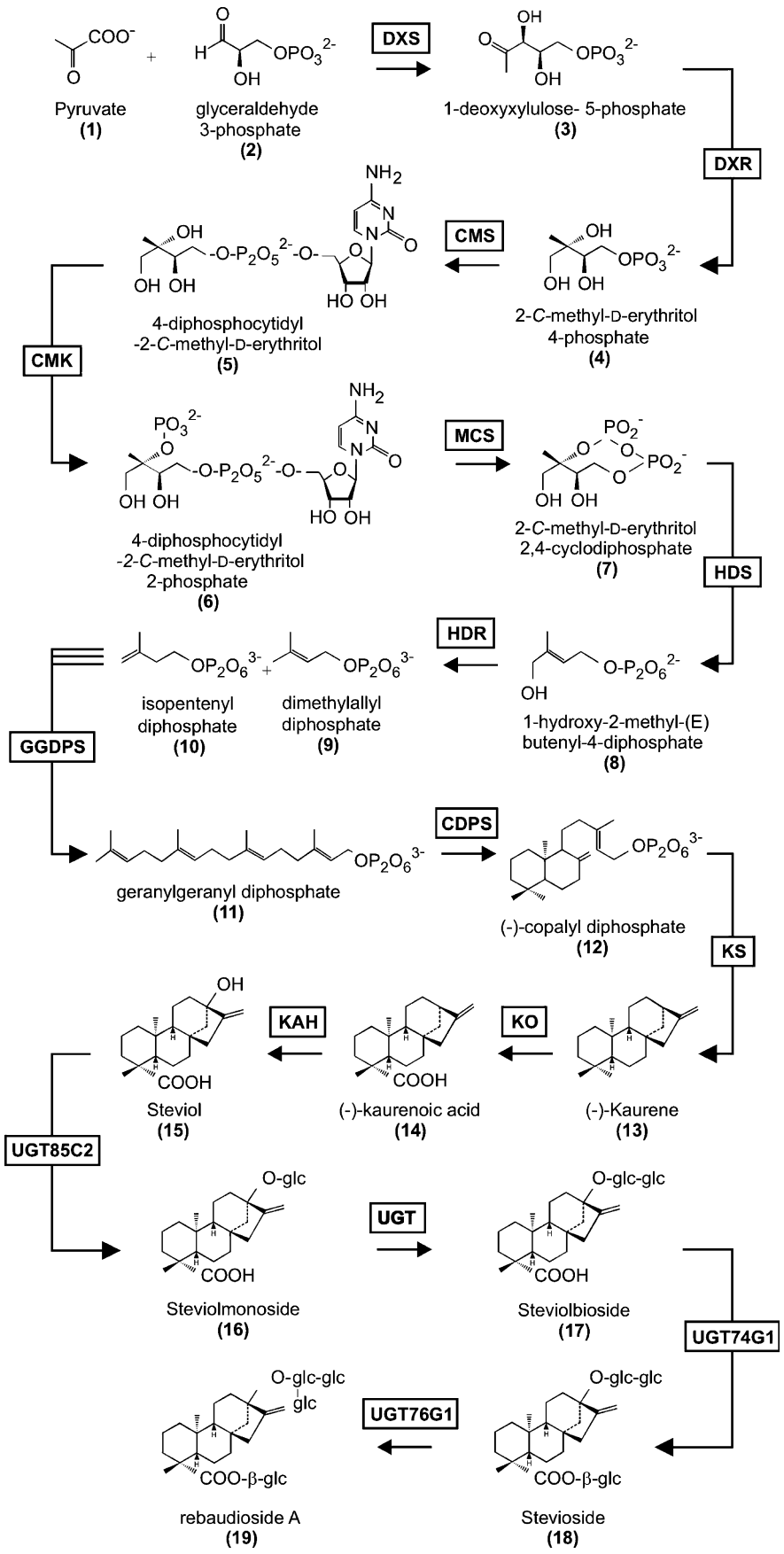

Fig. 1. The biosynthesis of steviol glycosides via the MEP pathway. Abbreviations are as in Table 1.

