Molecular studies in *Stevia rebaudiana* Bertoni: A Review

Abstract:

Stevia rebaudiana Bertoni belonging to family Asteraceae, is a plant native to the river valleys of Paraguay. Stevia is known to accumulate as high as 30% (w/w) of sweet steviol glycosides (SGs) in the leaf tissue. SGs are glucosylated derivates of a diterpenoid alcohol steviol. The SGs mixture includes stevioside, rebaudioside A, B, C, D, E and F and dulcoside A. Out of 230 species of genus *Stevia,* SGs have been reported only from *S. rebaudiana,* and *S. phlebophyla*. These glycosides are sweeter than common table sugar and are used as non-calorific sweetener in many countries of world. SGs have been reported to lower the postprandial blood glucose level of Type II diabetes patients and in reducing the blood pressure in mildly hypertensive patients. Due to these properties, *S. rebaudiana* has high socio-economic importance and biochemical and molecular research on this plant has gained pace. Based on the progressive research, this review summarizes the phytochemistry and pharmacological properties of *S. rebaudiana* , with special emphasis on molecular studies involving cloning of genes involved in SG biosynthesis, transgenic studies, RNAi experiments and miRNA profiling of this plant.

Keywords: *Stevia rebaudiana*, Natural sweetener, Stevioside, Secondary metabolism, Gene cloning, Transgenics, RNAi

Introduction

Stevia rebaudiana Bertoni belongs to the family *Asteraceae* within the tribe Eupatorieae, the largest plant family comprising 1620 genera and 23,600 species. It is one of 154 members of the genus Stevia and one of the only two species that produces sweet steviol glycosides (SGs) (Madan et al. 2010). It is also commonly known as Stevia, Sweet leaf, Sweet herb of Paraguay, Honey leaf and Candy leaf. Leaves of stevia are highly sweet, an observation which was brought to the attention of the scientific community over a hundred years ago (Gosling 1901; Bertoni 1905; Lewis 1992). The only limited research was conducted on the topic until 1931when two French chemists isolated the glycosides that give stevia its sweet taste (Bridel and Lavielle 1931a). Stevia is native to the valley of the Rio Monday in South America where it grows in sandy soils, acid infertile sand or muck soils whereas the first report of commercial cultivation of stevia was in 1964 in Paraguay (Katayama et al. 1976; Mitsuhashi et al. 1975; Lewis 1992). Since then it has been introduced as a crop in many countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada and several countries in Southeast Asia including India since 1990 (Lee et al. 1979; Donalisio et al. 1982; Goenadi 1983; Schock 1982; Saxena and Ming 1988; Brandle and Rosa 1992; Fors 1995). Its extracts are used today as a food additive and non-caloric sweetener by the Japanese and Brazilians . In the United States (US), however, its use is limited to supplement status only.

Morphology

It is a perennial semi-shrub approximately 30 cm in height possessing 3-4cm long elongate-lanceolate or spatulate shaped sessile leaves, with blunt-tipped lamina and serrate margin from the middle to the tip and entire below. The woody stem is weak-pubescent at bottom and rhizome possesses some secondary roots. Flowers are composite surrounded by an involucre of epicalyx and capitula are organized in loose, irregular, sympodial cymes. The flowers are light purple, pentamerous and fruit is a five-ribbed spindle-shaped achene (Katayama et al. 1976; Soejarto et al. 1982; Soejarto et al. 1983; Blumenthal 1996). The tiny white florets are perfect (hermaphrodite) having both male and female organs, borne in small corymbs of around two to six florets (Goettemoeller and Ching 1999). Stevia initiates flowering after a minimum of four true leaves must formed and takes more than a month to

pass through the various developmental flower stages and produce all its flowers (Taiariol 2004).

Botanical classification

Stevia comprises a group of shrubs that are found in mountain regions and annual and perennial herbs (Robinson 1930). Although there are about 230 species in the genus, *S. rebaudiana* and *S. phleobophylla* were found to be the only two species that gave the sweet essence (Soejarto et al. 1983).

