DOI: 10.5281/zenodo.10606764

Mini review

Unveiling the evolution of iridoid biosynthesis in the genus *Nepeta*: a mini review

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Accepted: 1 September 2023

Summary. The genus *Nepeta*, belonging to the Lamiaceae family, encompasses a diverse group of plants with significant biological activities attributed mainly to their iridoid compounds. This review provides a comprehensive analysis of recent research on iridoid biosynthesis, regulation, and evolutionary aspects within the *Nepeta* genus. The biological activities of *Nepeta* species, including repellent, phytotoxic, antimicrobial, and cytotoxic effects, have been extensively investigated, highlighting the potential applications of iridoids. Over the past decade, significant progress has been made in elucidating the molecular basis of iridoid biosynthesis and regulation, thanks to advancements in transcriptomics, genomics, and metabolomics. The presence of distinct chemotype groups within *Nepeta* has been revealed, characterized by their ability to produce both iridoid aglycones (nepetalactones) and glycosylated iridoids (IAs and IGs), exclusively produce IGs, or lack iridoids. The identification of key enzymes involved in iridoid biosynthesis, such as geraniol synthase (GES) and iridoid synthase (ISY), has played a crucial role in understanding the pathway. Furthermore, the evolutionary aspects of the iridoid biosynthesis loss in some of the *Nepeta* taxa, and the association of iridoid presence and content with the expression levels of specific genes, have been investigated. However, several areas remain to be explored, including the final steps of iridoid aglycones biosynthetic branch, the production of iridoid glucosides, the role of transcription factors in fine-tuning of iridoid biosynthesis, and the intricate interplay between biosynthetic enzymes. Continued research in these areas will deepen our understanding of iridoid metabolism in *Nepeta* and unlock their full potential in various fields, including pharmaceuticals, agriculture, and natural product-based industries.

Keywords: iridoid biosynthesis, iridoid synthase, *Nepeta*, nepetalactone.

INTRODUCTION

The Lamiaceae, commonly known as the mint family or Labiatae, is a cosmopolitan family of flowering plants with a substantial global presence. With approximately 236 genera and an estimated species count ranging from 6900 to 7200, the Lamiaceae family represents a rich and diverse group. Among this family, notable genera such as *Salvia*, *Scutellaria*, *Stachys*, *Plectranthus*, *Hyptis*, *Teucrium*, *Vitex*, *Thymus*, and *Nepeta* contribute to its prominence (Tamokou et al. 2017). As the sixth largest family of angiosperms, the Lamiaceae hold significant cultural and economic value for humans (Harley et al. 2004).

A remarkable attribute of the Lamiaceae family is its extensive assortment of specialized metabolites, encompassing monoterpenes predominated by iridoids, and sesquiterpenes. Iridoids are noncanonical monoterpenes characterized by a cyclopentane or cyclopentene ring fused to a cyclohexane ring. In plants, they are primarily synthesized in glycosylated form (e.g., aucubin), while the volatile aglycones (e.g., nepetalactones) are a rare occurrence. Furthermore, iridoids exhibit remarkable structural diversity, with variations in

Published online: 5 February 2024
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the number and position of functional groups, such as hydroxyl, carbonyl, and methyl groups (Yamane et al. 2010; Ludwiczuk et al. 2017). These compounds play pivotal roles in nature, performing functions such as antimicrobial activities, defense mechanisms against herbivores, and attraction of pollinators. In addition to their ecological significance, these compounds also hold substantial value for human well-being, serving as resources for health-related applications, food production, and agricultural practices.

The complete elucidation of the iridoid biosynthetic pathway remains an ongoing scientific pursuit, with multiple facets that require further exploration. A comprehensive understanding of the intricate evolutionary and biochemical processes governing iridoid biosynthesis is essential to unlock their industrial potential. Notably, within the Lamiaceae family, specific species demonstrate remarkable proficiency in iridoid production, making this family an excellent candidate for evolutionary-based investigations aimed at unraveling the underlying mechanisms of iridoid biosynthesis. While a large number of Lamiaceae subfamilies do not produce iridoids, notable exceptions include Ajugadaceae and Lamioideae, which are rich in iridoids. Interestingly, within one of the largest clades of Lamiaceae, Nepetoideae, iridoid production has been lost; however, iridoid biosynthesis has been re-established in the genus *Nepeta* (Duplais et al. 2020).