the issue of their safety unresolved (Planas and Kuc, 1968; Pezzuto et al., 1985). The two most recent safety reviews differ in their interpretation of the existing literature with respect to the toxic effects of steviol glycosides. Geuns (2000) concluded that *Stevia* and stevioside have no effect on mammalian reproduction or fertility, are safe for use as sweeteners and that they are acceptable for both diabetic and phenylketonuria patients. Huxtable (2002), on the other hand, was more guarded, citing the absence of non-rodent animal studies, the effect of glycosides on energy metabolism and the potential for steviol to be mutagenic as some of the factors that create uncertainty with respect to toxicity. Huxtable (2002) has pointed out that the formation of mutagenic metabolites of steviol by the liver is not understood and that key questions regarding the formation of steviol in the human gut are unanswered, thereby leaving the issue of mutagenicity unresolved. He discounted arguments of traditional use and stated that more definitive toxicity studies are required before either stevioside or rebaudioside A can be declared safe for human consumption. Following those reviews, clinical evidence emerged that suggested stevioside can reduce blood glucose levels in type II diabetics and blood pressure in mildly hypertensive patients (Hsieh et al., 2003; Gregersen et al., 2004). Since then Geuns et al. (2007) have shown that steviol or related metabolites do not accumulate in the human body and that at least in healthy human subjects pure stevioside taken at a dose of 750 mg/day had no effect on either blood pressure of insulin levels. In addition to being an approved sweetener in many countries, the World Health Organization has now recognized that stevioside is not genotoxic and assigned a temporary acceptable daily intake for steviol glycosides of 0–2 mg/kg body weight (Beneford et al., 2006). Given the impact that SGs have had on society, and the plant's remarkable metabolic capability the study of their biosynthesis is more than warranted.

2. *Stevia rebaudiana* – origin and distribution

Stevia is native to the Amambay region of Northeastern Paraguay and has been reported to occur in neighboring parts of Brazil and Argentina as well (Soejarto, 2002). Although *Stevia* continues to be a rare plant in its native habitat, agricultural production in South America and Asia, and ornamental use in Europe and North America have made its occurrence in the world perhaps more common than it ever was in the past. *Stevia rebaudiana* belongs to the Asteraceae family and it and *Stevia phlebophylla* are the only members of the 230 species in this genus to produce steviol glycosides (Kinghorn and Soejarto, 1985). The only other non-*Stevia* species found to have SGs is *Rubus chingii*, a member of the Rosaceae native to China, which contains rubusoside, a SG that is not found in *S. rebaudiana* (Tanaka et al., 1981). There has been very little investigation into the adaptive role of SGs, but there is some evidence that stevioside and its derivatives have a deterrent effect on aphid feeding suggestive of a classical role in chemical defense against pests (Nanayakkara et al., 1987; Wink, 2003). Other work has shown that the sweetness of SGs attracts aquatic animals, which when extrapolated to herbivorous land animals, could be associated with negative effects on survival and fitness of *Stevia* (Harada et al., 1993). That being said it simply may be that SGs have no real adaptive role and instead they are part of some unselected chemical diversity whose contribution to fitness and therefore the propagation of the species is as a sweetening agent. The attraction to humans has allowed *Stevia*, an otherwise obscure species, to spread throughout the world (Firn and Jones, 2003).

3. The MEP pathway

The configuration of steviol (**15**) was resolved more than thirty years ago and the work that followed concluded that steviol was synthesized from kaurene (**13**), via the mevalonate pathway (Mosettig et al., 1963; Ruddat et al., 1965; Bennett et al., 1967; Hanson and White, 1968). Like the synthesis of many diterpenes, however, it was later demonstrated using *in vivo* labeling with [1-^{13}C]glucose and NMR spectroscopy that the precursors of steviol are actually synthesized via the plastid localized methylerythritol 4-phosphate (MEP) pathway (Fig. 1; Lichtenhalter, 1999; Totté et al., 2000). The fact that Ruddat et al. (1965) were unable to show incorporation of labeled [2-^{14}C]sodium mevalonate into steviol is now not surprising, but there was incorporation of [2-^{14}C]sodium acetate and later work conducted by Hanson and White (1968) did succeed in demonstrating incorporation of labeled [2-^{14}C]sodium mevalonate. These latter results are indicative of a potential role for the cytosolic pathway and therefore the compartmentalization of the two pathways in *Stevia* may not be absolute. Like *Arabidopsis* and tobacco, there could be Ametabolic cross-talk@ between the plastid and cytosolic pathways in *Stevia* (Laule et al., 2003; Hemmerlin et al., 2003).