Some other related species of *S. rebaudiana* are *Stevia bertholdii, Stevia anisostemma, Stevia tunguraguensis, Stevia micrantha, Stevia ovata, Stevia plummerae etc.*

Origin and antiquity

The plant has a long history of medicinal use by the natives of Guaraní since more than 1,500 years and commonly called by them as *ka'ahe'ê* which ("sweet herb") (Misra et al. 2011). Guaraní people of Paraguay used stevia as a sweetener in and various foods including *yerba mate*, medicinally as a cardiac stimulant and as a treatment for obesity, hypertension, heartburn etc. (Tanvir et al. 2005). The leaves have been traditionally used since years in both Brazil and Paraguay to sweeten tea and medicines (Misra et al. 2011). In 1899 Swiss botanist, Moisés Santiago Bertoni in his research first described the plant and its sweet taste in detail (Bertoni and Santiago 1899). The plant′s generic name i.e. Stevia was given in memory of 'Petri Iacobi Stevii', botany professor of Valencia, Spain by Cavanilles (King and Robinson 1987). Further, stevia was named by Dr. M.S. Bertoni in honor of renouned Paraguayan chemist Dr. Rebaudi (Gosling 1901). Standardized extracts of stevia began to be utilized commercially for sweetening the foods and beverages in Japan in mid-1970s as a substitute for several banned synthetic sweeteners. By 1987, stevia extracts occupied 41% of the sweetener market in Japan (Kinghorn and Soejarto 1985). Currently, the largest and most diverse use of stevia remains in Japan, where it is used primarily in sweetening the alcoholic beverage, *"soju"* (Seon, 1995).

Cultivation

For many years the global stevia market was limited by regulatory constraints. However, the major breakthrough came in June 2008 when WHO/FAO Expert Committee on Food Additives (JEFCA) stated that SGs was safe for use in food and beverages. The international market for high-potency sweeteners during 2013 was reported to be of 4,100 tonnes by volume equal to US$304 million by value with U.S. and Japan being the leading economies of the world with 60% share in global stevia consumption while China is the world leader in production and extraction of stevia leaves with 80% share.

S. rebaudiana is a semi-humid and easily cultivable subtropical plant. The crop could be transplanted in February or March and seeds collected in the late summer. Flowering should occur between 54-104th day following transplantation under these conditions, further depending on the day length sensitivity of the cultivars used for seed production. Additionally, when the plants are grown under long days the concentration of stevioside in the leaves of Stevia increases (Metiver and Viana 1979). There are many commercially available products of stevia in both liquid and powder form in U.S. e.g. Fructevia, Nustevia, Sweet Leaf, Stevita, Truvia etc. However, in India it is commercially available under tradenames i.e. So Sweet, Sweetal, Sugarfree Herbvia Cheeni Kum, Honey Leaf etc.

Phytochemistry of stevia

S. rebaudiana contains eight ent-kaurene glycosides (Kinghorn et al. 1984) in its leaves, the concentrations of which widely vary depending on both genotype and production environment (Brandle and Telmer 2007). SGs are the glucosylated derivatives of diterpenoid alcohol steviol. Further, stevioside and rebaudioside A are the two major glycosides found in stevia which are mainly responsible for the sweet taste of the leaves of the stevia plant. Sweetness indices of these and other compounds ranges between 30 to 300 times higher than that of sucrose (Crammer and Ikan 1987) and are used as non-calorific sweeteners in various countries of the world. Moreover, the sweetness quality is superior to that of sugar in terms of mildness and refreshment (Kinghorn and Soejarto 1989). They are heat-stable, pH-stable and further do not ferment (Brandle 2004). Furthermore, when ingested, they do not even induce a glycemic response, which makes them attractive as natural sweeteners primarily to diabetics and others on carbohydrate-controlled diets. In

terms of weight fraction, the four major SGs found in the stevia plant tissue include stevioside, rebaudioside A, rebaudioside C and dulcoside A whereas rebaudioside B, D, and E are present in very limited quantities; however, rebaudioside B is suspected as a by-product of the isolation technique (Brandle 2004). Many secondary metabolites other than sweet-tasting glycoside constituents have been isolated from stevia (Kinghorn and Soejarto 1985; Philips 1987). In addition to SGs, stevia contains a complex mixture of sterols, triterpenoids, essential oils, flavonoids and a number of labdane-type diterpenes have also been identified in stevia.