Within this mini review, we are focusing on recent advances in the elucidation of iridoid biosynthetic pathways and its regulation within the genus *Nepeta*. We will explore the phytochemical and bioactivity characteristics of this genus, as well as the evolution and discovery of key enzymes of iridoid biosynthesis.

GENUS *NEPETA* – PHYTOCHEMICAL CHARACTERISTICS AND BIOLOGICAL ACTIVITIES

The genus *Nepeta*, belonging to the subfamily Nepetoidae and the tribe Nepetae (Takhtajan 2009), is one of the largest genera within the Lamiaceae family. Its name is derived from the ancient name of Nepi, a city located in present-day Italy (Etruria) – Nepet or Nepete (Marin and Tatić 2004). With over three hundred known species, *Nepeta* is primarily composed of herbaceous perennials, although some species are annuals. The distribution of *Nepeta* species encompasses a wide range, including Central and Southern Europe, the Middle East, Central and Southern Asia, and parts of Africa (Formisano et al. 2011). The highest diversity and abundance of *Nepeta* species are found in two main regions. The first is Southwest Asia, with particular presence in Turkey and Iran. The second area includes the western Himalayas, including the Hindu Kush (Formisano et al. 2011). Iran, in particular, serves as the center of origin for 75 species within the *Nepeta* genus, with 54% of them being endemic to the region (Jamzad et al. 2003). These regions exhibit a rich botanical heritage and play a significant role in the overall diversity and distribution of *Nepeta* species.

Over the past few decades, extensive research has been conducted on the diverse biological activities of *Nepeta* species. The essential oils derived from species such as *N. cataria*, *N. faassenii, N. rtanjensis*, and *N. parnassica* have demonstrated repellent properties against various harmful insects, including cockroaches (*Blattella germanica, Periplaneta americana)*, flies (*Musca domestica*), fleas, and mosquitoes (*Culex* spp.) (Peterson et al. 2002; Schultz et al. 2004; Amer and Mehlhorn 2006; Ghaninia et al. 2008; Zhu et al. 2009; Birkett et al. 2011; Gkinis et al. 2014; Sparks et al. 2017; Reichert et al. 2019). Additionally, several *Nepeta* species, such as *N. nuda, N. faassenii, N. curviflora, N. juncea,* and *N. rtanjensis,* have demonstrated phytotoxic effects against well-known weed species (Formisano et al. 2011; Dmitrović et al. 2015; Kordali et al. 2015; Nestorović Živković et al. 2016; Dragoeva et al. 2017; Shekari et al. 2022). Moreover, a significant number of *Nepeta* species have exhibited potent antimicrobial activity against respiratory, digestive, and skin infections, as well as foodborne pathogens (Formisano et al. 2011; Salehi et al. 2018; Aničić et al. 2021; Sharma et al. 2021; Amighi et al. 2023). Notably, essential oils derived from *N. glomerata* (Rigano et al. 2011), *N. rtanjensis* (Skorić et al. 2017), and *N. mahanesis* (Amirzadeh et al. 2022) have demonstrated remarkable cytotoxic effects against various types of human cancer cells.

Nepeta species exhibit a wide range of biological activities attributed to iridoids, including aglycones (nepetalactones) and glucosylated iridoids with unique stereochemistry found exclusively in this plant group. Through a comprehensive literature survey, distinct taxonomic groups within the *Nepeta* genus have been identified: 1) taxa producing both IAs and IGs (chemotype A), 2) taxa exclusively producing IGs (chemotype B), and 3) taxa lacking iridoids (chemotype C). It should be noted that reports of taxa producing only IAs (chemotype D) should be interpreted cautiously as their IG content has not been extensively analyzed (Formisano et al. 2011; Süntar et al. 2018).

