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Review

Revisiting the 'Morita II' Paradigm in *Stevia rebaudiana*: Genetic Bottlenecks, Steviol Glycoside Biology and Precision Breeding Pathways

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Abstract

Stevia rebaudiana Bertoni is a strategically important perennial crop because it is the main botanical source of steviol glycosides, a group of high-intensity, non-caloric sweeteners increasingly demanded by the global food and beverage industry. Despite the rapid expansion of stevia cultivation, commercial production remains strongly dependent on a narrow genetic base, particularly on clonally propagated cultivars such as 'Morita II', which has long served as the industrial benchmark because of its favourable rebaudioside A profile and processing consistency. This dependence has raised concerns about limited adaptive capacity, genetic erosion and restricted long-term breeding progress. In this review, we provide an integrated and critical synthesis of current knowledge on the genetic diversity of *S. rebaudiana*, the biosynthetic and regulatory architecture of steviol glycosides, and the conventional and emerging strategies available for crop improvement. Unlike previous reviews, this article explicitly connects domestication-driven genetic bottlenecks, wild germplasm mobilisation, metabolic pathway regulation, advanced analytical phenotyping and precision breeding into a single systems-oriented framework. We examine the roles of wild germplasm, somaclonal variation, polyploidy, molecular markers, omics-assisted approaches and transgene-free genome editing as complementary tools to broaden the stevia breeding base while preserving industrial quality standards. We finally propose an integrative roadmap for the sustainable genetic improvement of stevia, positioning 'Morita II' not as an endpoint, but as a benchmark within a broader diversification strategy.

Keywords: *Stevia rebaudiana*; steviol glycosides; genetic bottleneck; Morita II; pre-breeding; micropropagation; genome editing; marker-assisted selection; precision breeding

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1. Introduction

Stevia rebaudiana Bertoni is a perennial herbaceous species of the family Asteraceae that has attracted worldwide attention due to its exceptional capacity to accumulate steviol glycosides (SGs) in the leaves, a group of diterpenoid compounds with intense sweetening power and negligible caloric value [1,2]. The major glycosides, stevioside and rebaudioside A, are approximately 250–300 times sweeter than sucrose and have become key ingredients in the formulation of low-calorie and sugar-free food and beverage products [3]. The global expansion of stevia cultivation over the last three decades has been driven by the growing

demand for natural sweeteners and by the regulatory approval of steviol glycosides as safe food additives in major international markets [4].

At present, stevia is cultivated on a commercial scale in Asia, the Americas and parts of Europe, with China leading global production, followed by countries such as Paraguay, Brazil, the United States and Colombia [5]. However, despite this rapid geographical expansion and market consolidation, the agronomic performance of the crop remains highly variable and strongly dependent on genotype × environment interactions [6]. Early flowering induced by photoperiod, sensitivity to water stress and soil conditions, moderate biomass yields, and increasing incidence of foliar and stem diseases continue to limit productivity and economic stability in many producing regions [7–9].

Beyond these agronomic constraints, a more structural and less visible limitation underlies most of the current challenges faced by the crop: the extremely narrow genetic base of cultivated *S. rebaudiana*. Modern stevia production relies predominantly on a small number of clonally propagated cultivars, among which 'Morita II' has become the industrial standard due to its high rebaudioside A content and relatively stable performance [10,11]. However, this genetic uniformity is the result of a severe domestication and founder bottleneck, in which only a minute fraction of the genetic diversity present in wild Paraguayan populations was captured and subsequently fixed through vegetative propagation [12–14]. Molecular studies based on SSR, ISSR and high-throughput genotyping approaches have consistently shown that elite cultivars retain only a small proportion of the allelic richness observed in wild and landrace populations [12,14–16].

'Morita II' was originally developed through a targeted clonal selection programme based on a limited subset of Paraguayan germplasm, with emphasis on maximising rebaudioside A content and ensuring phenotypic uniformity for industrial use. The relative scarcity of similarly successful cultivars likely reflects not only the specificity of that original selection process, but also the narrow founder base of cultivated stevia, the predominance of vegetative propagation, and the historically limited use of recurrent recombination-based breeding strategies in the crop [17,18].

The agronomic consequences of this genetic erosion are increasingly discussed as a potential constraint on long-term resilience in cultivated stevia [15,16]. In particular, concerns have been raised about the response of elite clonal cultivars to foliar pathogens such as *Septoria steviae* and soilborne fungi such as *Agroathelia rolfsii*, especially under humid and intensive production conditions [7,19,20]. Evidence from comparative studies conducted in specific environments, including Latin American production systems, suggests that 'Morita II' may exhibit greater disease progress and associated yield penalties than more genetically diverse materials. These reports highlight the possible risks of large-scale clonal monoculture, but should be interpreted as context-dependent evidence rather than as a universally established response across all stevia-growing regions [20,21].

In parallel, remarkable progress has been achieved in the understanding of the biosynthesis and regulation of steviol glycosides. The plastidial diterpenoid pathway leading to steviol formation has been elucidated, and key UDP-glycosyltransferases, such as UGT74G1, UGT76G1 and UGT85C2, have been identified as major determinants of glycoside composition and sweetness profile [4,10–12]. In particular, the high expression of UGT76G1 in 'Morita II' explains its superior capacity to accumulate rebaudioside A relative to other genotypes [11,12]. Advances in analytical chemistry, especially liquid chromatography coupled to mass spectrometry, now enable accurate and high-throughput profiling of complex glycoside mixtures, facilitating the integration of metabolic phenotyping into breeding and selection schemes [14,15,21,22].

Concurrently, a wide range of breeding and biotechnological tools has been progressively incorporated into stevia improvement programmes. These include classical

selection and recurrent hybridisation, in vitro culture and micropropagation, exploitation of somaclonal variation, induced polyploidy, and, more recently, the application of functional markers, omics-assisted selection and genome editing or gene activation systems [8,16,20,23,24]. The availability of high-quality reference genomes and long-read assemblies has further accelerated the identification of candidate genes involved in steviol glycoside biosynthesis and its regulation [11,16]. Nevertheless, most of these approaches have so far been applied in a fragmented manner, often without a unified strategy aimed at rebuilding the genetic base of the crop while preserving the industrial quality standards required by the sweetener market.

In this review, we provide a comprehensive and integrative overview of the biological, genetic and technological foundations of *Stevia rebaudiana* improvement. Specifically, we (i) analyse the origin and agronomic consequences of genetic bottlenecks in cultivated stevia, with special emphasis on the 'Morita II' paradigm; (ii) summarise current knowledge on the biosynthesis, regulation and analytical characterisation of steviol glycosides; (iii) review conventional and modern breeding strategies, including in vitro technologies, polyploidy and precision biotechnology; and (iv) discuss emerging opportunities to combine wild germplasm, functional genomics and genome editing into coherent breeding roadmaps. By integrating these perspectives, we aim to outline a realistic and forward-looking framework for the sustainable genetic improvement of this strategic sweetener crop.

2. *Stevia rebaudiana* and the 'Morita II' Paradigm

2.1. Origin, Domestication and Global Dissemination of Stevia rebaudiana

Stevia rebaudiana is native to the Amambay region in northeastern Paraguay and adjacent areas of southern Brazil, where it occurs as a wild or semi-domesticated perennial species in grassland and forest-edge ecosystems [1,2]. For centuries, indigenous Guaraní communities used its leaves as a natural sweetener and medicinal plant, long before its scientific description and commercial exploitation [1,3]. The isolation and chemical characterisation of its sweet principles at the beginning of the twentieth century marked the starting point of its scientific and agronomic domestication [3].

The first systematic attempts to cultivate stevia outside its centre of origin were carried out in Japan in the mid-twentieth century, followed by gradual expansion to other Asian countries, the Americas and, more recently, Europe [4,5]. This global dissemination was not based on broad sampling of the natural genetic diversity of the species, but rather on a very limited number of founder accessions selected primarily for their sweetening profile and ease of vegetative propagation [8,23]. As a consequence, the worldwide cultivated stevia germplasm represents only a narrow genetic subset of the diversity present in wild Paraguayan populations [7,10,21].

Importantly, this wild diversity has not disappeared. It is still represented in natural and semi-domesticated populations, and part of it is conserved through in situ and ex situ germplasm initiatives, particularly in Paraguay and Brazil, although its systematic mobilisation in breeding programmes remains limited.

The domestication trajectory of stevia thus differs from that of most major crops, which typically underwent long periods of recombination and selection. In stevia, the combination of recent domestication, clonal propagation and strong directional selection for steviol glycoside composition resulted in a rapid fixation of a few elite genotypes and a severe reduction in effective population size [10,11,21]. This process laid the foundation for the current production system, which is highly standardised but genetically fragile.

2.2. Development and Global Adoption of the Cultivar 'Morita II'

Among Morita-derived breeding materials, the cultivar 'Morita II' became the most influential and widely adopted commercial selection, and is therefore the primary focus of this review. This cultivar was developed in the 1970s by the Japanese company Morita Kagaku Kogyo Co. through a clonal selection programme based on wild Paraguayan accessions, with the specific aim of increasing the proportion of rebaudioside A (Reb A) relative to stevioside [16,25]. This objective was driven by sensory considerations, as Reb A exhibits a cleaner sweet taste and reduced bitter aftertaste compared to stevioside [1].

The stabilised clone, later commercialised as 'Morita II', combined a relatively favourable agronomic architecture (semi-erect growth, larger leaves) with a Reb A content typically ranging between 3.5% and 5.5% of leaf dry weight and an improved Reb A/stevioside ratio [3]. These characteristics, together with its high clonal uniformity, made 'Morita II' particularly attractive for industrial-scale cultivation and processing, where chemical consistency of the raw material is a critical requirement.