Research status

Initially the major hurdle in commercializing stevia was the ban on its use as a food additive in food products in the U.S., though its usage as a dietary supplement was accepted by the Food and Drug Administration in 1995 (Bespalhok-Filho and Hattori 1997). In India, the plant was introduced at the University of Agricultural Sciences, Bangalore, in the late 1990s, and studies on its adaptability were drafted (Yadav et al. 2011). Additionally, stevia has been suggested to pose various beneficial effects on human health and is considered to possess anti-oxidant, anti-hyperglycemic, anti-tumour/ anti-inflammatory, anti-rotavirus, anti-hypersensitive, antidiarrheal, antimicrobial and immunomodulatory activities (Madan et al. 2010). Further, stevioside has been reported to lower the postprandial blood glucose level of Type II diabetes patients and even blood pressure in mildly hypertensive patients (Hsieh et al. 2003; Gregersen et al. 2004). SGs being important compounds of stevia responsible for imparting most of the above mentioned medicinal properties to the plant, so the molecular study done on this plant were mainly centered on understanding the biosynthesis and regulation of the genes involved in SG biosynthesis. SGs are diterpenoids whose biosynthetic pathways share four steps in common with the gibberellic acid formation. The convergence of genomics and plant biochemistry has led to the rapid elucidation of the genes coding for the various important enzymes in the biosynthetic pathway. Functional characterization of the enzymes coded by those genes is showing that the first committed step in the pathway is the synthesis of the aglycone steviol and further various glycosides found in the leaf tissue actually result from the elaboration of steviol by a number of glucosyltransferases (Brandle and Telmer 2007). A lot of work has been done on the

ecology, importance of the plant, its production necessities and the agronomic and management aspects of the plant to be grown as a crop and its potential as an alternate source to cane sugar (Skarla et al. 2004; Ramesh et al. 2006). Studies showed that stevia could actually replace some or all of the sugar in recipes without significantly affecting the visual acceptability or physical characteristics of the food product (Kerzicnik et al. 1998) but further studies on the safety of stevia are strongly recommended to determine its potential for serving as a sugar substitute. Vast study on its biological, agro-techniques, bioproduct extraction, phytochemical and toxicological aspects have been executed on *S. rebaudiana* (Madan et al. 2010).

At the molecular level *S. rebaudiana* remained unexplored for a while but then lot of research groups got attracted in unraveling the molecular circuitry of *S. rebaudiana* due to the exciting sweetening properties the plant. Recent innovations in small-molecule analysis have to make the ability to rapidly identify and quantify numerous compounds, and this vast data is creating new opportunities for further understanding stevia metabolism and for stevia metabolic engineering. The main purpose of this review is to summarize the recent literature for the improvement of stevia to provide a baseline for additional improvement. Further research needs to be carried out to develop stevia as a potential crop by developing improved varieties with higher yield and quality through various plant breeding methods and biotechnological approaches.

Expressed Sequence Tag studies

Expressed sequence tags (EST) are a powerful tool to reveal certain important parameters including gene expression patterns, gene regulation, and sequence diversity. It is clear that *S. rebaudiana* has committed a vast portion of its total metabolism to their synthesis, making *S. rebaudiana* an able candidate for an EST-based gene discovery effort. The pioneering work towards gene discovery started with the advent of ESTs, which provided a new approach to gene discovery in plant secondary metabolism. So, in order to create a resource for gene discovery and for increasing the understanding of steviol glycoside biosynthesis, 5,548 ESTs were sequenced from *S. rebaudiana* leaf cDNA library. A significant portion of the ESTs was solely for standard leaf metabolic pathways whereas diterpene metabolism in *S. rebaudiana* represented only 1.1% of the total

transcripts. This study also identified candidate genes for 70% of the known steps in the steviol glycoside pathway out of which kaurene oxidase, ranked the 8th most abundant EST in the collection. The repository of ESTs greatly facilitated the identification of candidate genes and further increased understanding of diterpene metabolism (Brandle et al. 2002). Recently after the introduction of next generation sequencing, ESTs have further become an effective means of gene discovery in metabolic pathways (Sterky et al. 1998; Ohlrogge and Benning 2000).

Linkage map and molecular marker studies

Although Compositae is one of the largest family of flowering plants, there has been little research involving molecular markers as the family possesses very few major crop species (Kesseli and Michelmore 1996). Yao et al. (1999) conducted the pioneer detailed genetic study which proved to be sole research ever conducted in this genus by constructing the genetic linkage map of *S. rebaudiana* utilizing segregation data obtained from a pseudotest-cross F_1 population. The first genetic linkage map for *S. rebaudiana* was constructed by Yao et al. (1999) based on RAPD (Random Amplified Polymorphic DNA) markers which proved to be useful for those interested in developing marker-assisted selection procedures for providing a foundation for those who are interested in understanding genome organization in stevia. A lot of 183 RAPD markers were sreened to finally assemble them into 21 linkage groups covering a distance of 1389 cM in totality, with an average distance between each marker of 7.6 cM. Construction of the stevia genetic linkage map has laid a strong foundation to relate genes involved with economically important traits to the RAPD markers. Newly cloned cDNAs from stevia genes involved in glycoside biosynthesis, such as kaurene synthase, were mapped using cleaved amplified polymorphic sequence markers for a better understanding of the genomic organization of secondary metabolism (Richman et al. 1998; Konieczny and Ausubel 1993).