Nepetalactones (NL), highly volatile compounds known for their hallucinogenic effect on the cat family (Felidae), are dominant in the synthesis of most *Nepeta* species (chemotype A). They can exist as four different diastereoisomers with a 7*S* configuration (Liblikas et al. 2005), with only one being predominantly produced. Nepetalactone serves as a valuable chemotaxonomic marker for *Nepeta* species (Mišić et al. 2015). Based on the classification by Liblikas et al. (2005), the diastereoisomers include *cis,trans*-NL (4aα,7α,7aα), *trans,cis*-NL (4aα,7α,7aβ), *cis,cis*-NL (4aβ,7α,7aβ), and *trans,trans*-NL

(4aβ,7α,7aα). The first part of the name refers to the 4a-7a configuration at the ring junction, while the second part denotes the 7a-7 bond configuration (Liblikas et al. 2005). Notably, the R configuration at the 7 C-atom has only been found in *Nepeta elliptica* (Bottini et al. 1987). In nature, nepetalactone can exist in the form of eight different stereoisomers, including four diastereoisomers and their enantiomers, exhibiting diverse biological activities dependent on subtle differences in hydrogen bonding (Formisano et al. 2011; Mišić et al. 2015).

IRIDOID BIOSYNTHETIC PATHWAY IN THE GENUS *NEPETA*

In the past decade, significant breakthroughs have occurred in our understanding of the molecular basis of iridoid biosynthesis and regulation in the *Nepeta* genus, thanks to advancements in transcriptomic, genomic, and metabolomic techniques. Regarding the initial steps of iridoid biosynthesis, it is reasonable to assume that they are similar to the basic iridoid pathway, which has been extensively studied in *Catharanthus roseus*, a species known for producing the iridoid glucoside secologanin and its terpenoid indole alkaloid derivatives. Experiments using labeled [1-^{13}C]-glucose in *C. roseus* cell cultures and ^{13}C-NMR analysis demonstrated that carbon isotopes incorporated into secologanin originate from the methylerythritol phosphate (MEP) biosynthetic pathway (Contin et al. 1998). The enzyme geranyl diphosphate synthase (GPPS), responsible for the condensation of dimethylallyl pyrophosphate (DMAPP) and isopentenyl diphosphate (IPP) to form the C10 monoterpene base, has been immunolocalized in the leaf plastids of *Arabidopsis thaliana* and *C. roseus* (Bouvier et al. 2000; Thabet et al. 2012). Considering these findings and the knowledge gained from other iridoid-rich species, it can be assumed that plastidial IPP, produced via the MEP pathway, serves as the initial biogenic precursor for nepetalactone biosynthesis.

In the subsequent step of the biosynthetic pathway, geraniol synthase (GES) catalyzes the removal of the pyrophosphate group from geranyl diphosphate (GPP) (Iijima et al. 2004). GES was first isolated from the glandular trichomes of basil (*Ocimum basilicum*) and subsequently characterized in other Lamiaceae species, including *Perilla citriodora*, *P. frutescens*, and *C. roseus* (Iijima et al. 2004; Ito et al. 2007; Simkin et al. 2013). GES exhibits a catalytic mechanism similar to other terpene synthases, involving the addition of a hydroxyl group to the intermediate carbocation rather than hydrolysis of the phosphoester bond (Iijima et al. 2004).

Following GES activity, geraniol is hydroxylated at the C10 position by geraniol-10-hydroxylase/geraniol-8-oxidase (G10H/G8O), an enzyme belonging to the cytochrome P450 monooxygenase superfamily (Collu et al. 2001). G8O represents a unique example of an endoplasmic reticulum-bound cytochrome P450 enzyme, which has been successfully isolated and purified, leading to the partial identification of its amino acid sequence (Collu et al. 2001). The purification of G8O was accomplished using a cell culture of *C. roseus,* and it was observed that the expression of the gene encoding this enzyme was enhanced upon the addition of the plant hormone methyl jasmonate to the substrate (Collu et al. 2001). This enzyme has been purified from *Nepeta racemosa* leaves and is localized in the glandular trichomes on the leaf surface (Hallahan et al. 1998).