Following its commercial release in Japan in 1977, 'Morita II' rapidly became the reference cultivar for stevia production in multiple regions, including the United States, China and several Latin American countries [26]. In Colombia, for example, its introduction in the mid-1990s displaced earlier materials that were considered less suitable under local production conditions, including greater susceptibility to foliar disease. However, this historical advantage should be interpreted comparatively and within that specific replacement context; it does not imply that 'Morita II' is broadly disease-resistant under current intensive monoculture systems [19].

2.3. Molecular Basis of the 'Morita II' Phenotype

At the molecular level, the distinctive sweetening profile of 'Morita II' is closely linked to the regulation of the terminal steps of steviol glycoside biosynthesis. In particular, this cultivar exhibits high expression levels of the UDP-glycosyltransferase gene UGT76G1, which catalyses the conversion of stevioside into rebaudioside A, thereby shifting the metabolic flux towards sweeter and less bitter compounds [3].

Comparative transcriptomic and functional analyses have confirmed that variation in the expression and/or allelic composition of key UGT genes, including UGT74G1, UGT76G1 and UGT85C2, is a major determinant of glycoside composition among stevia genotypes [4,7,10,21]. In 'Morita II', directional selection for high Reb A content appears to have fixed a particularly favourable regulatory configuration of this pathway, explaining its long-standing industrial dominance.

However, this biochemical specialisation came at a genetic cost. Molecular marker analyses based on SSR, ISSR and SNP datasets have consistently shown that 'Morita II' retains only a small fraction—approximately 15%—of the allelic diversity present in wild Paraguayan populations [10,21]. This extreme narrowing of the genetic base reflects both the limited number of founder genotypes and the subsequent decades of strict clonal propagation.

2.4. The 'Morita II' Paradox: Industrial Standardisation Versus Genetic Vulnerability

The global success of 'Morita II' illustrates a classical paradox in crop domestication and improvement: the same genetic uniformity that ensures industrial standardisation also generates systemic vulnerability. On the one hand, the widespread adoption of a single, highly homogeneous cultivar simplifies agronomic management, harvesting and industrial processing, and guarantees a stable and predictable sweetener profile [4]. On the other hand, it drastically reduces the adaptive capacity of the crop at the population level.

Evidence from comparative studies suggests that reliance on a highly uniform clonal cultivar may increase phytosanitary risk under certain environments and management systems. In particular, concerns have been raised about the possible susceptibility of 'Morita II' to foliar pathogens such as *Septoria steviae* and to soilborne fungi such as *Agroathelia (Athelia) rolfsii*, as reported in specific studies and production contexts [19,27–29]. These findings are relevant because they illustrate how large-scale clonal uniformity may compromise resilience, but they should be interpreted as context-dependent evidence rather than as definitive proof of universal cultivar failure.

From an evolutionary and breeding perspective, the effective population size of a clonal cultivar such as 'Morita II' is extremely small, often below 150 individuals when estimated from molecular data [10]. Such reduced effective population sizes favour genetic drift, the accumulation of deleterious mutations and the progressive erosion of fitness-related traits, even in the absence of strong external stresses [11,21].

2.5. Implications for Future Stevia Improvement

The dominance of 'Morita II' has undoubtedly accelerated the global adoption of stevia as a commercial crop, but it has also locked the production system into a narrow genetic corridor. Breaking this dependency requires a deliberate and strategic effort to diversify the genetic base of cultivated stevia by reintroducing variation from wild germplasm, landraces and novel breeding populations, while preserving or even improving the sweetener quality standards demanded by the market.

In this context, 'Morita II' may be more productively regarded as a useful benchmark of industrial quality than as the final stage of stevia improvement. From this perspective, broadening the genetic base of the crop through wild germplasm, pre-breeding and recombination-based pipelines appears to be a promising strategy to complement, rather than simply replace, the current cultivar paradigm. The challenge for the coming decade is therefore not necessarily to abandon 'Morita II', but to reduce overdependence on a single clonal standard by developing a more diversified and resilient portfolio of high-quality stevia cultivars.

3. Genetic Diversity in *Stevia rebaudiana*

Genetic diversity represents the foundation of adaptive potential, evolutionary resilience and breeding progress in any crop species. In *Stevia rebaudiana*, a species with a relatively recent and atypical domestication history, the current structure of genetic variation is the direct result of strong founder effects, clonal propagation and intense directional selection for steviol glycoside composition [30–32]. This combination has produced a paradoxical situation in which a globally successful crop is supported by an exceptionally narrow genetic base.

3.1. Genetic Bottlenecks and Founder Effects in Cultivated Stevia

The commercial expansion of *S. rebaudiana* beyond its centre of origin in Paraguay relied on a very limited number of founder accessions, most of which were subsequently multiplied vegetatively [18]. This demographic history generated a severe genetic bottleneck, characterised by a drastic reduction in allelic richness and effective population size in cultivated materials.

Molecular surveys based on SSR and EST-SSR markers have consistently revealed high apparent heterozygosity values but concentrated in a restricted set of alleles, indicating a shallow but internally redundant diversity structure [7]. For instance, Cosson et al. analysed 145 genotypes with 18 EST-SSR loci and found an average of 12 alleles per locus and high polymorphic information content (PIC), while still revealing strong genetic homogeneity

within cultivated groups and limited differentiation among elite cultivars [17,31,33]. This pattern reflects an early capture of diversity followed by prolonged clonal fixation.

High-throughput genotyping approaches have painted an even more restrictive picture. Studies based on genotyping-by-sequencing (GBS) and ISSR markers have reported expected heterozygosity (He) values as low as 0.19–0.25 and PIC values between 0.18 and 0.32 in commercial collections from Asia and the Americas [10,21,34]. Moreover, comparative analyses have shown that artificial selection and vegetative propagation can reduce polymorphism levels by more than 60% relative to sexually reproduced or wild-derived populations [21].

One of the most striking examples of this genetic narrowing is the cultivar 'Morita II'. Based on multilocus molecular data, its effective population size (Ne) has been estimated to be below 150 individuals, and it retains only about 15% of the allelic diversity present in wild Paraguayan populations [7]. Such extremely reduced effective population sizes favour genetic drift, limit recombination and increase the long-term risk of inbreeding depression and mutational load, even in clonally propagated systems [13,19].

3.2. Sources of Genetic Variability for Broadening the Stevia Gene Pool

Overcoming the current genetic narrowness of cultivated stevia requires the identification and strategic mobilisation of alternative sources of variability. Three major reservoirs of genetic novelty can be distinguished: wild germplasm, somaclonal and epigenetic variation induced in vitro, and targeted or semi-targeted genomic interventions.

3.2.1. Wild Populations and Germplasm Collections

Wild and semi-domesticated populations of *S. rebaudiana* from Paraguay and southern Brazil represent the primary and most valuable reservoir of untapped genetic diversity [31,35]. Comparative population genetic studies have shown that landraces and wild accessions harbour substantially higher allelic richness and a large number of private alleles absent from commercial cultivars. For example, Cosson et al. reported an average of 3.7 private alleles per locus in Argentine and Cuban landraces, compared with only 1.8 in elite cultivars [7]. Genetic differentiation between wild Paraguayan populations and cultivated materials from Mexico or Asia often reaches F_ST values above 0.35, indicating deep divergence and substantial diversity loss during domestication [7,36,37].

The conservation and systematic characterisation of this diversity in ex situ and in situ germplasm banks, such as those established in Paraguay and Brazil, is therefore a strategic priority [36]. Additional surveys in regions such as Malaysia, Brazil and the Andean countries have confirmed the existence of promising materials with high genetic variability and potentially valuable agronomic and metabolic traits, including stress tolerance, favourable plant architecture and alternative glycoside profiles [30,38].

3.2.2. Somaclonal and Epigenetic Variation Induced by In Vitro Culture

In vitro culture and micropropagation, while primarily used for the rapid multiplication of elite genotypes, also constitute a significant source of genetic and epigenetic variation in *S. rebaudiana* [4,39–41]. Early studies already reported substantial changes in DNA methylation patterns in regenerated plants, with up to 26% of CpG/CHG sites showing differential methylation relative to donor plants [41]. More recently, epitranscriptomic analyses in autotetraploid lines revealed a 27–30% increase in m^6A peaks in promoter regions of genes associated with steviol glycoside biosynthesis, suggesting complex layers of regulatory variation induced during tissue culture and polyploidisation [42].

Structural genomic variation also appears to be stimulated in vitro. Cosson et al. detected the activation of transposable elements in clonal lines, pointing to an increased rate of mutation and genome restructuring under tissue culture conditions [7]. From

a phenotypic perspective, several studies have shown that between 13% and 17% of regenerated plants exhibit significant changes in the stevioside/Reb A ratio and in total glycoside content, as determined by HPLC analyses [4,39,40]. Although such variation must be carefully screened to eliminate undesirable traits, it provides a practical route to locally broaden the genetic and phenotypic spectrum without sexual recombination.

3.2.3. Emerging Genomic Resources and Targeted Genome Manipulation

The recent availability of high-quality reference genomes for *S. rebaudiana*, generated using long-read sequencing technologies from Pacific Biosciences (PacBio HiFi; Menlo Park, CA, USA) and Oxford Nanopore Technologies plc (Oxford, UK), has opened a new phase in stevia genetics and breeding [11,43]. These assemblies have enabled the precise identification and structural characterisation of key genes in the steviol glycoside pathway, including UGT76G1, UGT85C2 and UGT74G1, as well as upstream diterpene biosynthesis genes.

Building on these resources, first proof-of-concept studies using CRISPR-based systems have been reported. For instance, the application of a DNA-free dCas9-VP64 activation system in protoplasts resulted in an approximately 27-fold increase in UGT76G1 expression, accompanied by the co-activation of UGT85C2, with the aim of redirecting metabolic flux towards higher rebaudioside A accumulation [44]. Although stable gene knock-out lines targeting enzymes such as ent-kaurene oxidase or ent-kaurenoic acid hydroxylase have not yet been reported, transgenic overexpression of SrKAH has already produced lines with up to 88% increases in total steviol glycoside content without obvious developmental penalties [45].