Until recently, population genetic analysis in *S. rebaudiana* has been performed using various inconsistent markers such as FLP, RAPD and ISSRs (Yao et al. 1999; Heikal et al. 2008; Hassanen and Khalil 2013) exhibiting significant limitations such as very low reproducibility rate and further inability to detect heterozygous individual which lead to underestimation of recessive allele frequency in a population leading to a bias in the

estimation of genetic diversity (Nybom 2004) making them less preferred markers (Kalia et al. 2011).

In contrast, microsatellite markers have a sharp edge over other markers due to their added advantages including codominant nature, hyper-variability, genomewide occurrence, robustness and their ability to establish markers–trait association (Provan et al. 2001; Chung et al.2006). Such markers remained unveiled until 2014 in Stevia, limiting its diversity characterization and genetic advancement through breeding. The pioneer work towards exploring the microsatellite repertoire was focussed on the development of microsatellite markers from publicly available EST data and further evaluation of their potential for diversity characterization of Stevia germplasm. A total of 2977 unigenes representing 2.58 Mb of sequence were predicted from 5548 publicly available ESTs of *S. rebaudiana*. Out of these ninety-nine unigenes containing 107 SSRs were predicted. Microsatellite frequency was noticed to be one in every 26 Kb of nonredundant EST data representing the first ever set of functionally relevant SSR markers that have proven to be an addition to the repertoire of unique EST-SSR markers available in Asteraceae. Furthermore, in future hypervariability potential of novel microsatellite markers can be explored through genetic mapping and genetic diversity studies in Stevia (Bhandawat et al. 2014).

Cloning of genes involved in steviol biosynthesis

SGs are synthesized through SGs biosynthesis pathway and stored in the vacuole. SGs biosynthesis can be described as three major stages: (1) the plastid 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway providing, isopentenyl diphosphate (IPP) and dimethylalyl diphosphate (DMAPP), acting as universal isoprenoid precursors; (2) the condensing steps producing aglycone, steviol from IPP and DMAPP; (3) the glucosylation of steviol yielding a mixture of different steviol glycosides. Earlier mevalonate pathway had been postulated as an exclusive source of kaurene skeleton of sweet glycosides present in stevia. However incorporation experiments have revealed the role of MEP pathway in the formation of the diterpene skeleton (Totté et al. 2000). The MEP pathway starts with the condensation of glyceraldehydes-3 phosphate and pyruvate, yielding 1-deoxy-D-xylulose-5 phosphate by enzyme 1-deoxy-D-xylulose-5 phosphate synthase (DXS) (Sprenger et al. 1997). In the next committed step 1-deoxy-D-xylulose-5 phosphate is further reduced and

isomerized to 2-C-methyl-D-erythritol-4-phosphate by 1-deoxy-D-xylulose-5 phosphate reductoisomerase (DXR) (Takahashi et al. 1998). 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol synthase (IspD) transfers cytidine monophosphate moiety from cytidine triphosphate to MEP, converting MEP into 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol in the presence of divalent cations Mg^{2+}, Mn^{2+} and Co^{2+} (Rohdich et al. 1999; Richard et al. 2001). 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol is further phosphorylated at carbon no. 2 by 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol kinase (IspE) in an ATP-dependent phosphorylation step affording 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol 2-phosphate (Lüttgen et al. 2000). 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate synthase (IspF) convert 4-(cytidine 5′ diphospho)-2-C-methyl-D-erythritol 2-phosphate into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate through intra-molecular attack by a phosphate (Kishida et al. 2003). Enzyme (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (IspG) catalyzes the conversion of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate into (E)-4-hydroxy-3-methylbut-2-enyl diphosphate, direct precursor for IPP and DMAPP synthesis (Hecht et al. 2001). Finally IPP/DMAPP synthase (IspH), a key enzyme reduces (E)-4-hydroxy-3-methylbut-2-enyl diphosphate and converts it into a 5:1 fraction of IPP and DMAPP (Adam et al. 2002). Also IPP isomerase (IDI) can catalyze the conversion of IPP to DMAPP.