The enzyme 8-hydroxygeraniol oxidoreductase (8HGO) plays a crucial role in iridoid biosynthesis by catalyzing the oxidation of 8-hydroxygeraniol, an acyclic monoterpene alcohol, to form 8-oxogeranial, the common precursor of all iridoids. Initially, 8HGO was isolated from suspension cultures of *Rauvolfia serpentina* (Apocynaceae) (Ikeda et al. 1991). Subsequently, an enzyme with similar functionality was identified in *N. racemosa*, which was shown to utilize geraniol, nerol, citronellol, and their hydroxylated derivatives as substrates (Hallahan et al. 1995). The presence of 8HGO activity in the leaves of *N. racemosa* further supported the hypothesis of nepetalactone synthesis via the general iridoid pathway. Additionally, 8HGO has been identified in *C. roseus* (Miettinen et al. 2014; Krithika et al. 2015). The enzymatic activity of 8HGO leads to the formation of 8-oxogeranial, a dialdehyde monoterpene that serves as a substrate for the synthesis of cyclic monoterpenoid iridodiols.

The subsequent step in iridoid biosynthesis is catalyzed by iridoid synthase (ISY), an enzyme first isolated from *C. roseus*. ISY converts 8-oxogeranial to *cis*,*trans*-nepetalactol and *cis*,*trans*-iridoidial, which exist in equilibrium (Geu-Flores et al. 2012). The gene encoding CrISY was identified in the transcriptomic database of *C. roseus* based on expression pattern similarities with other genes involved in secologanin biosynthesis (Geu-Flores et al. 2012). Sequence analysis of ISY revealed a significant resemblance to known progesterone-5β-reductases, which belong to the short-chain reductase family. Furthermore, ISY activity is dependent on the cofactor NADPH, further confirming its classification as a short-chain reductase. In addition to *C. roseus*, iridoid synthase has been identified in other iridoid-producing species such as *Olea europea*, *Antirrhinum majus*, *Swertia mussotti*, *Plantago major*, *Vaccinium corymbosum,* as well as in *Nepeta cataria* and *N. mussinii* (Alagna et al. 2016; Kries et al. 2017; Sherden et al. 2017; Xiang et al. 2017; Fellows et al. 2018; Lawas et al. 2023).

Recent discoveries have shown that in *Nepeta* species, the reduction and cyclization of 8-oxogeranial are two separate reactions, with iridoid synthases solely mediating

its reduction (Lichman et al. 2019a, 2019b). Following reduction, the acyclic reactive enolate is not catalyzed by ISY for cyclization but is released as a reaction product. These findings suggest that, under *in vitro* conditions, spontaneous cyclization occurs in the solvent with the buffer serving as an acid catalyst (Lichman et al. 2019a). The final step in nepetalactone synthesis is mediated by an NAD-dependent enzyme specific to the *Nepeta* genus, called nepetalactol-related short-chain dehydrogenase/reductase (NEPS) (Lichman et al. 2019b). Among the known NEPS enzymes, NEPS1 is the best-characterized one. The NEPSs and latex protein-like genes (MLPL) act in combination to control the formation of nepetalactone stereoisomers (*cis,cis*-, *cis,trans*-, *trans,cis*- and *trans,trans*-) (Lichman et al. 2019a, 2019b). However, the enzymes responsible for the formation of dehydronepetalactone, other nepetalactone derivatives, as well as the subsequent steps of the iridoid glucosides branch are still unknown, representing an intriguing area for future research.

Apart from studies with biochemical assays of enzymes involved in the biosynthesis of iridoids, their functional roles *in planta* have not been fully validated. Recent progress has been made in a study that employed virus-induced gene silencing (VIGS) to provide evidence that the biosynthetic enzymes GES, ISY, and MLPL play a crucial role in nepetalactone biosynthesis in *N. cataria* (Palmer et al. 2022).

A comprehensive study by the Mint Evolutionary Genomics Consortium (2018) investigated the evolution of iridoid biosynthesis within the Lamiaceae family. Analysis of gene expression levels of orthogroups encoding enzymes involved in the iridoid biosynthetic pathway revealed two distinct expression clusters: Group I, consisting primarily of non-iridoid-producing species, and Group II, consisting primarily of iridoid-producing species. The presence or absence of iridoids was found to be significantly associated with the expression levels of genes known to be involved in iridoid biosynthesis, particularly GES and ISY. The pivotal roles of GES and ISY in determining the presence or absence of iridoids within the *Nepeta* genus was recently confirmed (Aničić et al. 2023).