The plant gene for the first step in the MEP pathway, deoxyxylulose-5-phosphate (DXP) synthase (DXS), which leads to the synthesis of DXP (**3**) from pyruvate (**1**) and glyceraldehyde 3-phosphate (**2**) was first cloned and characterized in mint (Lange et al., 1998). Once synthesized, DXP can either be used for the production of vitamins like thiamin or in the MEP pathway for isoprenoid synthesis (Julliard and Douce, 1991). The DXS amino acid sequence is highly conserved among plant species, which enabled Totté et al. (2003) to design primers for RT-PCR and clone the DXS gene from *Stevia*. The *Stevia* DXS gene was then used in a complementation assay with the *E. coli* strain MC4100 *dxs::CAT* to confirm its function (Fig. 1; Table 1; Totté et al., 2003). In the next step in the pathway, DXP reductoisomerase (DXR) reduces and rearranges the DXP chain to form 2*C*-methyl-D-erythritol 4-phosphate **(4)**. The plant gene for this first committed step in the pathway was first cloned from *Arabidopsis* and mint

Table 1
Genes involved in the biosynthesis of steviol glycosides, their gene bank accession numbers and comparison at the protein level to their *Arabidopsis* orthologs

Gene	Acc. No.	Activity	% Identity/Similarity to Acc No.
DXS	AJ429232	Established by Totté et al. (2003)	74/86 to Q38854
DXR	AJ429233	Established by Totté et al. (2003)	79/89 to AAF73140
CMS	DQ269452	Putative	82/91 to NP_565286.1
CMK	DQ269453	Putative	70/80 to NP_180261.1
MCS	DQ631427	Putative	77/87 to NP_850971
HDS	DQ768749	Putative	82/92 to AAM19840
HDR	DQ269451	Putative	79/89 to AAN87171
GGDPS	DQ432013	Putative	63/80 to P34802
CDPS	AF034545	Established by Richman et al. (1999)	53/69 to NP_192187
KS	AF097310	Established by Richman et al. (1999)	52/69 to AAC39443
KO	AY364317	Established by Humphrey et al. (2006)	60/79 to NP_197962
KAH	–	Established by Brandle et al.(unpublished)	52/72 to NP_188087.1
UGT85C2	AY345978	Established by Richman et al. (2005)	47/67 to NP_173652.1
UGT74G1	AY345982	Established by Richman et al. (2005)	44/63 to NP_973682.1
UGT76G1	AY345974	Established by Richman et al. (2005)	45/62 to NP_196207.1

Deoxyxyulose-5-phosphate synthase (DXS), deoxyxyulose-5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS) and 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), geranylgeranyl diphosphate synthase (GGDPS), copalyl diphosphate synthase (CPS), kaurene synthase (KS), kaurene oxidase (KO), kaurenoic acid 13-hydroxylase (KAH).

by Lange and Croteau (1999), and like DXS the plant ortholog of DXR had a transit peptide sequence that had the typical characteristics of plastid targeting and the amino acid sequence was highly conserved. Using RT-PCR again, Totté et al. (2003, Fig. 1; Table 1); cloned the DXR gene from *Stevia* and confirmed its activity in a complementation assay using *E. coli* strain MC4100 *dxr::TET*. The genes for the next 5 steps in the MEP pathway have been cloned and characterized in Arabidopis and other plant species, which has led to the cloning and sequencing of MEP pathway genes in many other species including *Stevia* (Rohdich et al., 2000a; Rohdich et al., 2000b; Hsieh and Goodman, 2006; Querol et al., 2002; Guevara-Garcia et al., 2005). Although they are uncharacterized, complete sequences from *Stevia* identified as putative 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS) and 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR) that catalyze the next five steps in the MEP pathway have been deposited in GenBank (Fig. 1; Table 1).