Geranylgeranyl diphosphate synthase (GGDPS), in three discrete steps carries out the condensation of three molecules of IPP and one molecule of DMAPP to form geranylgeranyl diphosphate (McGarvey and Croteau 1995). The geranylgeranyl diphosphate pool serve as common precursor for many terpenoids including phytol moiety of chlorophylls, providing substrates for protein prenylation, carotenoids and of gibberellin biosynthesis. Geranylgeranyl diphosphate is converted into copalyl diphosphate by copalyl diphosphate synthase (CDPS) by a protonation-initiated cyclization. Kaurene is produced from copalyl diphosphate by an ionization dependant cyclization with the help of kaurene synthase (KS) (Richman et al. 1999). Kaurene oxidase (KO), a cytochrome P450 mono-oxygenase oxidizes ent-kaurene into ent-kaurenoic acid (Humphrey et al. 2006), which serves as a common precursor for both Gibberellin and SGs biosynthesis. Kaurenoic acid hydroxylase (KAH), another cytochrome P450 mono-oxygenase oxidizes ent-kaurenoic acid into steviol (Hanson and White 1968). Since both gibberellin and SGs biosynthesis

pathways have common steps up to the synthesis of ent-kaurenoic and given that GA concentrations in stevia tissues are similar to those found in other plants, their role in secondary metabolism probably depends on spatial as well as temporal expression patterns that keep their activity separate from that involved in GA synthesis. Glucosylation of steviol at C13 and C19 hydroxyl groups by various glucosyltransferases (GTs) leads to the synthesis of different SGs present in stevia leaves (Shibata et al. 1995). Three GTs involved in steviol glycoside biosynthesis have been reported (Richman et al. 2005). The initial leap of research was majorly dominated by the rapid elucidation of the genes coding for the various enzymes in the biosynthetic pathway and further functional characterization of the enzymes coded by these genes (Brandle and Telmer 2007).

Pioneer studies initiated with the cloning and characterization of a UDP-glucose flavonoid glucosyltransferase (srUFGT) in *S. rebaudiana* (Ma et al. 2003) followed by the enzymes catalyzing the first two steps of 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) (Totte et al. 2003).

Down the discovery line, Richman et al. (2005) isolated full-length cDNAs for 12 of the UGTs (Uridine diphosphate glycosyltransferase), cloned them into an expression vector, for producing recombinant enzymes in *E. coli*. Various studies including in vitro glucosyltransferase activity enzyme assay and thin layer chromatography were used to separate the products. HPLC and LC-ES/MS were then used to further define those reaction products which suggested that steviol UGTs act in a regioselective manner and in turn proposed a modified pathway for the synthesis of rebaudioside A from steviol.

Additionally, for revealing the functional genomics of three UGTs quantitative real-time polymerase chain reaction and reversed phase HPLC methods were used to estimate the transcript levels of downstream genes contributing to the biosynthesis of SGs. It was found that in same daylength condition, SG concentration and the transcript levels of the three UGT genes were higher in upper leaves as compared in lower leaves and accumulated more in plants growing under short-day conditions indicating a significant correlation between UGT85C2 transcription and total SG accumulation in the upper leaves. This further confirmed that the glycosylation of steviol to form steviolmonoside is the rate-

limiting step in the glycosylation pathway of SGs. However, in the upper leaves, a relatively high accumulation of rebaudioside A as compared to stevioside was also observed, but without any correlation with the transcription of UGT76G1 (Mohammed et al. 2011).

As fifteen genes are involved in the formation of SGs (stevioside and rebaudioside A). Of the various genes involved in the SG biosynthetic pathway, SrDXR, SrCPPS, SrDXS, SrKS, SrKO and also three glucosyltransferases i.e. SrUGT85C2, SrUGT74G1 and SrUGT76G1 were reported from stevia. Additionally seven full-length cDNA sequences namely, SrMCT, SrCMK, SrMDS, SrHDS, SrHDR, SrIDI and SrGGDPS were cloned in 2012 followed by expression analysis of all the above fifteen genes in parallel with SGs content analysis. SGs content was found to be highest in the leaves at 3rd node position as compared to the leaves at other node positions and further SGs accumulation was maximum in leaf tissue followed by stem and root, and the pattern of expression of all the fifteen genes was also found to be similar. Additionally, expression studies were carried out with certain hormone treatments including gibberellin (GA(3)) treatment which significantly up-regulated the expression of SrMCT, SrCMK, SrMDS and SrUGT74G1, on the other hand methyl jasmonate and kinetin treatment exhibited opposite effect i.e. down-regulated the expression of all the fifteen genes involbed in the pathway (Kumar et al. 2012).

As discussed above, within the 12 UGTs of *S. rebaudiana* so far elucidated, the functional genomics of three UGTs-UGT76G1, UGT74G1 & UGT85C2 in stevioside synthesis was studied. Recently, a UGT gene of *S. rebaudiana* named UGTSr showing similarity with UGT76G1 was structurally analyzed and further the functional role of the recombinant UGTSr in the synthesis of rebaudioside A was assured. Inspite of the similarity of nucleotide with UGT76G1, the gene UGTSr shows 48 SNPs and 39 associated amino acid substitutions with significant variation in the secondary and tertiary structure of the protein supporting its functional stability and ascertaining its specificity from other UGTs of *S. rebaudiana* ensuring a novel status to it from other UGTs of *S. rebaudiana* (Madhav et al. 2013).