These early results should currently be interpreted as proof-of-concept advances rather than as field-ready breeding outputs. They illustrate the potential of genomic and epigenomic engineering to fine-tune metabolic traits and generate novel functional variation in stevia, but stable regeneration, multi-generation assessment and field validation remain limited or, in several cases, have not yet been reported. This translational gap remains one of the main bottlenecks preventing the practical deployment of precision interventions in the crop.

3.3. Agronomic Consequences of Limited Genetic Diversity

The agronomic impact of genetic uniformity in *S. rebaudiana* is most clearly manifested in its phytosanitary vulnerability. In Asia, foliar disease outbreaks in commercial nurseries have reached incidences of up to 15%, while in Europe and the Americas, rapid disease progression of Septoria leaf spot has been documented using the area under the disease progress curve (AUDPC) as a quantitative indicator [28,46].

Detailed quantitative evidence for the phytosanitary consequences of limited diversity has been reported in specific comparative studies, particularly for Septoria leaf spot. In such studies, the clonal cultivar ‘Morita II’, often grown under highly uniform conditions, showed higher AUDPC values and faster disease progression than some wild-derived accessions or more genetically heterogeneous materials [28]. Under those experimental conditions, this translated into measurable foliar damage and reductions in steviol glycoside yield. Although these results should not be overgeneralised across all environments, they provide an important empirical example of how narrow genetic backgrounds may increase vulnerability and reduce buffering capacity under pathogen pressure.

Taken together, the current genetic base of cultivated stevia is both narrow and structurally fragile, which severely constrains long-term genetic gain and resilience. The main reservoirs of diversity that can be mobilised to rebuild this base, together with their potential contributions and limitations from a breeding perspective, are summarised in Table 1.

Table 1. Main sources of diversity currently available or potentially exploitable for *Stevia rebaudiana* improvement, their expected breeding value, major target traits, associated risks or limitations, recommended capture strategies, evidence level, and key supporting references. The table is intended as an integrative breeding-oriented synthesis and should therefore be interpreted as a strategic framework rather than as an exhaustive inventory.

Diversity Source	What It Contributes (Breeding Value)	Key Target Traits	Main Risks/Limitations	How to Capture/Use It (Recommended Actions)	Evidence Level/Readiness	Key References
Wild germplasm (Paraguay/adjacent regions)	New alleles; broad adaptive potential; pathway/regulatory variants	SG profile diversification; abiotic stress tolerance; disease resistance	Linkage drag; photoperiod/flowering; agronomic instability	Core collections; pre-breeding; introgression + marker support; multi-environment evaluation	Medium–High (needs harmonised sampling)	[30,36,38]
Landraces/farmer-selected materials	Locally adapted allele combinations; "intermediate domestication"	Yield stability; resilience; phenology	Poor documentation; heterogeneity	Passporting + genotyping; build mini-core; use as bridge parents	Medium	[7,30,38]
Elite clones ('Morita II' paradigm)	High Reb A performance baseline; industry compatibility	Quality standardisation; biomass in specific environments	Genetic bottleneck; fragile resilience; limited long-term gain	Use as recurrent parent only within broadened base; avoid over-reliance	High (but constrained)	[10,21]
Tissue culture-derived lines/somaclonal variants	Novel phenotypes; epigenetic/regulatory shifts	Leaf biomass; SG ratios; stress responsiveness	Instability; off-types; epigenetic drift	Tight QC: genetic fidelity + chemoprofile; select stable lines over cycles	Medium	[4,39–42]
Polyploid/cytotype variation (where applicable)	Altered dosage/regulation; potential SG yield shifts	SG content; vigour	Fertility issues; segregation complexity	Cytotyping; controlled crossing; evaluate trait stability	Low–Medium (con-text-dependent)	[42,47–49]
Segregating populations (recombination-based)	Break linkage blocks; create new trait stacks	Multi-trait improvement	Long cycles; need phenotyping capacity	Structured populations + MAS/GS; replicated G × E trials	Medium–High (if infrastructure exists)	[16,25,50]
Pangenome-informed diversity (future-ready)	Presence/absence and structural variants beyond single reference	Complex traits; resilience	Data/analysis-intensive	Build multi-line pangenome + functional annotation; allele mining	Emerging but high value	[11,43,51,52]

4. Breeding Strategies in *Stevia rebaudiana*

The improvement of *Stevia rebaudiana* represents a particularly challenging breeding problem, as it must reconcile three partially conflicting objectives: (i) increasing and stabilising biomass yield, (ii) optimising steviol glycoside composition and total content, and (iii) restoring genetic diversity and adaptive capacity in a crop historically shaped by clonal propagation and strong directional selection [12,50]. Over the last decades, a broad spectrum of breeding strategies has been explored, ranging from classical selection schemes to advanced biotechnological and genomic approaches.

4.1. Conventional Breeding: Selection, Hybridisation and Recurrent Improvement

Early stevia breeding programmes relied primarily on phenotypic selection within heterogeneous seed-derived populations and on the evaluation of segregating progenies obtained from controlled or open pollination [25,50]. Although *S. rebaudiana* is an obligate outcrosser with a sporophytic self-incompatibility system, the exploitation of sexual reproduction has been historically limited by low and unstable seed set, low germination percentages, strong environmental effects on flowering, and the rapid re-fixation of elite phenotypes through vegetative propagation [50].

Nevertheless, several studies have demonstrated that substantial genetic variability exists for key agronomic and biochemical traits in sexually reproduced populations. Quantitative analyses have reported broad ranges for plant height, leaf area, dry biomass and total steviol glycoside content, as well as significant transgressive segregation for the rebaudioside A/stevioside ratio in F_1 and F_2 progenies [7,50]. Estimates of broad-sense heritability for total glycoside content and Reb A proportion typically range between 0.45 and 0.70, indicating a moderate to high genetic control and substantial potential for selection response [16,50].

Recurrent selection schemes based on half-sib and full-sib families have been proposed as a practical way to gradually improve complex traits while maintaining a broader genetic base than in purely clonal systems [25,50]. In such schemes, selected individuals are recombined at each cycle, and superior clones are simultaneously identified for potential direct release or for use as parents in subsequent cycles. Although long-term experimental data are still scarce, modelling studies suggest that recurrent selection could significantly increase both mean performance and genetic variance over a few breeding cycles, provided that sufficiently large effective population sizes are maintained [7,50].

4.2. Biotechnological Approaches: In Vitro Culture, Polyploidy and Genetic Transformation

In vitro culture has played a dual role in stevia improvement programmes: as a tool for the rapid and disease-free multiplication of elite genotypes, and as a source of novel genetic and epigenetic variation [4,50]. Protocols based on nodal segments, leaf explants and apical meristems have been optimised for several cultivars, achieving multiplication rates sufficient for large-scale commercial deployment [50]. At the same time, somaclonal variation—discussed in Section 3 as a source of genetic and epigenetic novelty induced during in vitro culture—has also been considered a secondary breeding resource in stevia, particularly for generating exploratory variation in plant architecture, biomass allocation and steviol glycoside composition.

Induced polyploidy represents another classical biotechnological approach to stevia improvement. Colchicine- or oryzalin-induced autotetraploid and mixoploid lines have been generated and characterised in several studies [47,48]. These polyploids often exhibit increased leaf size, thicker stems and, in some cases, higher total steviol glycoside content compared to their diploid counterparts [47–49]. Moreover, recent epigenomic analyses have revealed extensive reprogramming of DNA methylation and m^6A RNA methylation

landscapes in tetraploid lines, particularly in genes associated with diterpenoid and glycoside metabolism, suggesting complex regulatory effects beyond simple gene dosage [47]. However, polyploidisation can also negatively affect fertility and growth rate, making careful phenotypic screening indispensable before any practical use.

Genetic transformation has been primarily used as a functional genomics tool in stevia rather than as a direct breeding strategy. Stable overexpression of pathway genes such as SrKAH (ent-kaurenoic acid hydroxylase) has resulted in transgenic lines showing up to 80–90% increases in total steviol glycoside content without major morphological alterations [12]. Similarly, heterologous expression of regulatory genes and transcription factors has been explored to modulate flux through the diterpenoid pathway [12]. Although these approaches clearly demonstrate the manipulability of steviol glycoside biosynthesis, regulatory constraints and consumer acceptance issues currently limit their direct deployment in commercial varieties.

4.3. Towards Precision Breeding: Molecular Markers, Omics and Genome Editing

The progressive integration of molecular tools into stevia breeding has paved the way towards more precise and knowledge-driven improvement strategies. A wide array of DNA markers, including RAPD, ISSR, AFLP, SCoT and SSR, has been developed and applied for germplasm characterisation, genetic diversity analysis and, to a lesser extent, parent selection [7,50]. More recently, functional markers directly targeting genes of the steviol glycoside pathway, such as UGT76G1, have been proposed as tools for marker-assisted selection (MAS) aimed at improving glycoside composition rather than overall content [12].

The availability of high-quality reference genomes and transcriptome datasets has further accelerated the identification of candidate genes, regulatory elements and structural variants associated with key traits [50]. These resources open the door to quantitative trait locus (QTL) mapping, genome-wide association studies (GWAS) and genomic selection approaches, which, although still in their infancy in stevia, have transformed breeding programmes in many other crops.

Genome editing technologies, particularly CRISPR/Cas-based systems, represent the most recent and potentially transformative addition to the stevia breeding toolbox. While stable gene knock-out lines have not yet been widely reported, proof-of-concept experiments using DNA-free dCas9-VP64 activation systems have demonstrated the feasibility of precisely upregulating key pathway genes such as UGT76G1 and UGT85C2, achieving strong increases in transcript abundance and suggesting a viable route to fine-tune glycoside profiles without introducing foreign DNA [44]. In a regulatory context that increasingly distinguishes between transgenic and gene-edited crops, such transgene-free approaches may become particularly attractive for industrial stevia improvement.