COMPARATIVE IRIDOID PROFILING AND CO-EXPRESSION ANALYSES IN *NEPETA* TAXA: EXPLORING REGULATORY MECHANISMS OF IRIDOID BIOSYNTHESIS

In systems biology, the assumption holds true that genes involved in the same biological process, i.e. the biosynthetic pathway of a specific specialized metabolite, are co-regulated and co-expressed through a common regulatory system. This exemplifies the principle of "guilt-by-association", which suggests that genes within the same pathway exhibit similar expression patterns in response to specific environmental conditions (Wisecaver et al. 2017; Zhang et al. 2018). Based on this principle, gene co-expression correlations have frequently been utilized to identify unknown genes related to specialized metabolism in plants, leveraging transcriptomic data and expression profiling techniques. Subsequently, reverse genetic and biochemical experiments are typically conducted to validate the functions of these candidate genes (Zhang et al. 2018). This approach has successfully revealed regulatory mechanisms and structural genes in various species, including *Arabidopsis thaliana*, *Cannabis sativa*, *Papaver somniferum*, *Solanum lycopersicum*, and *C. roseus* (Higashi et al. 2013; Zhang et al. 2018).

Following this principle, a comparative iridoid profiling and co-expression analysis of iridoid-related biosynthetic genes and transcription factors (TFs) were conducted in chemodiverse *Nepeta* taxa. The analyses were focused on the plants' exposure to environmental stresses (e.g., dehydration, pathogens) or elicitors (MeJA) to elucidate regulatory mechanisms and key genes determining plant productivity and factors influencing the presence/absence of iridoids or specific groups of iridoids (IAs and IGs) in these plants.

Two chemically diverse *Nepeta* species, *N. rtanjensis* Diklić & Milojević (rich in *trans*,*cis*-NL and DNL) and *N. argolica* Bory & Chaub. subsp. *argolica* (predominantly producing *cis*,*trans*-NL), were subjected to PEG-induced dehydration stress in an experimental setting under *in vitro* conditions (Aničić et al. 2020). Concurrently with the initial decrease in NL content, a reduction in transcript levels of the majority of 10 NL-related biosynthetic genes and some of the 5 transcription factors (TFs) was observed. Both species employed similar strategies in response to severe dehydration stress, with *N. rtanjensis* demonstrating greater efficiency in maintaining NL levels in tissues. The results suggest a coordinated regulation of NL biosynthesis at the gene expression level, with the trichome-enriched MYC2 and YABBY5 TFs identified as potential positive regulators. Manipulating the expression of these TFs could effectively influence the NL biosynthetic pathway, increasing the production of *cis*,*trans*-NL in *N. argolica* ssp. *argolica* and *trans*,*cis*-NL in *N. rtanjensis*. Furthermore, the elevated expression of the analyzed genes in glandular trichomes, the main sites of nepetalactone accumulation in *Nepeta*, provides further confirmation of their function (Aničić et al. 2018, 2020).

To explore the relationship between iridoid content and gene expression, we employed MeJA as a potent elicitor of specialized metabolites in *N. rtanjensis* and *N. nervosa* (non-iridoid producing species, chemotype C) (Aničić et al. 2023). MeJA treatment significantly increased major iridoid content in *N. rtanjensis*, with the most substantial increase observed for DNL, followed by *t*,*c*-NL and 1,5,9-*e*DLA. The expression

of targeted biosynthetic genes (BGs) and transcription factors (TFs) peaked 24 hours after MeJA application, gradually decreasing thereafter. Nevertheless, the overall expression levels of most BGs and TFs remained higher in MeJA-treated plants even after 72 hours. Pearson's correlation analysis revealed positive correlations among the majority of analyzed BGs and TFs, with the exception of *NrISY1* and *NrCOI1*, which exhibited negative correlations with other genes. These co-expression patterns suggest the involvement of analyzed *N. rtanjensis* genes in the same biosynthetic pathway and their transcriptional regulation. In contrast, MeJA treatment did not induce iridoid accumulation in leaves of iridoid non-producing *N. nervosa.* However, it did induce the expression of most analyzed BGs at 24 and 72 hours. Pearson correlation analysis demonstrated positive correlations among all analyzed BGs and TFs, indicating their transcriptional regulation. Interestingly, putative *NnGES* transcripts were not detected, implying a significant constraint in iridoid biosynthesis for this species.