Further, various enzymes of the MEP pathway, 2*C*-methyl-d-erythritol 2,4-cyclodiphosphate synthase (MDS) and (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) are the key enzymes involved in catalyzing important conversions in the pathway. SteviaMDS (SrMDS) and (SrHDR) successfully rescued MDS and HDR lethal mutant strain depicting SrMDS and SrHDR to be a functional gene showing diurnal variation. To identify the regulatory elements, upstream region of the genes were cloned and putative cis-acting elements were identified by in silico analysis. Data suggested the role of light and IAA in regulating expression levels of SrMDS (Kumar et al. 2012) and involvement of GATA box in light mediated gene regulation of SrHDR in stevia (Kumar and Kumar 2013) respectively.

Furthermore, the expression studies of above described fifteen MEP pathway genes in stevia showed remarkable higher levels during hardening as observed in micropropagated plants when they were allowed to harden for one month during which complete transcriptional profiling of candidate genes was carried out. Although at the initial phase of hardening there were higher transcript levels of candidate genes but the highest stevioside content was found after 30 days of hardening, suggesting the role of translational/posttranslational regulatory mechanisms. Out of 15 genes, 9 were up-regulated to approximately two-fold or greater whereas, nine genes expressed at higher levels after 30 days than in controls. Moreover, these transcriptional differences were strongly correlated with a significant increase in stevioside content from 0- (11.48%) to 30- (13.57%) day-old plants (Modi et al. 2014).

RNA interference (RNAi) studies

However, the genetic regulation involved in SG biosynthesis was not understood until RNA interference (RNAi) based Agrobacterium mediated transient gene silencing (AMTS) approach was executed. SrKA13H, and three SrUGTs (SrUGT85C2, SrUGT74G1 and SrUGT76G1) genes encoding ent-kaurenoic acid-13 hydroxylase and further three UDP glycosyltransferases of the SG biosynthesis pathway were silenced in *S. rebaudiana* to understand the underlying molecular mechanism and their association with gibberellins. RNAi mediated AMTS of SrKA13H and three SrUGTs has considerably reduced both the

expression of targeted endogenous genes as well as the total SG accumulation. However, whereas gibberellins (GA3) content significantly enhanced on AMTS of SrUGT85C2 and SrKA13H. Hence, SrKA13H and SrUGT85C2 both were identified as regulatory genes influencing the carbon flux between SG and gibberellin biosynthesis. This study further also ascertained the existence of an alternative route for biosynthesis of SG′s (Guleria and Yadav 2013).

Transgenic studies

Further major advancements in elucidation of specific gene functions in *S. rebaudiana* was finally achieved through introduction of transgenic plants. Transgenic plant production in *S. rebaudiana* has been reported both through direct regeneration from leaf and indirect regeneration via elite somaclones generation . Transfer of GUS-positive transgenic as well as four somaclones to glasshouse acclimatization has been successfully carried out. Inter-simple sequence repeat (ISSR) profiling of transgenic as well as somaclonal plants showed a total of 113 bands out of which 43.36 % were monomorphic and 56.64 % were polymorphic. Transgenic plants were found to be closer to mother plant, while based on steviol, stevioside, and rebaudioside A profile, somaclone S_2 was considered to be the best as it showed maximum variability in ISSR profiling (Khan et al. 2014).

Additionally as both SG and gibberellin's biosynthetic routes are divergent branches of methyl erythritol-4 phosphate (MEP) pathway so, SrUGT85C2 might be significantly influencing gibberellin content. Hence transgenic *Arabidopsis thaliana* (*A. thaliana*) overexpressing SrUGT85C2 cDNA from *S. rebaudiana* was generated to check its effect on gibberellin accumulation and related plant growth parameters. The transgenics showed a remarkable decrease of 78-83% in GA3 content and further downregulated expression of genes encoding important MEP pathway enzymes including geranyl geranyl diphosphate synthase (GGDPS), copalyl diphosphate synthase (CDPS), kaurenoic acid oxidase (KAO), chlorophyll synthetase and chlorophyll a oxygenase along with a typical gibberellin deficient phenotype i.e. stunted hypocotyl length, reduced shoot growth and a significant fall in relative water content. Furthermore , transgenics also showed significant reduction of 17-37% and 64-76% reduction in photosynthetic pigments chlorophyll a and chlorophyll b

contents, respectively which may be responsible for the noteworthy decrease in plant biomass (Guleria et al. 2014).