From a strategic perspective, the future of stevia breeding is likely to depend on the intelligent combination of these approaches. Classical recombination-based breeding is essential to rebuild genetic diversity and generate new allele combinations, while molecular and genomic tools can dramatically increase selection efficiency and precision. In this integrative framework, the objective is not merely to further optimise existing clonal cultivars, but to design breeding pipelines capable of delivering genetically diverse, resilient and high-quality cultivars adapted to a wide range of production environments.

5. Steviol Glycosides: Biosynthesis, Regulation and Metabolic Engineering

Steviol glycosides (SGs) constitute the defining metabolic trait of *Stevia rebaudiana* and the biochemical basis of its economic value. More than 40 structurally related SGs have been identified to date, differing in the number, type and position of sugar moieties attached to the steviol backbone [49,53]. Among these, stevioside and rebaudioside A (Reb A) dominate the leaf glycoside profile of most commercial cultivars, accounting together for 70–90% of total SG content [54]. Understanding the biosynthetic origin, regulation and plasticity of this pathway is therefore central to any rational improvement strategy.

5.1. The Steviol Glycoside Biosynthetic Pathway

The biosynthesis of steviol glycosides integrates the plastidial methylerythritol phosphate (MEP) pathway with cytosolic and endoplasmic reticulum-associated reactions of diterpenoid metabolism and glycosylation [1,45]. The early steps are shared with gibberellin biosynthesis and involve the conversion of pyruvate and glyceraldehyde-3-phosphate into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via the MEP pathway, followed by the formation of geranylgeranyl diphosphate (GGPP) [45].

GGPP is then cyclised by ent-copalyl diphosphate synthase (CPS) and ent-kaurene synthase (KS) to produce ent-kaurene, which is subsequently oxidised by a series of cytochrome P450 monooxygenases, including ent-kaurene oxidase (KO) and ent-kaurenoic acid hydroxylase (KAH), to yield steviol [1,2]. Steviol is finally glycosylated at the C13 and C19 positions by a small family of UDP-dependent glycosyltransferases (UGTs), producing the diverse spectrum of steviol glycosides found in stevia leaves [1,55].

Among these enzymes, UGT85C2 catalyses the first glycosylation step at C13, UGT74G1 adds a second glucose moiety, and UGT76G1 is responsible for the key conversion of stevioside into rebaudioside A by adding an additional glucose at the C13-linked sugar chain [1,55]. The relative activities and expression levels of these UGTs are major determinants of both total SG content and glycoside composition. A consolidated overview of the steviol glycoside biosynthetic pathway, including the main enzymes, corresponding genes, subcellular localisation and functional roles, is provided in Appendix A (Table A1).

5.2. Genetic and Regulatory Control of Glycoside Accumulation

Transcriptomic and expression analyses have shown that the accumulation of SGs in *S. rebaudiana* leaves is tightly regulated at the transcriptional level and displays strong developmental and genotypic specificity [1,56,57]. In particular, the high Reb A content characteristic of 'Morita II' is closely associated with elevated expression of UGT76G1, which shifts metabolic flux away from stevioside towards sweeter glycosides [10,58].

Beyond UGTs, several upstream genes in the MEP and diterpenoid pathways, including DXS, DXR, CPS, KS, KO and KAH, have also been shown to influence total SG accumulation when overexpressed or differentially regulated [3,59]. For example, transgenic overexpression of SrKAH resulted in increases of up to 88% in total SG content, indicating that steviol supply can be a limiting factor in the pathway [60].

Regulatory networks controlling SG biosynthesis are still incompletely understood, but emerging evidence points to the involvement of transcription factors from the MYB, bHLH and WRKY families, as well as to epigenetic modulation via DNA methylation and RNA methylation processes [11,61,62]. The latter is particularly relevant in polyploid and tissue culture-derived lines, where extensive epigenomic reprogramming has been observed in genes related to diterpenoid metabolism [63].

Environmental factors further modulate SG accumulation and composition. Light intensity, photoperiod, nitrogen availability and water status have all been reported to

influence both total SG content and the relative proportions of individual glycosides, partly through effects on photosynthate supply and gene expression patterns [2,64]. This strong genotype × environment interaction underscores the need to integrate metabolic phenotyping into multi-environment breeding trials.

5.3. Analytical Platforms for Steviol Glycoside Profiling

Accurate and reproducible quantification of steviol glycosides is a prerequisite for both fundamental research and breeding applications. High-performance liquid chromatography (HPLC) with UV or diode-array detection has long been the standard method for SG analysis and remains widely used for routine screening [1,65,66]. However, the increasing complexity of breeding materials and the need to resolve minor glycosides have driven the adoption of more advanced techniques.

Ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) now offers superior sensitivity, resolution and throughput, enabling the simultaneous quantification of more than 15 individual SGs in a single run [67]. Hydrophilic interaction liquid chromatography (HILIC) and two-dimensional LC approaches have further improved the separation of closely related glycoside isomers [14,56].

In addition, emerging analytical platforms such as ambient mass spectrometry, near-infrared spectroscopy (NIRS) calibrated against LC data, and even nanopore-based sensing technologies are being explored as rapid, low-cost phenotyping tools for large breeding populations [68]. The integration of such high-throughput phenotyping platforms with genomic and transcriptomic data is expected to greatly accelerate the identification of genotype–metabolite associations in stevia.

5.4. Metabolic Engineering and Synthetic Biology Approaches

Beyond conventional breeding, metabolic engineering has been actively explored as a means to optimise SG production and composition. In planta approaches include the stable overexpression of pathway genes, as discussed above, as well as the manipulation of regulatory genes and transport processes [3,59]. However, regulatory and societal constraints limit the commercial deployment of transgenic stevia cultivars in many markets.

In parallel, heterologous production of steviol glycosides in microbial and plant-based expression systems has emerged as a complementary strategy. Yeast and *E. coli* strains engineered with combinations of stevia pathway genes have been shown to produce steviol and several glycosides at industrially relevant titres, opening the door to fermentation-based production platforms [44]. While these systems are primarily of interest to the sweetener industry rather than to crop improvement per se, they also provide powerful experimental platforms to dissect pathway regulation and enzyme function.

From the perspective of crop breeding, the most promising direction is likely the convergence of metabolic engineering and precision breeding. Genome editing and gene activation technologies offer the possibility to fine-tune endogenous pathway genes in elite genetic backgrounds without introducing foreign DNA, thereby combining the predictability of metabolic engineering with the regulatory advantages of non-transgenic approaches [12]. Such strategies could enable the targeted redesign of glycoside profiles, for example by increasing the proportion of next-generation sweeteners such as rebaudioside D and M, while simultaneously preserving or improving agronomic performance.

6. Micropropagation and Clonal Deployment

The commercial success of *Stevia rebaudiana* has been closely linked to the widespread adoption of clonal propagation systems, which ensure genetic uniformity, rapid multiplication and chemical consistency of planting material [14,69]. In a crop dominated by a

few elite genotypes—most notably 'Morita II'—micropropagation has become not only a propagation technique, but a structural component of the entire production chain, from nursery establishment to industrial-scale field deployment. This section is not intended to provide an exhaustive inventory of all published micropropagation protocols for *Stevia rebaudiana*. Instead, the literature discussed here was selected according to its relevance to breeding pipelines, clonal scale-up, phytosanitary quality, genetic fidelity and, where available, links between in vitro propagation and steviol glycoside-related outcomes.

6.1. In Vitro Propagation Systems and Scaling-Up

Early protocols for stevia micropropagation were based on nodal segments and apical meristems cultured on Murashige and Skoog (MS) medium supplemented with cytokinins such as BAP or kinetin, achieving high shoot multiplication rates and reliable rooting [41,70,71]. Subsequent optimisation efforts have explored alternative explants (leaf discs, internodes), growth regulator combinations and temporary immersion bioreactor systems to further increase multiplication efficiency and reduce production costs [72–74].

Temporary immersion systems (TIS) and other liquid-culture platforms have proven particularly effective for large-scale propagation, allowing better control of nutrient uptake, gas exchange and hyperhydricity while significantly increasing the number of plantlets produced per unit time [72,74]. In some systems, multiplication rates exceeding 10–12 shoots per explant per subculture cycle have been reported, making micropropagation economically viable even for medium-sized commercial operations [69,72].

Acclimatisation remains a critical step in the micropropagation pipeline. Although stevia plantlets generally exhibit high survival rates during transfer to ex vitro conditions, careful control of humidity, light intensity and substrate composition is required to minimise losses and ensure uniform field establishment [14,71].

6.2. Phytosanitary Quality and the Role of Clean Plant Programmes

One of the major advantages of micropropagation is the possibility to produce pathogen-free planting material. Meristem culture and thermotherapy-based sanitation protocols have been successfully applied to eliminate systemic pathogens and to establish clean plant stocks of elite stevia cultivars [69,75]. This is particularly relevant in a crop highly susceptible to foliar and soilborne diseases, where the use of infected planting material can rapidly compromise entire production cycles [69].

Several countries have therefore integrated stevia micropropagation into broader clean plant or certified nursery programmes, ensuring traceability and phytosanitary quality from the initial in vitro stock to the final field-transplanted plantlets [69,76]. In such systems, micropropagation is not merely a multiplication tool, but a central pillar of disease management and production stability.

6.3. Genetic Stability, Somaclonal Variation and Quality Control

Despite its many advantages, clonal propagation via tissue culture is not genetically neutral. As discussed in Section 3, in vitro culture can induce both genetic and epigenetic changes, collectively referred to as somaclonal variation [75,76]. In stevia, molecular analyses have detected activation of transposable elements, changes in DNA methylation patterns and occasional structural genomic rearrangements in long-term cultured lines [69,75].