Biotic stresses have been recognized for their role in elevating iridoid production in plants, contributing to plant protection against insects and pests. In our unpublished study, *N. sibirica* plants were treated with two *Trichoderma* strains, specifically *T. viride* and *T. harzianum*. This induced heightened levels of iridoids in *N. sibirica* leaves, and coordinated over-expression of a majority of biosynthetic pathway genes and TFs.

FUNCTIONAL CHARACTERIZATION OF IRIDOID SYNTHESES IN IRIDOID-PRODUCING AND NON-PRODUCING *NEPETA* SPECIES

Iridoid synthases (ISY) belong to atypical-extended short chain dehydrogenases (SDR) (Qin et al. 2016). These enzymes are functionally and structurally overlapping with progesterone-5β-reductases (P5βR), which is why they are collectively named "PRISE" (Petersen et al. 2016). It is assumed that iridoid synthase activity of the "PRISE" enzyme is a characteristic of flowering plants and had developed early in evolution, while the function of P5βR is evidently older in evolution, because functional P5βRs were also discovered in gymnosperm (Rudolph et al. 2015). After CrISY was first discovered in *C. roseus* (Geu-Flores et al. 2012), iridoid synthases have been functionally characterized in various iridoid-producing species (Alagna et al. 2016; Kries et al. 2017; Xiang et al. 2017; Lawas et al. 2023), as well as in *N. cataria* and *N. mussinii* (Sherden et al. 2017). It has been shown that ISYs from *Nepeta,* although greatly structurally similar, are found in two functionally distinct isoforms – family 1 that contains enzymes with high P5βR and very limited ISY activity, and family 2 that is represented by active ISYs that catalyze complete conversion of substrate to *cis,trans*-NL (Sherden et al. 2017; Lichman et al. 2019a). The emergence of this phenomenon can be attributed to the duplication of a "promiscuous" ancestor gene within the PRISE group, characterized by low ISY activity. Over time, selective pressures have driven the neofunctionalization of these enzymes, ultimately establishing iridoid biosynthesis in *Nepeta* species (Lichman et al. 2019b).

The association between the number and presence of ISY isoforms and the occurrence of iridoids has been demonstrated in Lamiaceae (Mint Evolutionary Genomics Consortium 2018). In the genus *Nepeta*, ISYs have a unique role and position in iridoid biosynthesis, as they catalyze the crucial initial step and act as key regulators in the bifurcation towards the formation of nepetalactones and iridoid glycosides. Given these characteristics, ISY is believed to play a significant role in elucidating the molecular basis of iridoid biosynthesis in iridoid-producing and iridoid-lacking species within the *Nepeta* genus. To address this, a recent study was conducted to functionally characterize ISY isoforms in two iridoid-producing species, namely *N. rtanjensis* (chemotype A, rich in *trans,cis*-NL) and *N. sibirica* (chemotype A, predominantly *cis,trans*-NL), as well as an iridoid-lacking species, *N. nervosa* (chemotype C) (Fig. 1) (Aničić et al. 2023).

The nucleotide sequences encoding ISYs, namely NrISY2 from *N. rtanjensis*, NsISY from *N. sibirica,* and NnISY from *N. nervosa*, were found to exhibit high similarity to functionally characterized ISYs from other species in the NCBI database. Phylogenetic analysis of the amino acid sequences demonstrated that all ISYs clustered together with publicly available and characterized ISYs from various species. Specifically, NrISY2 was closely related to characterized iridoid synthases, such as NcISY2 from *N. cataria* and NmISY2 from *N. mussinii,* both belonging to family 2 (Sherden et al. 2017). Previous studies have shown that recombinant family 2 enzymes are active isoforms, effectively converting 8-oxogeranial to *cis,trans*-nepetalactol in assays conducted *in vitro* (Sherden et al., 2017). On the other hand, NsISY and NnISY formed a distinct branch alongside family 1 isoforms from *N. cataria* (NcISY1) and *N. mussinii* (NmISY1).