Another case study involving transgenic technology involved SrUGT74G1 a UDP-glycosyltransferase which is responsible for the conversion of steviolbioside into stevioside in *S. rebaudiana* leaves. Transgenic *A. thaliana* with overexpressing SrUGT74G1 cDNA from Stevia were generated to check the probability of stevioside biosynthesis in them. However, stevioside accumulation was not much evident in transgenics and showed no change in GA3 content on SrUGT74G1 overexpression. At the same time, the study revealed the significant role of Stevia SrUGT74G1 gene in trichome branching pattern, greatly enhanced vegetative growth, scavenging potential, and seed yield by catechin accumulation in transgenic *Arabidopsis* (Guleria and Yadav 2014).

Next generation sequencing (NGS) studies

MicroRNAs (miRNAs) belongs to a family of small RNA (sRNA) population that regulates the gene expression and plays a vital role in plant development, metabolism, signal transduction and stress response. In a pioneer study involving miRNAs, only seven conserved miRNA families, including miR414, miR169, miR319, miR414, miR164, miR167 and miR398 were identified from a computational analysis of EST sequence data and stem-loop RT-PCR analysis (Guleria and Yadav 2011). Extensive studies on miRNAs have been performed in different plants such as *A. thaliana*, *Oryza sativa* etc. and volume of the miRNA database, mirBASE, has been increasing on day to day basis. Several studies have been carried out for understanding molecular mechanism involved in biosynthesis of these glycosides, however, thorough information about miRNAs was still lacking in *S. rebaudiana* until the advent of deep sequencing of sRNAs combined with transcriptomic data which proved to be a powerful tool for identifying conserved as well as novel miRNAs irrespective of availability of genome sequence data. To identify miRNAs in *S. rebaudiana*, sRNA library was constructed and sequenced using Illumina genome analyzer II. Based on sequence similarity, 100 miRNAs belonging to 34 highly conserved families and 12 novel miRNAs were identified. Putative target genes were predicted for most conserved and novel miRNAs. The predicted targets are mainly mRNA encoding enzymes regulating essential plant metabolic and signaling pathways. These results provided information on

stevia miRNAs and their targets building a foundation for future studies to understand their roles in key stevia traits (Mandhan et al. 2012). An extension to this extensive effort in elucidation of miRNAs in *S. rebaudiana* was done recently in 2015 with the unraveling of 12 more novel miRNAs (Mandhan and Singh 2015).

Additionally, the genomics of Stevia, a non-model species, remains uncharacterized. The recent introduction of RNA-Seq, another NGS technology, provided a platform further to expand the identification of Stevia genes via in-depth transcript profiling without a reference genome. A total of 143 UGT unigenes were predicted, some of which might be involved in SG biosynthesis. Thus, transcriptome data obtained from this study yielded new insights into the process of SG accumulation in *S. rebaudiana*(Chen et al. 2014).

Structural studies

Structural studies in *S. rebaudina* initiated with the discovery of eight ent-kaurene glycosides, including dulcosides A, rebaudiosides A-E, steviobioside and stevioside (Bridel and Lavielle 1931b; Khoda et al. 1976; Kinghorn et al. 1984) and further structures of some diterpene glucosides were determined using 13 C NMR (Yamasaki et al.1976). In addition to the sweet diterpenoid glycosides, several other types of diterpenes have been isolated from Stevia. Out of them, first to be characterized were jhanol and austroinulin, and 6- O-acetylaustroinulin (Sholichin et al. 1980). Additional labdane-type diterpenoids, (Melis 1992) sterebins E, F, G and H, were isolated from leaves and their structures were elucidated on the basis of various studies (Oshima et al. 1988). Further, rebaudioside A and steviobioside have been isolated by HPTLC methods. The sweet diterpenoid glycoside, rebaudioside F, was isolated from leaves and further its structure was established by various chemical and spectral studies (Starratt et al. 2002). The absolute configuration of steviol and isosteviol was established, through a series of correlations over 20 years after the initial work of Bridel and Lavielle (Mosettig and Nes 1955; Dolder et al. 1960; Djerassi et al. 1961; Mosettig et al. 1963). Six new labdane-type, sterebins I-N, non-glycosidic diterpenes, were isolated from the leaves of *S. rebaudiana* and their structures, were elucidated based on various spectroscopic and chemical studies (McGarvey et al. 2003)

Recently, in the direction of structural studies, isolation and complete structure of an isomer of rebaudioside D and rebaudioside M, known as rebaudioside D2 (Prakash et al.