From a production perspective, this represents a double-edged sword. On the one hand, somaclonal variation can generate novel phenotypes with altered steviol glycoside profiles or improved agronomic traits, which may be exploited as breeding material [14,69]. On the other hand, uncontrolled variation can compromise the uniformity and quality standards required by the industry, particularly when the objective is to faithfully reproduce a cultivar such as 'Morita II' [69,76].

Consequently, modern stevia micropropagation systems increasingly incorporate molecular and biochemical quality control steps. The use of SSR or SNP markers to verify genetic identity, combined with periodic HPLC or LC–MS profiling of steviol glycosides, allows early detection of off-types and metabolic deviants before large-scale field deployment [69,76]. Because clonal deployment will remain indispensable for rapid dissemination, in vitro platforms must be evaluated not only for multiplication efficiency but also for genetic fidelity and, where relevant, chemotype stability. Representative basal media, PGR combinations and morphogenic outputs reported for *Stevia rebaudiana* are summarised in Table 2.

Table 2. Basal media, PGR combinations and morphogenic responses in *Stevia rebaudiana* relevant to clonal scale-up and breeding pipelines. Data are reported as in the original sources and are selected for relevance to multiplication, rooting, elongation and callus induction; where available, links to steviol glycoside (SG) outcomes are indicated.

Basal Medium/ System	Cytokinin (mg L^{-1})	Auxin (mg L^{-1})	Stage	Response (Reported Metric)	Reference
MS full-strength	BAP 3.0	—	Multiplication	16 ± 2 shoots/explant	[77]
½ MS	—	IBA 0.5	Rooting	90% ± 3% rooting	[71]
¼ MS	KIN 3.0 + NAA 0.1	—	Elongation	+25% shoot length; ↑Reb-A 11%	[54]
BIT® (liquid MS)	BAP 2.0	NAA 0.5	Multiplication	8 shoots/plant/4 weeks	[74]
MS + Gelrite	TDZ 0.1	IAA 0.05	Multiplication	22 ± 3 shoots/explant	[78]
Gamborg B5 (liquid)	BA 1.0	IAA 0.1	Multiplication	12 ± 1.5 shoots; Reb-A 8% DW	[79]
MS + 2,4-D	2,4-D 2.0	KIN 0.2	Callus	96% explants forming callus	[80]

Temporary immersion bioreactors (BIT®/RITA®) using liquid MS with short immersion pulses (30–60 s every 6–8 h) can reduce agar and labour inputs by ~25–40% while increasing the shoot/cost ratio. Recent optimisation trends include replacing sucrose with maltose (2–3%), using silver nanoparticles (1–5 ppm) as selective biocides under high microbial pressure, and applying response surface methodologies to reduce fine-tuning trials.

6.4. Clonal Deployment at Regional and Industrial Scales

In many stevia-producing regions, particularly in Asia and Latin America, micropropagation has become the dominant source of planting material for commercial fields [13,73,74,76]. This has enabled rapid expansion of cultivated areas and facilitated the dissemination of elite cultivars with predictable performance and quality.

However, the large-scale clonal deployment of genetically uniform material also reinforces the systemic risks discussed in Sections 2 and 3. Extensive monoclonal plantations increase the vulnerability of the production system to emerging pathogens and to unexpected environmental stresses, as has been repeatedly observed in other clonally propagated crops such as banana, potato and cassava [80,81].

From a strategic perspective, micropropagation should therefore be viewed as a powerful enabling technology rather than as a substitute for genetic diversification. In a forward-looking breeding framework, it will remain indispensable for the rapid multiplication and dissemination of new cultivars, but these cultivars should ideally represent a diversified portfolio of genotypes rather than successive generations of a single clonal lineage.

7. Molecular Tools for Diversity Analysis and Breeding

The transition from descriptive to predictive and design-oriented breeding in Stevia rebaudiana critically depends on the availability and intelligent use of molecular and genomic tools. Over the last two decades, a wide array of marker systems and omics platforms has been progressively incorporated into stevia research, providing unprecedented resolution for the analysis of genetic diversity, population structure and the molecular basis of key agronomic and metabolic traits [4,15,40,82].

7.1. DNA Marker Systems for Genetic Diversity and Germplasm Characterisation

Early molecular studies in stevia relied on dominant marker systems such as RAPD and ISSR, which offered a rapid and cost-effective means to assess genetic variability in cultivated and wild populations [4,15,36]. These approaches consistently revealed moderate to high levels of polymorphism in wild germplasm and a much narrower diversity in elite cultivars, thereby providing the first quantitative evidence of the domestication bottleneck discussed in previous sections [4,15,82].

Subsequently, more robust and informative marker systems were developed, including AFLP, SCoT and, most importantly, simple sequence repeats (SSRs) and expressed sequence tag-derived SSRs (EST-SSRs) [7,83–85]. SSRs have become the reference markers for stevia diversity studies due to their high polymorphism, co-dominant inheritance and reproducibility. Large-scale surveys based on 15–20 SSR loci have revealed clear genetic structuring between wild, landrace and cultivated materials and have enabled the estimation of key population genetic parameters such as allelic richness, expected heterozygosity and effective population size [5,36,85,86].

Beyond diversity assessment, SSR and related markers have also been used for clonal identity verification and nursery quality control, helping to detect off-types and unintended mixtures in commercial propagation systems [36,85]. A comparative overview of the main molecular marker systems used in *S. rebaudiana*, together with their typical applications and analytical scope, is provided in Appendix B (Table A2).

7.2. Functional Markers and Candidate Gene Approaches

While neutral markers are invaluable for describing population structure, breeding decisions ultimately require information on loci directly controlling target traits. In stevia, the progressive elucidation of the steviol glycoside biosynthetic pathway has enabled the development of functional or gene-targeted markers associated with key enzymes, particularly UDP-glycosyltransferases [34,87].

For example, polymorphisms in the UGT76G1 gene, which plays a central role in determining the rebaudioside A/stevioside ratio, have been shown to correlate with glycoside profiles in segregating populations and diverse germplasm panels [87]. Such markers can be used in marker-assisted selection (MAS) schemes to pre-select seedlings with a higher probability of expressing favourable sweetener profiles, thereby reducing the cost and time associated with extensive biochemical phenotyping.

Similar candidate gene approaches have been proposed for upstream pathway genes such as CPS, KS, KO and KAH, as well as for regulatory genes identified through transcriptomic analyses [1,44,45]. Although MAS in stevia is still at an early stage, these developments lay the groundwork for a more targeted and efficient breeding process.

7.3. High-Throughput Genotyping, Omics and Systems-Level Approaches

The advent of next-generation sequencing (NGS) technologies has profoundly changed the scale and scope of genetic analysis in stevia. Genotyping-by-sequencing (GBS) and related reduced-representation approaches now enable the simultaneous interrogation of thousands of SNPs across the genome, providing much higher resolution for population structure analysis and for the detection of genotype–phenotype associations than classical marker systems [88–91].

In parallel, transcriptomic, proteomic and metabolomic datasets have become increasingly available, particularly for leaf tissues and for contrasting genotypes or developmental stages [92–94]. These omics resources have been instrumental in identifying differentially expressed genes, co-expression networks and regulatory modules associated with steviol glycoside accumulation and stress responses.

The logical next step is the integration of these layers of information into systems-level frameworks. Approaches such as quantitative trait locus (QTL) mapping, genome-wide association studies (GWAS) and, eventually, genomic selection (GS) offer the possibility to move from single-gene to genome-wide prediction models for complex traits such as total glycoside content, biomass yield and disease tolerance [51,90,95]. Although such approaches have not yet been widely implemented in stevia, the recent availability of high-quality reference genomes and dense marker datasets makes their application technically feasible in the near future [11,43,51,52].

7.4. From Molecular Diagnosis to Predictive Breeding

From a strategic standpoint, molecular tools should not be viewed as isolated technologies, but as components of an integrated breeding pipeline. Neutral markers are essential for managing and broadening genetic diversity, functional markers can accelerate the fixation of key quality traits, and genome-wide markers and omics data can ultimately support predictive models of performance across environments.

In the context of stevia, such an integrated approach is particularly important, given the historical over-reliance on clonal propagation and the urgent need to rebuild a more diverse and resilient genetic base. By combining classical recombination-based breeding with modern molecular diagnostics and prediction tools, it should be possible to transition from the current paradigm of clonal optimisation to a genuinely evolutionary and sustainable improvement strategy. Taken together, these approaches constitute an integrated toolbox spanning clonal identity control, diversity management, locus discovery, predictive modelling and precision intervention. For clarity, the main molecular, genomic and analytical platforms currently supporting stevia improvement, together with their decision-support roles, prerequisites and limitations, are summarised in Table 3.

Table 3. Molecular, genomic, and analytical toolbox for rebuilding the genetic base and enabling precision breeding in *Stevia rebaudiana*. Approaches are organised by the primary breeding decision supported, from clonal identity control and diversity management to locus discovery, genomic prediction and precision intervention.