4. Terpene cyclases

At the end point of the MEP pathway, the resulting isopentenyl diphosphate (**10**) and dimethylallyl diphosphate (**9**) are converted to geranylgeranyl diphosphate (**11**, GGDP) by the plastidic prenyltransferase GGDP synthase via three successive condensation reactions (Fig. 1; McGarvey and Croteau, 1995). A *Stevia* gene sequence identified as putative GGDP synthase has been deposited in GenBank (Table 1). Like all diterpenes, steviol is synthesized from GGDP, first by protonation-initiated cyclization to copalyl diphosphate (CDP) by CDP synthase (CPS). Next, kaurene is produced from CDP by an ionization dependant cyclization catalysed by kaurene synthase (KS). These enzymes from the GA biosynthetic pathway have been identified and characterised from a number of plant species including *Stevia* (reviewed in Hedden and Phillips, 2000; Richman et al., 1999). Northern analysis of gene expression in *Stevia* showed high levels of expression of both CDPS and KS in leaves and in particular in fully expanded leaves. *In situ* hybridization using CDPS and KS as probes revealed that transcripts of both the genes only occur in leaf parenchyma (Richman et al., 1999). From that we concluded that the early steps in the pathway are limited to green tissue, a result consistent with the observation that SGs are only found in tissues that either have or had chloroplasts, like leaves, stems and sepals (Bondarev et al., 2003). This is further supported by the fact that the MEP pathway is limited to plastids and by subcellular localization experiments that showed the signal peptides from CDPS and KS in *Arabidopsis* target the enzymes to the chloroplast (Lichtenhalter, 1999; Helliwell et al., 2001). Since both CDPS and KS are involved in the synthesis of gibberellic acid (GA), and given that GA concentrations in *Stevia* tissues are similar to those found in other plants (1.2 μg/kg fresh weight), their role in secondary metabolism probably depends on spatial and temporal expression patterns that keep their activity separate from that involved in GA synthesis (Richman et al., 1999; Alves and Ruddat, 1979).

5. P450s

Kaurene is then oxidized in a three step reaction to kaurenoic acid **(14)**, by kaurene oxidase (KO) a P450 mono-oxy-

genase that also functions in GA biosynthesis (Bennett et al., 1967; Helliwell et al., 1999). Among the candidate ESTs in our collection, *Stevia* KO was the most highly represented and like KS it was duplicated in the *Stevia* genome (Brandle et al., 2002; Humphrey et al., 2006). A full length KO cDNA from our collection was expressed in yeast and, using GC–MS with selected ion monitoring, we showed that it could convert kaurene to kaurenoic acid (Humphrey et al., 2006). It was 58% identical to the Arabidopsis KO and was classified in the same family as CYP701A5. *Stevia* KO was found to be highly expressed in leaves, succulent stems, flowers and seedling shoots, in a manner similar to that found for CDPS and KS (Humphrey et al., 2006). Therefore, like KS and CDPS, KOs role in SG biosynthesis depends on an expression pattern that separates it from that used in GA biosynthesis and in addition the regulation of GA synthesis appears to be downstream from KO at the stage of dioxygenase activity (Davidson et al., 2005; Fleet et al., 2003). We constructed fusions with KO and GFP and following bombardment into tobacco leaf guard cells and co-localization with YFP-HDEL, an endoplasmic reticulum marker, showed that Stevia KO is localized to the ER (Humphrey et al., 2006). This result was different from *Arabidopsis* KO, which localizes to the chloroplast and showed that trafficking of intermediates to the ER and the link between the chloroplast and the ER involves KS instead of KO in *Stevia* (Helliwell et al., 2001). While it is tempting to speculate that duplication of KO in the *Stevia* genome led to specialization in the enzymes, we have no evidence to support that hypothesis.

Steviol biosynthesis diverges from gibberellin biosynthesis with the hydroxylation of kaurenoic acid by kaurenoic acid 13-hydroxylase (KAH) (Hanson and White, 1968; Kim et al., 1996). This is the first committed step in the synthesis of steviol glycosides and the enzyme is of significant interest for use in biotechnology. It was reported to have been partially purified and an N-terminal sequence was described (Kim et al., 1996); however, efforts to use that sequence to clone a fragment of the gene in our lab and those reported by others failed to produce any meaningful sequence, casting doubt on its validity (Brandle unpublished; Geuns, 2003). In fact Geuns (2003) reported the cloning of fructose biphosphate aldolase (FBPA) using PCR primers designed using the published N-terminal sequence. Our searches of the *Stevia* EST collection using the N-terminal sequence also led to FBPA. The enzyme activity reported by Kim et al. (1996) was in the stroma of the chloroplast, which is somewhat inconsistent with our finding that KO, the biosynthetic step before KAH is located in the ER (Humphrey et al., 2006). Kim et al. (1996) concluded that the enzyme was a homotetramer with a subunit size of about 39 kDa, very similar to FBPA which is homotetramer with 37 kDa subunits and can also be resident in the chloroplast. In addition, the purified enzyme was not used to confirm the activity, so it is quite possible that their protein purification work led them not to KAH but instead to FBPA. Recently we screened 5 candidate cytochrome P450s from our EST collection (Brandle et al., 2002) and found one, when expressed in the WAT21 strain of *S. cerivisae* (Pompon et al., 1996), that was capable of converting kaurenoic acid to steviol (Brandle and Richman unpublished).