The phylogenetic analysis of previously characterized ISYs along with NrISY2, NsISY, and NnISY revealed a significant level of sequence homology among them (Aničić et al. 2023). However, it is important to note that phylogenetic clustering alone does not provide absolute certainty regarding the enzymatic activity of these enzymes. Therefore, to further investigate their functionality, heterologous expression in *Escherichia coli* was employed, and the enzymatic activity of the resulting recombinant proteins was analyzed

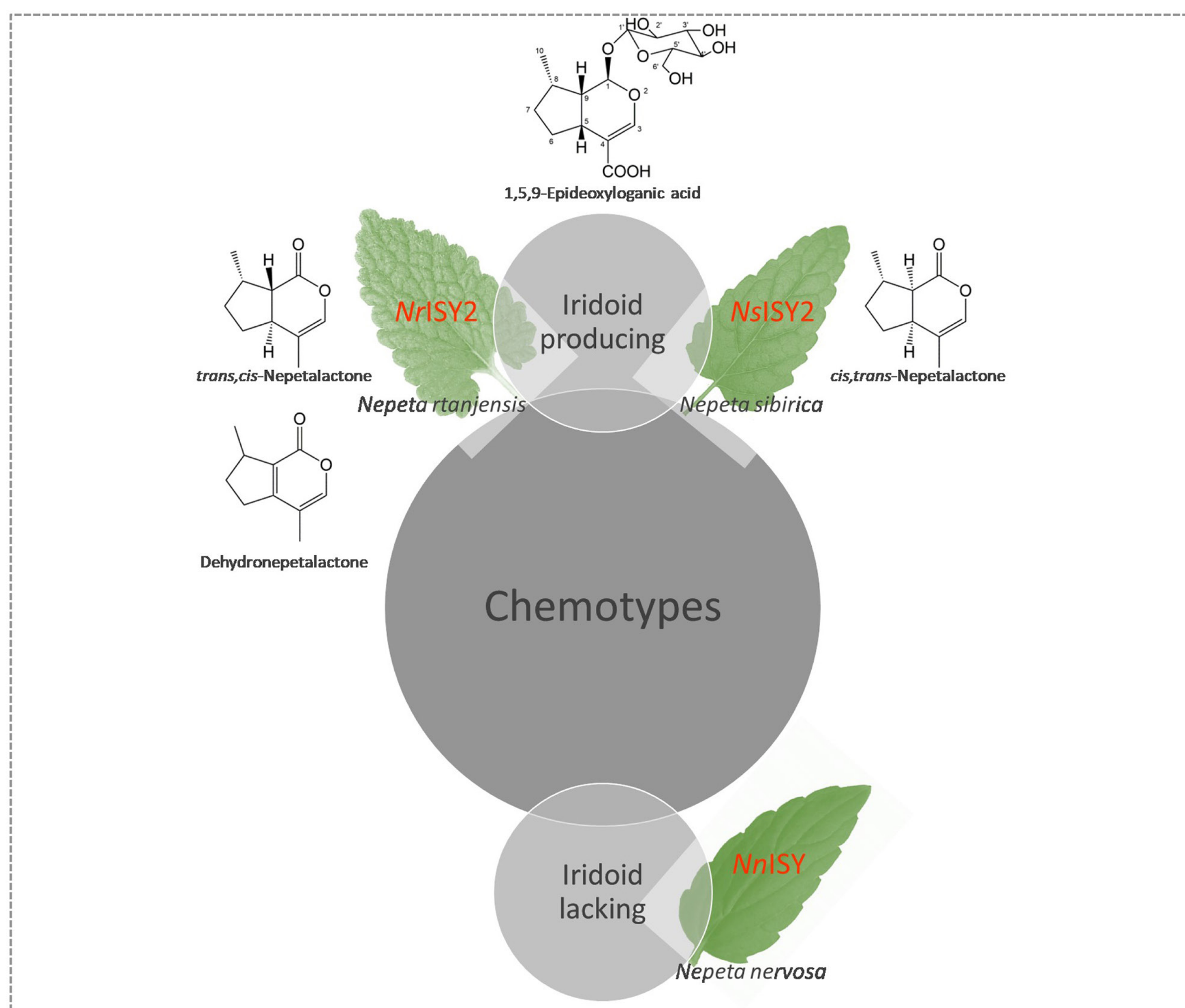

Fig. 1. Unraveling the enzymatic properties of iridoid synthases sheds light on the chemical diversity within *Nepeta* genus. Illustration of diverse *Nepeta* chemotypes from which iridoid synthases are isolated and functionally characterized. Abbreviations: *Nr*ISY2 –iridoid synthase 2 from *N. rtanjensis*, *Ns*ISY2 - iridoid synthase 2 from *N. sibirica, Nn*ISY –iridoid synthase from *N. nervosa*.

using *in vitro* assays. The reaction products were structurally confirmed through GC-MS and NMR analyses. Initially, ISY sequences were amplified and cloned into the expression vector pRSET-A, utilizing restriction enzymes. The resulting constructs were then used to transform *E. coli* BL21 (DE3) bacterial strains. Optimal conditions for bacterial culture growth were determined by varying parameters such as temperature, isopropyl ß-D-1-thiogalactopyranoside (IPTG) concentration for induction, incubation time post-induction, and the speed of the rotary mixer. This approach led to a high yield of recombinant proteins via heterologous expression in *E. coli.* Following successful protein expression, affinity purification using histidine tags was performed. SDS-PAGE analysis, Coomassie Brilliant Blue staining, and immunoblot detection using anti-His antibodies confirmed the presence of a single major band at the expected molecular weight of 42 kD, representing the monomeric form of the recombinant proteins (Aničić et al. 2023).

Enzyme reaction mixtures containing recombinant proteins, 8-oxogeranial as the substrate, and NADPH as the cofactor, along with a control assay lacking the proteins of interest (NrISY2, NsISY, and NnISY), were extracted using hexane and subjected to GC-MS analysis. By comparing the GC-MS spectra and retention indices of reaction products and commercially available 8-oxogeranial and *cis*,*trans*-nepetalactol, as well as utilizing the Wiley Mass Spectral Database (Registry of Mass Spectral Data, Palisade Corporation, Newfield, NY, USA), peaks corresponding to reaction