Approach/Platform	Data Type	Primary Breeding Decision Supported	Typical Stevia Use-Case	Critical Prerequisites	Main Pitfalls/QC Notes	Readiness
RAPD/ISSR	Dominant markers	Exploratory diversity screening	Rapid contrasts among wild vs. cultivated; first-pass clustering	Basic PCR setup; replicate runs	Limited reproducibility across labs; weak comparability; not ideal for MAS	Medium (screening)
AFLP/SCoT	Dominant/gene-targeted	Fine fingerprinting; structure	Higher-resolution diversity surveys; structure in poorly characterised panels	Standardised protocol; scoring QC	Not optimal for allele dosage; still limited for predictive breeding	Medium
SSR/EST-SSR	Co-dominant markers	Clonal identity control + diversity management	Core collections; parent selection; nursery QC	Reference panel; controls; allele binning	Panel-dependent resolution; genotyping error must be monitored	High
SNP genotyping (GBS/ddRAD)	Genome-wide	Genome-level diversity; population structure	Bottleneck diagnosis; diversity rebuilding; relations-hip matrices	Bioinformatics; filtering pipeline	Missing data; batch effects; needs strict QC thresholds	Medium–High
QTL mapping	Markers + segregating populations	Locus discovery (structured)	Biomass, phenology, stress/disease traits in biparental material	Adequate population size; replicated phenotyping	Low power if N small; context-dependent loci under G × E	Medium
GWAS	Dense SNPs + diversity panel	Locus discovery (diverse)	Trait architecture beyond SG pathway; candidate prioritisation	Large panel; structure correction	Spurious associations; needs independent validation	Medium
Genomic selection (GS)	Genome-wide SNPs + training set	Predict breeding values	Multi-trait improvement across environments	Training population; harmonised phenotyping; G × E models	Prediction collapses if phenotype noise is high; requires scale	Emerging–Medium
Functional/candidate markers (e.g., UGTs, KO/KAH)	Targeted variants/haplotypes	Accelerate fixation of quality alleles	SG composition (Reb A/Stev; Reb D/M); pathway-informed selection	Validated links; multi-env checks	Often polygenic; background dependence; avoid over-claiming causality	Medium–High
Transcriptomics/regulatory profiling	RNA-seq (±small RNA)	Identify regulatory modules; targets	TF/miRNA modules; stress–SG crosstalk; candidate ranking	Sampling design; replication; batch control	Tissue/time specificity; not directly deployable without marker conversion	Medium
Metabolite profiling	HPLC/UHPLC–MS/MS	Direct quality phenotype	SG chemotyping for selection and release; validate MAS/editing	Standards; reference controls; QC pipeline	Stage-dependent profiles; throughput/cost constraints	High
Genome editing/gene activation	CRISPR/dCas systems	Functional validation; trait tuning	Validate targets; adjust flux/regulation	Efficient transformation/regeneration	Regulatory/IP; genotype dependence; off-target checks	Medium
Integrated release QC	SSR/SNP + chemoprofile	Prevent off-type scale-out	Clean-plant programmes; deployment integrity	Thresholds; routine sampling cadence	Without routine QC, clonal drift/off-types propagate widely	High

Readiness reflects feasibility in current stevia programmes, accounting for infrastructure requirements and transformation bottlenecks. For clonal deployment, routine identity checks (SSR/SNP) combined with periodic SG chemotyping are recommended as mandatory quality-control steps.

8. An Integrative Model: From Genotype to Sweetness

The biological and agronomic performance of *Stevia rebaudiana* emerges from a complex, hierarchical interaction between genetic variation, regulatory networks, metabolic pathways and environmental modulation. As discussed in the previous sections, neither steviol glycoside accumulation nor yield, stress tolerance or disease resistance can be understood—or improved—by considering isolated genes or traits. Instead, stevia must be viewed as an integrated biological system in which genotype, epigenetic state, transcriptional regulation, metabolic fluxes and physiological processes are tightly interconnected [11,42,52]. In this framework, chemical profiling (HPLC/UHPLC–MS/MS) and genetic identity control (SSR/SNP) should be treated as mandatory release-gating criteria rather than auxiliary analytical steps [36].

The value of this integrative model is not merely descriptive. It provides a decision-support framework for stevia improvement in which each layer contributes a distinct breeding function: diversity inputs expand the available allelic repertoire; regulatory and metabolic analyses help identify tractable determinants of sweetener quality, adaptation and resilience; precision interventions increase the efficiency and specificity of selection or trait modification; and field validation ensures that candidate materials are assessed under realistic production conditions before large-scale deployment. In this sense, the framework is intended to support the transition from clonal optimisation around a narrow genetic base to a more diversified, evidence-driven and iterative breeding strategy.

8.1. A Multi-Layered Framework Linking Genome, Regulation and Metabolism

At the base of the system lies the genomic layer, which encompasses both the structural diversity inherited from wild populations and the severe narrowing imposed by domestication and clonal propagation (Sections 2 and 3). This layer defines the allelic repertoire available for key genes involved in diterpenoid and glycoside metabolism (e.g., DXS, CPS, KS, KO, KAH, UGT85C2, UGT74G1, UGT76G1) as well as for genes controlling development, stress responses and defence [11,43,96,97].

Above this static genomic template operates a dynamic regulatory layer, including transcription factors, chromatin modifications, DNA methylation and RNA methylation processes. Increasing evidence indicates that epigenetic and transcriptional regulation plays a central role in shaping the quantitative output of the steviol glycoside pathway, particularly in polyploid and tissue culture-derived lines [42,44,62]. This regulatory layer provides both robustness and plasticity, allowing the same genotype to express different metabolic phenotypes under contrasting environmental or developmental conditions.

The metabolic layer integrates these regulatory signals into actual biochemical fluxes. Carbon fixed through photosynthesis and channelled via the MEP pathway is partitioned between primary metabolism, gibberellin biosynthesis and steviol glycoside production. The relative activity of competing branches, as well as the efficiency of glycosylation steps mediated by UGTs, ultimately determines both total SG content and glycoside composition [3,45].

8.2. Phenotype as an Emergent Property: Integrating Yield, Quality and Resilience

From a systems perspective, agronomic phenotype should be regarded as an emergent property of this multi-layered network rather than as the direct output of a few major genes. For example, the high rebaudioside A content of 'Morita II' is not solely the result of elevated UGT76G1 expression, but also depends on adequate steviol supply, sufficient photosynthate availability, coordinated regulation of upstream pathway genes and a developmental context that favours leaf biomass accumulation [45,55,98].

Similarly, disease susceptibility and stress responses are shaped by the interaction between a limited genetic defence repertoire (Sections 2 and 3), regulatory responsiveness and the plant's overall physiological status. The observed trade-offs between high metabolic specialisation and reduced resilience in clonally propagated stevia illustrate the systemic nature of these constraints [9,13,54].

8.3. Implications for Breeding: From Trait Stacking to System Design

This integrative view has profound implications for breeding strategy. Traditional approaches based on the incremental stacking of individual traits—such as higher Reb A content, larger leaves or delayed flowering—are unlikely to yield stable long-term gains if they do not simultaneously address the underlying system architecture and genetic base of the crop [12,16,25,50].

Instead, breeding should aim at system design, i.e., the construction of genotypes in which:

1. The genomic layer is sufficiently diverse to provide adaptive potential and buffering capacity.
2. The regulatory layer is tuned to ensure robust yet flexible expression of key metabolic pathways.
3. The metabolic layer is optimised for both high flux towards desirable glycosides and efficient use of resources.
4. The physiological layer supports stable biomass production across variable environments.

In practical terms, this means combining classical recombination-based breeding to rebuild diversity (Section 4) with molecular diagnostics and precision interventions (Sections 5 and 7), rather than relying on any single approach in isolation.

8.4. A Conceptual Roadmap from Genotype to Sweetness

In this model, genetic diversity sets the boundaries of what is biologically possible, regulatory networks determine how much of this potential is realised in a given context, and metabolic pathways translate regulatory states into measurable chemical outputs. Environmental factors and management practices modulate each layer, further reinforcing the need for multi-environment and systems-level evaluation.

Figure 1 summarises this integrated framework as a cyclic, systems-level breeding model, in which diversity exploration, regulatory and metabolic interrogation, and field-based multi-environment phenotyping are connected through a Precision Biotechnology Hub. Importantly, chemical profiling (HPLC/UHPLC–MS/MS) and genetic identity control (SSR/SNP) are treated as mandatory release-gating criteria rather than auxiliary analytical steps, ensuring that both chemotype stability and clonal integrity are enforced before large-scale deployment.

Such a framework provides not only a conceptual synthesis of current knowledge, but also a practical guide for future research and breeding efforts. It highlights why the mere optimisation of 'Morita II'-like genotypes is inherently limited and why the long-term sustainability of stevia cultivation depends on a deliberate transition towards genetically diverse, systemically designed cultivars.