6. Glucosyltransferases

The aglycone steviol has two hydroxyl groups, one attached to the C-19 of the C-4 carboxyl and the other attached to the C-13, both of which in theory can be glycosylated. Shibata et al. (1991) used 13-*O*- and 19-*O*-methylsteviol as substrates for crude *Stevia* leaf enzyme extracts to determine which active group is glucosylated first (Fig. 1). They found that only 19-*O*-steviol could serve as a substrate and concluded that synthesis of SGs starts with the glucosylation of the 13-hydroxyl of steviol, which produces steviolmonoside (**16**, Fig. 1). The next step is the glucosylation of the C-2′ of the 13-*O*-glucose of steviolmonoside, which results in the production of steviolbioside (**17**, Fig. 1). Stevioside is then produced by the glycosylation of the C-19 carboxyl of steviolbioside (Fig. 1). Shibata et al. (1991) attempted to use the steviol 13,19 bisglucoside rubusoside as a substrate for the crude enzyme fractions, which failed and confirmed their conclusion that the C-19 is glucosylated after the glucosylation of the C2′ of the C13-glucose of steviolmonoside. Rebaudioside A is then synthesized by glucosylation of the C-3′ of the C-13-*O*-glucose. When it is used as a substrate, rebaudioside A gives no product, indicating it is the terminal step in the pathway. The tri-glycoside stevioside and the tetra-glycoside rebaudioside A typically represent the majority of the steviol glycosides present in *S. rebaudiana* leaves (Kinghorn and Soejarto, 1985). As mentioned earlier, rhamnosylated glycosides can also be formed by addition of a UDP rhamnose moiety to steviolmonoside and in genotypes enriched in rebaudioside C, the C2′ of the C13-glucose can be xylosylated to form rebaudisode F (Starratt et al., 2002).

Plant UDP-glycosyltransferases (UGTs) are a divergent group of enzymes that transfer a sugar residue from an activated donor to an acceptor molecule. The transfer of activated sugars like UDP-glucose to aglycone acceptor molecules helps to stabilize, detoxify and solubilize metabolites and is often the end point of secondary product pathways. Plant UGTs were thought to be promiscuous; however, evidence is mounting that demonstrates that this broad substrate specificity is limited by a regio-specificity (Hansen et al., 2003; Lim et al., 2003) and in some cases UGTs have been shown to be highly specific (Fukuchi-Mizutani et al., 2003). Given the existence of thousands of acceptors, and only about 100 different UGTs in any given plant, the idea of one enzyme for one substrate is questionable and some degree of multifunctionality must exist. Using our EST collection and an *E. coli* based activity assay, we screened 12 of 17 candidate glucosyltrasferases in our collection (Richman et al., 2005). Three of

the four UGTs (UGT85C2, UGT74G1 and UGT76G1) involved in the synthesis of stevioside and rebaudioside A were subsequently identified and characterised in our laboratory. The addition of the C13-glucose to steviol is catalyzed by UGT85C2, the C19-glucose by UGT74G1 and finally glucosylation of the C3' of the glucose at the C13 position is catalyzed by UGT76G1. We reasoned that the absence of full length clones for the remaining candidates meant that they were rare transcripts and probably not components of the pathway. Since then we did clone the five other candidate UGTs and confirmed that none were involved in steviol glycoside synthesis. The UGT responsible for the synthesis of steviolbioside from steviolmonoside has not yet been identified, but we have produced an additional 6500 ESTs and have nine new candidates.