2014a) and rebaudioside M2 (Prakash et al. 2014b) respectively were established. Furthermore, two new diterpene glycosides rebaudioside KA and 12-α-[(2-O-β-d-glucopyranosyl-β-d-glucopyranosyl)oxy]ent-kaur-16-en-19-oic acid β-d-glucopyranosyl ester has been isolated from a commercial extract of the leaves of *S. rebaudiana* and their structures were elucidated based on comprehensive 1D- and 2D-NMR studies (Ibrahim et al. 2014).

Seperation and quantification studies

Stevia′s commercial exploitation increased with the advent of several analytical techniques like droplet counter (Fullas et al. 1989) , current chromatography (Kinghorn et al. 1982), thin layer chromatography (Kinghorn et al. 1984; Nikolova-Damyanova et al. 1994) over pressured layer chromatography and capillary electrophoresis (Liu and Li 1995) have been employed to estimate the distribution and level of sweet diterpenoid glycosides in *S. rebaudiana.*

Levels of stevioside (Ahmed et al. 1980) have also been determined both enzymatically (Sakamoto et al. 1977) and by near infrared reflectance spectroscopy (Nishiyama et al. 1992) in several plant strains. Another highly sensitive reversed-phase HPLC method has been introduced for the determination of steviol from *S. rebaudiana* using dihydroisosteviol (DHISV) as an internal standard (Minne et al. 2004). Recently, research in this direction has been made simpler with the introduction of water-only extraction processes in place of the older solvent extraction technology (Yadav et al. 2010).

As the simultaneous separation of steviol and SGs is a challenge because of differences in their polarity and chemical structure which was recently achieved by LC with UV detection using a mixed-mode RP weak anion exchange chromatography column. The method was used to authenticate SGs in several samples of Stevia plant material as well as for quantification of SGs in various dietary supplements containing Stevia (Jaworska et al. 2012).

Further, an HPTLC method was validated in terms of precision, accuracy, specificity, and robustness and can be useful for routine analysis of the sweeteners for simultaneous estimation of stevioside (STE) and rebaudioside-A (REB-A) in the leaves of *S. rebaudiana* .

The content of STE and REB-A in leaf extract was estimated to be 6.94 and 6.35%, respectively (Londhe and Nanware 2013).

For matching up to the increasing consumer demand for natural food ingredients, SGs, have recently been approved as food additives in the European Union. As regulatory constraints demand sensitive methods to analyze the SGs in foods and beverages, a HILIC-MS/MS method was developed for the accurate and reliable quantitation of the major steviol glycosides stevioside, rebaudiosides A-F, steviolbioside, rubusoside, and dulcoside A by utilizing the suitable internal standards. This quantitation provides a base for the optimization of various breeding and postharvest downstream processing of Stevia plants to produce both sweet as well as least bitter tasting Stevia extracts (Well et al. 2013).

Vision for the future

A new vision statement for the future of *S. rebaudiana* research included the most important goal of sequencing of the genome followed by detailed characterization of cellular, physiological, and developmental pathways and make advances obtained through the Stevia genome project available to those working on other plants of the family Asteraceae. Technological innovations such as the use of NGS, DNA chips and microarrays to study global patterns of gene expression (Marshall and Hodgson 1998) should play an important role in *Stevia* research during this period. The more long-term goals to achieve include the determination of the functions and locations of key gene products identified through large-scale sequencing efforts, uncover mechanisms by which complex networks of gene products become established and localized, combine information on gene products with advances in plant physiology and biochemistry to establish a comprehensive picture of plant structure and function, and to resolve questions concerning its evolutionary relationships among eukaryotic organisms and the evolution of common cellular as well as developmental pathways (Meinke et al. 1997). Further, the unraveling of sRNA guided circuitry in stevia will further enhance the value of its gene and EST information and improve our ability to devise strategies to enhance certain essential features of stevia that are less amenable to functional genomics analysis leading to its enhanced nutritive value. The identification of miRNA and their targets will serve not only to help us learn more about the roles of miRNAs in stevia development and physiology but also to provide a framework for

further designing RNAi (RNA interference) based experiments for regulation of gene expression in this species.

Meeting these goals will place increasing demands on the development of databases designed to present massive amounts of information to *Stevia* experts and the diverse audience of biologists. With continued progress in genomics, biology, and database management, it nevertheless, appears likely that *Stevia* will soon become one of the model plants not only for understanding plant structure and function but also for addressing universal questions regarding the nature and origin of biological complexity.

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