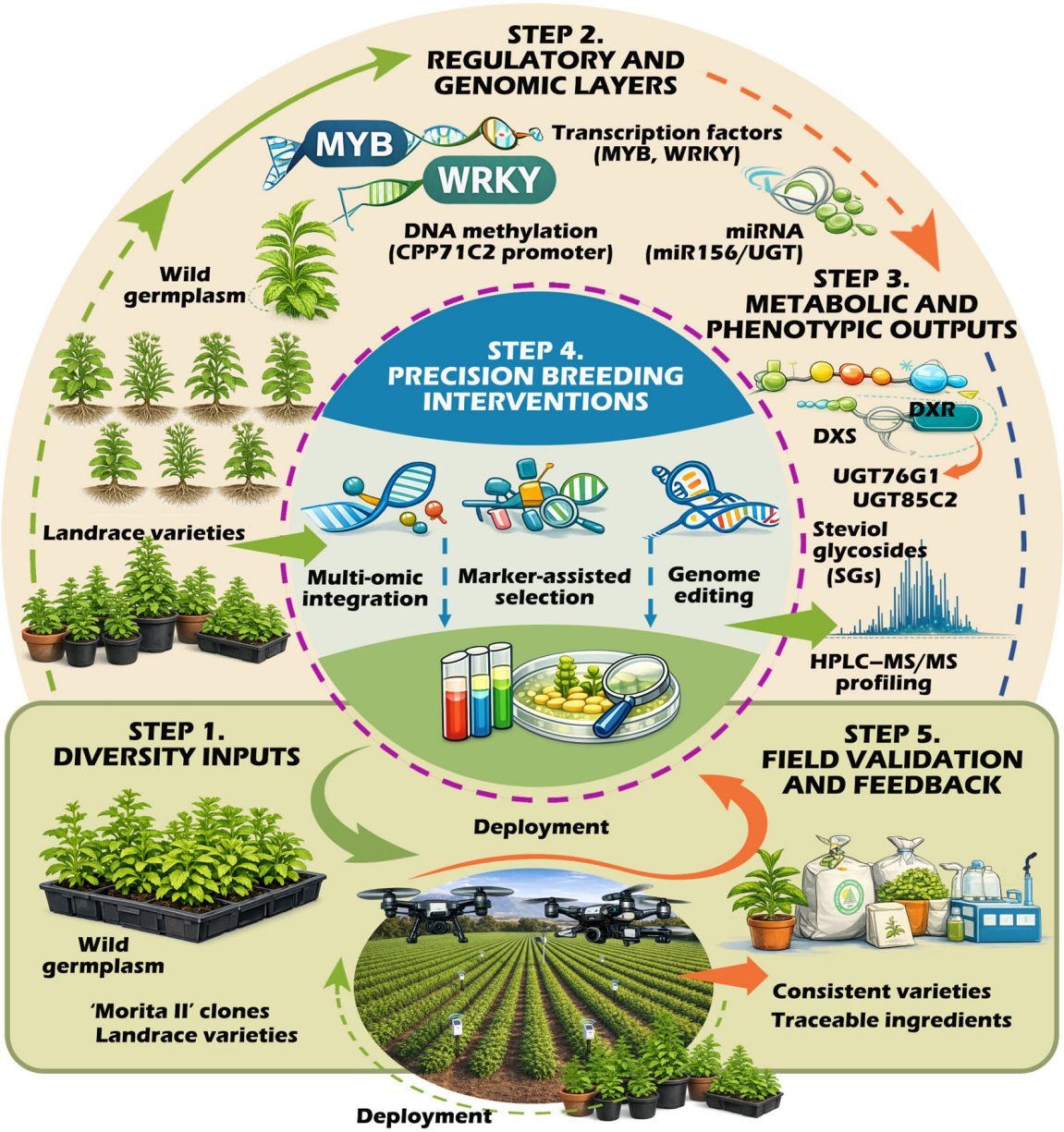

Figure 1. Five-step integrative breeding framework for *Stevia rebaudiana*. The model is organised as a sequential improvement pathway that begins with **Step 1, Diversity Inputs**, which incorporates wild germplasm, landrace varieties, and 'Morita II' clones as the main sources of variation and breeding reference material. **Step 2, Regulatory and Genomic Layers**, summarises the principal molecular determinants shaping trait expression, including transcription factors (e.g., MYB and WRKY), DNA methylation, and miRNA-mediated regulation. **Step 3, Metabolic and Phenotypic Outputs**, represents the translation of this regulatory architecture into pathway activity, steviol glycoside accumulation, and measurable phenotypic outcomes, supported by analytical profiling such as HPLC–MS/MS. **Step 4, Precision Breeding Interventions**, integrates multi-omic analysis, marker-assisted selection, and genome editing as complementary tools for targeted improvement. **Step 5, Field Validation and Feedback**, closes the cycle through deployment, agronomic evaluation, and product-oriented validation, feeding performance information back into subsequent breeding decisions. Together, the framework illustrates how diversity mobilisation, molecular regulation, metabolic phenotyping, and precision interventions can be integrated into an iterative strategy for the development of more resilient, traceable, and high-quality stevia cultivars. Solid arrows indicate the main directional flow of the framework, whereas dashed arrows and dashed outlines denote iterative, feedback, or conceptual relationships among modules. Colour differences are used to distinguish the major functional components of the model.

9. Future Perspectives and Research Gaps

Despite the remarkable progress achieved in the understanding of *Stevia rebaudiana* biology, metabolism and improvement over the last two decades, the crop remains in a paradoxical situation: it is globally commercialised, yet scientifically under-domesticated and genetically under-explored. Several critical knowledge gaps and strategic challenges must be addressed to move from a system based on clonal optimisation to one founded on evolutionary resilience and predictive breeding.

9.1. Rebuilding the Genetic Base: From Germplasm Exploration to Pre-Breeding

Perhaps the most urgent and structural challenge is the systematic reconstruction of the stevia genetic base. Although wild populations in Paraguay and adjacent regions have been repeatedly identified as reservoirs of untapped diversity, their representation in breeding programmes remains marginal [31,49,99,100]. Comprehensive and coordinated germplasm exploration, collection, conservation and characterisation efforts are still lacking at a global scale.

Future research should prioritise the development of core collections capturing the maximum allelic and phenotypic diversity with a minimum number of accessions, followed by pre-breeding programmes aimed at introgressing this diversity into agronomically usable genetic backgrounds. Such programmes are standard in major crops but are still in their infancy in stevia. Without this foundational step, all downstream molecular and biotechnological tools will continue to operate on a severely constrained genetic substrate.

9.2. From Single Reference Genomes to a Stevia Pangenome

The recent availability of high-quality reference genomes represents a major milestone [11,43,52], but it also exposes a new limitation: a single reference genome cannot capture the full spectrum of structural and presence–absence variation in a species with strong domestication bottlenecks and geographically structured wild populations [101,102].

A major unresolved question is how much of the *S. rebaudiana* pangenome is currently represented in wild collections, landraces and breeding materials, and how much functionally relevant variation remains uncaptured. At present, this cannot be quantified robustly because broad multi-line pangenomic resources for stevia are still emerging. Defining the fraction of structural and dispensable genomic diversity already conserved—and identifying what is still missing—should therefore be considered a strategic priority for future germplasm management and breeding design.

The construction of a stevia pangenome, incorporating multiple wild, landrace and elite genomes, would allow the identification of core and dispensable genes, structural variants and lineage-specific adaptations. Such information is essential to understand the genetic basis of metabolic diversity, stress tolerance and disease resistance, and to design informed strategies for allele mining and targeted introgression.

9.3. Dissecting Complex Traits: Beyond the Steviol Glycoside Pathway

To date, research has focused disproportionately on the steviol glycoside biosynthetic pathway, for understandable economic reasons. Although this limitation is not unique to stevia and is shared by many crop species, especially those relying on narrow breeding bases or strong market-driven trait prioritisation, *S. rebaudiana* represents a particularly illustrative case in which metabolic specialisation and industrial standardisation have advanced faster than genetic diversification. However, long-term crop sustainability depends on a much broader set of traits, including biomass accumulation, phenological stability, root system architecture, nutrient use efficiency and multi-stress tolerance.

These traits are inherently polygenic and strongly influenced by genotype × environment interactions. Their genetic dissection will require the implementation of multi-environment trials, high-throughput phenotyping platforms and integrative statistical frameworks such as GWAS and genomic selection. Without this shift, breeding efforts will remain largely confined to a narrow metabolic niche.

9.4. Precision Breeding in Practice: Technical and Regulatory Challenges

Genome editing and gene activation technologies offer unprecedented opportunities for precise manipulation of endogenous genes [44,103], but several practical challenges remain. On the technical side, efficient and genotype-independent transformation and regeneration systems are still needed for a wide range of stevia genetic backgrounds [15,104–106]. On the regulatory side, the acceptance and legal status of gene-edited crops vary widely among countries and remain in flux [103,107].

Future research should therefore pursue parallel tracks: (i) the development of robust, transgene-free editing and delivery systems, and (ii) the alignment of breeding strategies with regulatory and societal expectations, including transparency, traceability and risk assessment.

9.5. Integrating Omics, Environment and Management: Towards Predictive Agriculture

A final and overarching challenge is the integration of genetic and molecular knowledge with environmental and agronomic dimensions. Steviol glycoside accumulation, biomass yield and disease dynamics are all strongly modulated by light, temperature, water and nutrient availability [46,108,109]. Yet most genetic studies are still conducted under highly controlled or limited conditions [109–111].

The future of stevia improvement lies in predictive, systems-based agriculture, where genomic, transcriptomic and metabolomic data are combined with environmental covariates and crop models to forecast performance across diverse scenarios. From a sustainability perspective, this integration is relevant not only for improving prediction accuracy, but also for supporting the development of cultivar portfolios and management strategies that are more resilient, resource-efficient and less vulnerable to disease outbreaks and environmental instability. In this sense, predictive stevia breeding should be understood as part of a broader transition towards sustainable agriculture, where genetic diversification, improved input-use efficiency and more context-specific decision-making become central goals rather than secondary outcomes. Achieving this will require interdisciplinary collaboration between geneticists, physiologists, pathologists, agronomists and data scientists.

10. Conclusions

Stevia rebaudiana has transitioned in only a few decades from a locally used ethnobotanical resource to a globally strategic crop for the production of natural, non-caloric sweeteners. The reduced variability observed in cultivated stevia is, to some extent, an expected consequence of recent domestication combined with prolonged clonal propagation. However, in *S. rebaudiana* this narrowing appears particularly consequential because commercial production has become highly concentrated around a very limited number of elite genotypes, especially ‘Morita II’, thereby constraining adaptive breadth, long-term breeding flexibility and system-level resilience. In this context, genetic uniformity has supported industrial standardisation, but has also intensified concern over phytosanitary vulnerability and the limited long-term scope for diversification-based breeding progress.

By integrating evidence from population genetics, molecular biology, biochemistry, physiology and breeding science, we argue that the central challenge facing stevia improvement is not primarily metabolic, but genetic and systemic. The steviol glycoside pathway

is now comparatively well understood, and powerful analytical and molecular tools are available to fine-tune glycoside profiles. Yet, without a deliberate effort to rebuild and restructure the genetic foundation of the crop, these tools will continue to operate within a severely constrained design space.