Unlike their mammalian counterparts, which are localised to the ER membrane, most plant UGTs are thought to be soluble cytoplasmic enzymes, although this is supported by experimental evidence for only a few plant UGTs (e.g. Achnine et al., 2005). We fused green fluorescent protein (GFP) to each of the three UGTs and examined the subcellular location of the fusion proteins using confocal microscopy (Humphrey et al., 2006). Using particle bombardment in tobacco and onion epidermis, and transgenic *Arabidopsis*, we demonstrated that the three UGTs involved in SG biosynthesis were located in the cytoplasm. If we assume at least some degree of substrate promiscuity, then it does not seem probable that all UGTs and all potential substrates exist in freely diffusing cytoplasmic solutions. Therefore, in addition to substrate specificity, various other mechanisms such as partitioning into subcellular compartments or metabolon formation may underlie the regulation of the multitude of glycosylation reactions. We explored the possibility of metabolon formation using co-expression of KO-GFP and each of the UGTs fused to either the yellow (YFP) or cyan (CFP) variant. There was no interaction between any of the three UGTs and KO (Humphrey et al., 2006), but metabolic channelling could be occurring and metabolon formation is quite possible. In support of that idea we showed that rubusoside can be synthesized *in vitro* using either recombinant enzymes or protein extracts from *Stevia* leaves, yet it is a glycoside that does not occur in *Stevia* and cannot used as a substrate for the synthesis of stevioside *in vivo*(Humphrey et al., 2006; Shibata et al., 1995). It must be that rubusoside synthesis is prevented by the preferred synthesis of steviolbioside through some form of complex that ensures the rapid channelling of steviolmonoside from UGT85C2 to an as yet undiscovered UGT responsible for the synthesis of steviolbioside. Such a complex could include or require KAH and we are investigating that possibility.

7. Transport to the vacuole

The final phase of glycoside accumulation is the translocation of glycosylated steviol out of the cytosol and into the vacuole. The central vacuole of the plant cell has long been known to be important for the sequestration of toxic secondary metabolites and xenobiotics away from sensitive metabolic processes within the cytosol (Martinoia et al., 2000). In *Stevia*, steviol glycosides are known to occur in the vacuole, but the mechanism by which they are trafficked into the vacuole is not yet understood (Mu-zuan et al., 1983). The intracellular trafficking pathways for low molecular weight phytochemicals in plants are poorly understood in general, but there is some evidence that phytochemical trafficking to the vacuole is vesicle-mediated (Grotewold, 2004). For example, in sorghum, anti-fungal anthocyanins have been shown to accumulate in small vesicular structures, thought to be ER-golgi derivatives, which then fuse with the central vacuole (Synder and Nicholson, 1990). In addition, Mitsuhashi et al. (2005) have shown that the vacuolar accumulation of phytic acid in *Arabidopsis* suspension cells is inhibited by brefeldin A, an inhibitor of vesicular trafficking. Vesicle-mediated trafficking of metabolites seems like a possible scenario for biosynthetic pathways which are physically associated with the ER or organized in ER-associated metabolons (Jorgensen et al., 2005). However, for enzymatic reactions which occur in the cytoplasm, such as what we have observed for the steviol-specific UGTs, small molecular weight products are likely to be directly transported into the central vacuole or other pre-vacuolar structures from the cytosol. Translocation of compounds across the selectively-permeable vacuolar membrane requires substrate-specific membrane-integral transporters that exploit the energy stored in the electrochemical gradient or are directly energized by the hydrolysis of ATP. Secondary carriers, energized by proton or ion gradients including those belonging to the Major Facilitator (MFS) (Pao et al., 1998) and Multidrug and Toxic Compound Extrusion (MATE) (Hvorup et al., 2003) families of transporters, have been implicated in the vacuolar uptake of endogenous metabolites such as isotevixin in barley (Klein et al., 1996), berberine in *Coptis japonica* (Otani et al., 2005), and vacuolar flavonoid accumulation in *Arabidopsis thalania* (Debeaujon et al., 2001). Although secondary carrier mediated uptake was long thought to be the exclusive means by which vacuolar transport of secondary metabolites occurred, recent work has revealed an equally important role for the ATP Binding Cassette (ABC) superfamily of transporters. ABC transporters are directly energized by the hydrolysis of ATP (Schmitt and Tampe, 2002) and have been shown to transport a diverse array of compounds across the vacuolar membrane in plants including glutathione-conjugated agrichemicals (Bartholomew et al., 2002; Rea et al., 1998; Klein et al., 1998), anthocyanins (Goodman et al., 2004) and flavone glucuronides (Klein et al., 2000). The energetics of vacuolar accumulation in *Stevia* need to be investigated to confirm a direct carrier-mediated mechanism and to identify the class of transporter involved in the uptake of steviol glycosides. Upon examination of our annotated EST collection, we have identified several novel