products were identified as *cis*,*trans*-nepetalactol. Further structural characterization of the compounds produced by enzyme activities was conducted using NMR analysis (^{1}H NMR and 2D ^{1}H-^{1}H COSY NMR spectroscopic techniques), which additionally confirmed the function of the enzymes.

The expression of functional iridoid synthases (ISYs) has been observed in both iridoid-producing species, *N. rtanjensis* and *N. sibirica,* as well as in the iridoid-lacking species, *N. nervosa* (Aničić et al. 2023). While *N. nervosa* possesses the genetic machinery for iridoid biosynthesis, it appears to have an inactive pathway, possibly due to the suppression or inactivation of certain early gene(s) in the biosynthetic pathway. These findings contribute to our understanding of the diversification and evolution of iridoid biosynthesis within the *Nepeta* genus. Further investigations into the underlying genetic and regulatory mechanisms are needed to unravel the specific factors responsible for the loss of iridoid production in *N. nervosa.*

CONCLUDING REMARKS

There have been very few investigations on the biosynthesis of iridoids and their remarkable evolutionary features, despite the growing number of studies on the biological functions of iridoids. However, significant progress has been made in the last decade in unraveling the biosynthesis and regulation of iridoids within the genus *Nepeta*. Many important discoveries have been made, shedding light on various aspects of the biosynthetic pathway. Yet, there are still important gaps in our understanding, particularly in the final steps of aglycone biosynthesis and the production of iridoid glucosides, as well as the involvement of transcription factors in fine-tuning the biosynthetic process. Further research is needed to fully elucidate these remaining aspects and uncover the complete picture of iridoid biosynthesis in *Nepeta*. Such knowledge will not only deepen our understanding of this unique class of compounds but also open up possibilities for their manipulation and utilization in various fields including pharmaceuticals, agriculture, and synthesis of natural product.

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