A sustainable future for stevia therefore requires a paradigmatic shift: from the incremental optimisation of a few elite clones towards the construction of genetically diverse, resilient and evolutionarily robust breeding populations. Such a shift, however, also entails the classical risk of genetic segregation and the partial disruption of favourable elite trait combinations, especially when sweetener quality, agronomic performance and adaptation must be improved simultaneously. For this reason, recombination-based breeding in stevia should be accompanied by careful recurrent selection, robust phenotyping and, where possible, marker-supported recovery and clonal fixation of superior recombinants. This implies the strategic mobilisation of wild germplasm, the establishment of pre-breeding pipelines, and the integration of classical recombination-based improvement with modern molecular diagnostics, omics platforms and precision biotechnology.

In this framework, genome editing, gene activation and metabolic engineering should be viewed not as isolated technological solutions, but as components of a broader system-design approach aimed at reshaping regulatory networks and metabolic fluxes within a diversified genetic landscape. Similarly, micropropagation and clonal deployment will remain indispensable for rapid dissemination, but must serve a dynamic and pluralistic cultivar portfolio rather than perpetuate genetic monoculture.

Ultimately, the long-term success of *Stevia rebaudiana* as a strategic crop will depend on the ability of the scientific and breeding communities to move beyond the "Morita II paradigm" and to embrace a truly integrative vision of crop improvement—one that combines genetic diversity, system-level understanding and precision intervention into a coherent and forward-looking strategy. In doing so, stevia can evolve from a metabolically specialised yet genetically fragile crop into a resilient, adaptable and scientifically mature agricultural system capable of meeting the demands of global health, sustainability and food security.

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Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Meaning
SGs	Steviol glycosides
Reb A	Rebaudioside A
MEP	Methylerythritol phosphate (pathway)
GGPP	Geranylgeranyl diphosphate
CPS	ent-Copalyl diphosphate synthase
KS	ent-Kaurene synthase
KO	ent-Kaurene oxidase
KAH	ent-Kaurenoic acid hydroxylase
UGT	UDP-glycosyltransferase
UGT74G1	UDP-glycosyltransferase 74G1
UGT76G1	UDP-glycosyltransferase 76G1
HPLC	High-performance liquid chromatography
UHPLC–MS/MS	Ultra-high-performance liquid chromatography–tandem mass spectrometry
AUDPC	Area under the disease progress curve
SSR	Simple sequence repeat
SNP	Single-nucleotide polymorphism
GWAS	Genome-wide association study
MAS	Marker-assisted selection
CRISPR	Clustered regularly interspaced short palindromic repeats

Appendix A. Steviol Glycoside Biosynthetic Pathway and Enzymatic Architecture

Appendix A.1. Enzymatic Components of the Steviol Glycoside Biosynthetic Pathway in Stevia rebaudiana

The steviol glycoside biosynthetic pathway integrates the plastidial MEP pathway, diterpene backbone biosynthesis, cytochrome P450-mediated oxidation steps and a set of cytosolic UDP-glycosyltransferases responsible for the structural diversification of steviol glycosides. Table A1 summarises the main enzymes involved, their corresponding genes, subcellular localisation and biological functions, providing a consolidated reference framework complementary to the pathway overview discussed in the main text.

Table A1. Main enzymes involved in steviol glycoside biosynthesis in *Stevia rebaudiana*.

Pathway Stage	Enzyme	Gene (Common Name)	Subcellular Localisation	Main Function in the Pathway	Key Role in SG Profile
MEP pathway	1-Deoxy-D-xylulose-5-phosphate synthase	SrDXS	Plastid	Initiates MEP pathway; controls carbon flux into isoprenoid biosynthesis	Controls precursor supply
MEP pathway	1-Deoxy-D-xylulose-5-phosphate reductoisomerase	SrDXR	Plastid	Second committed step of MEP pathway	Controls precursor supply
Diterpene core	ent-Copalyl diphosphate synthase	SrCPS	Plastid	Converts GGPP into ent-copalyl diphosphate	Commits flux to diterpene pathway
Diterpene core	ent-Kaurene synthase	SrKS	Plastid	Converts ent-copalyl diphosphate into ent-kaurene	Steviol backbone formation
Oxidation steps	ent-Kaurene oxidase	SrKO	Endoplasmic reticulum	Oxidises ent-kaurene to ent-kaurenoic acid	Required for steviol synthesis
Oxidation steps	ent-Kaurenoic acid hydroxylase	SrKAH	Endoplasmic reticulum	Converts ent-kaurenoic acid to steviol	Controls steviol availability

Table A1. *Cont.*

Pathway Stage	Enzyme	Gene (Common Name)	Subcellular Localisation	Main Function in the Pathway	Key Role in SG Profile
Glycosylation	UDP-glycosyltransferase	SrUGT85C2	Cytosol	First glycosylation at C13 of steviol	Initiates SG biosynthesis
Glycosylation	UDP-glycosyltransferase	SrUGT74G1	Cytosol	Adds second glucose moiety to steviol glycosides	Determines stevioside accumulation
Glycosylation	UDP-glycosyltransferase	SrUGT76G1	Cytosol	Converts stevioside into rebaudioside A	Key determinant of Reb A content

Appendix A.2. Functional Organisation and Regulatory Significance of the Pathway

The organisation of the steviol glycoside pathway reflects a hierarchical control structure. Upstream enzymes of the MEP and diterpene core pathway (DXS, DXR, CPS, KS) primarily regulate carbon flux and the total capacity for steviol production. The cytochrome P450 monooxygenases (KO and KAH) represent critical control points determining the availability of the steviol aglycone. Finally, the UDP-glycosyltransferases (UGTs) define the qualitative composition of the steviol glycoside pool by directing glycosylation patterns.

Among these, SrUGT76G1 plays a pivotal role in shaping the industrial sweetener profile by catalysing the conversion of stevioside into rebaudioside A, explaining the characteristic phenotype of elite cultivars such as 'Morita II'. Conversely, modulation of upstream enzymes such as SrKAH has been shown to significantly affect total steviol glycoside accumulation, highlighting the multi-level regulatory architecture of the pathway.

Appendix A.3. Use of the Pathway as a Breeding and Biotechnological Target

The enzymes listed in Table A1 constitute the primary molecular targets for:

i. functional marker development in marker-assisted selection,
ii. candidate gene approaches in association studies, and
iii. metabolic engineering and genome editing strategies aimed at fine-tuning both total steviol glycoside content and glycoside composition.

This consolidated enzymatic framework provides the biochemical and molecular foundation for the integrative breeding and precision biotechnology strategies discussed throughout the main text.

Appendix B. Molecular and Genomic Tools for Genetic Diversity Analysis and Breeding in *Stevia rebaudiana*

Appendix B.1. Molecular Marker Systems Applied in Stevia rebaudiana

Genetic diversity analysis and germplasm characterisation in *Stevia rebaudiana* have relied on a progressive succession of molecular marker systems, reflecting both the historical development of the field and the increasing resolution of available genomic tools. Table A2 provides a comparative overview of the main marker systems used, their typical applications and their relative analytical power.

Table A2. Molecular marker systems used in *S. rebaudiana* for diversity analysis, germplasm characterisation and breeding.

Marker System	Type of Marker	Main Applications	Resolution Level	Typical Use in Stevia	Strategic Value
RAPD	Dominant	Rapid diversity screening	Low	Early surveys of variability	Historical baseline, low cost
ISSR	Dominant	Population structure analysis	Medium	Cultivated vs. wild comparisons	Robust, simple implementation
AFLP	Dominant	High-density fingerprinting	Medium–high	Fine-scale diversity analysis	High multiplexing
SCoT	Dominant	Gene-targeted diversity analysis	Medium	Functional diversity surveys	Targets coding regions
SSR/EST-SSR	Co-dominant	Germplasm characterisation, identity control	High	Core collections, QC in nurseries	Reference markers in stevia
SNP/GBS	Co-dominant	GWAS, genomic studies	Very high	Genome-wide diversity and structure	Foundation for modern breeding

Appendix B.2. Functional Markers and Candidate Gene Approaches

Beyond neutral markers, increasing emphasis has been placed on the development of functional or gene-targeted markers associated with key components of the steviol glycoside biosynthetic pathway and with agronomically relevant traits. Polymorphisms in genes such as UGT76G1, UGT74G1 and SrKAH have been shown to correlate with variation in glycoside composition and total steviol glycoside content, making them suitable candidates for marker-assisted selection.

Such functional markers enable a shift from purely descriptive diversity analysis towards predictive breeding tools, in which seedlings or in vitro regenerants can be preselected for desirable metabolic profiles before extensive phenotypic evaluation. Although still at an early stage in Stevia rebaudiana, this approach represents a critical bridge between genomics and practical breeding.

Appendix B.3. From Classical Genotyping to Genome-Wide Approaches

The recent availability of high-quality reference genomes and long-read assemblies has opened the door to high-density, genome-wide analyses in stevia. Reduced-representation sequencing approaches such as genotyping-by-sequencing (GBS) and SNP arrays now make it possible to:

i. resolve fine-scale population structure,
ii. identify genomic regions affected by domestication and clonal selection bottlenecks, and
iii. perform genome-wide association studies (GWAS) for complex traits such as biomass yield, glycoside composition and disease tolerance.

In the medium term, these resources are expected to enable the implementation of genomic selection frameworks, in which breeding values are predicted from genome-wide marker profiles rather than from individual loci, marking a transition from marker-assisted to truly genome-enabled breeding in *Stevia rebaudiana*.

Appendix B.4. Strategic Role of Molecular Tools in Rebuilding the Stevia Genetic Base

From a strategic perspective, molecular and genomic tools are indispensable for managing the trade-off between clonal uniformity and genetic diversification. They provide the means to:

- Monitor and control genetic identity in clonal propagation systems,

- Quantify and manage genetic diversity in breeding populations,
- Track the introgression of wild alleles into elite backgrounds, and
- Support the rational design of genetically diverse yet industrially consistent cultivar portfolios.

In this sense, molecular tools should be viewed not merely as analytical instruments, but as core components of an integrated, diversity-oriented improvement strategy for *Stevia rebaudiana*.

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