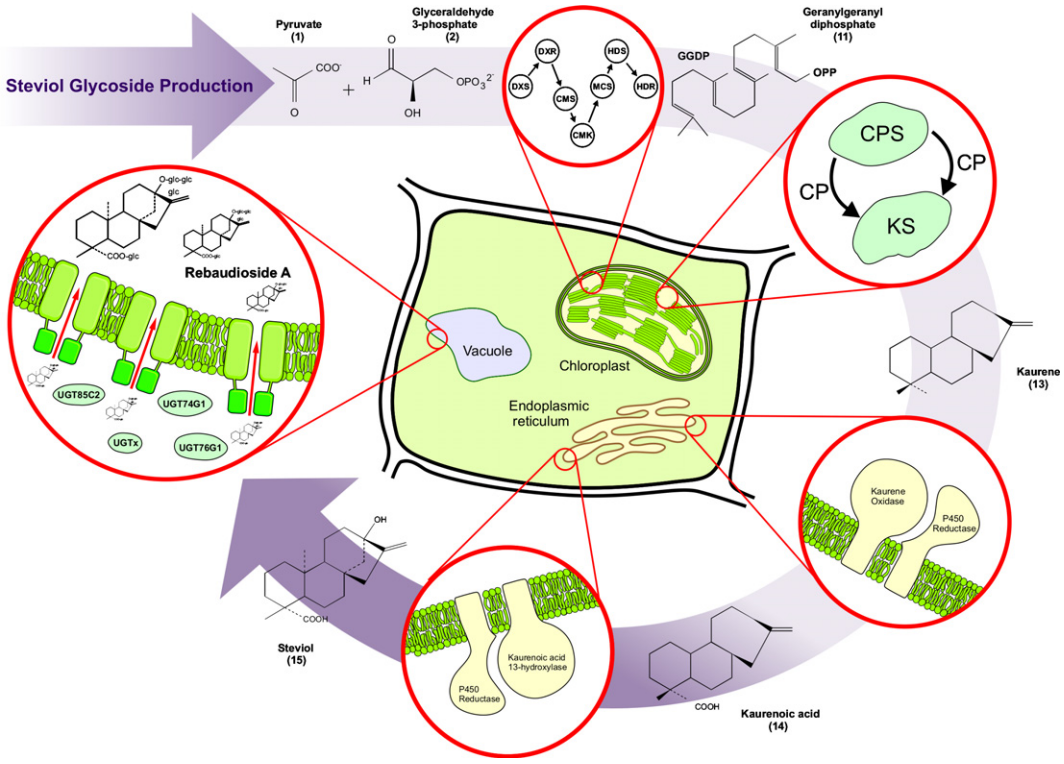

Fig. 2. A model of steviol glycoside biosynthesis showing the subcellular location of the components of the pathway.

genes coding for MFS, MATE and ABC transport-like proteins, one or more of which may be involved in glycoside transport. Candidate genes are currently being expressed in a heterologous system to assess transport activity and specificity.

8. Summary

It has been over 40 years since the first insights into the biosynthesis of steviol glycosides were published. No genes were isolated and only a limited understanding of the enzymes involved existed until 1999, when the two terpene cyclases in the pathway were characterized. As plant biochemistry and genomics converged over the next seven years all of the genes in the pathway, save one, were cloned and sequenced and many of those functionally characterized. The speed with which this has been accomplished by a relatively small number of labs is a clear testament to the power of genomics and is evidence of the synergy created by its convergence with the study of plant secondary products. We now know that steviol glycoside synthesis is restricted to green tissues, with all of the steps up to kaurene occurring in plastids, one of the two oxidation steps is located on the surface of the ER and glycosylation takes place in the cytoplasm (Fig. 2). New work should lead to an understanding of the vacuolar transport mechanisms and the identification of the only enzyme missing from the biosynthetic pathway.

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