Food & Medicine Homology

ISSN 3006-6867 (print); 3006-4252 (online)
https://doi.org/10.26599/FMH.2025.9420060

Review Article

Functional activities and biosynthesis of isothiocyanates in *Moringa oleifera* Lam. and Brassicaceae: an update

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Received: 8 July 2024 / **Revised:** 2 August 2024 / **Accepted:** 2 August 2024

Abstract: Isothiocyanates, secondary metabolites primarily derived from cruciferous plants, are known for their distinct flavors in food and diverse biological activities such as anti-tumor, anti-diabetic, neuroprotective, antibacterial, anti-inflammatory, and antioxidant properties. They are extensively utilized in food, medicine, and agriculture. Moringa oleifera Lam. has recently been recognized as a new food resource and has attracted increasing research attention. This plant belongs to the Brassicales order and the Moringa family. It was found to contain biologically active isothiocyanates such as sulforaphane and 4-(*α*-*L*-rhamnopyranosyloxy)-benzyl isothiocyanate (MIC-1). Considering the low content of natural products and the related environmental issues caused by chemical synthesis, synthetic biology presents a promising approach for the sustainable and efficient production of these compounds. Recently, the biosynthetic pathways of isothiocyanates in many plants have been elucidated, which can promote the heterologous production of isothiocyanates through microbial fermentation. This review comprehensively examines the bioactivity, bioavailability, and biosynthetic pathways of M. oleifera Lam. isothiocyanates, alongside recent advancements in molecular biology techniques aimed at enhancing their yield. By elucidating the functional significance of isothiocyanates, this review aims to provide a comprehensive understanding of their role and to support further developments in their utilization in functional foods. It also proposes strategies for achieving high-yield production of these valuable compounds, offering insights that bridge theoretical knowledge with practical applications in the field.

Keywords: *Moringa oleifera* Lam.; isothiocyanates; biological activities; biosynthesis; yield

Citation: Guo Z. Y., Dai C., Xie J., et al. Functional activities and biosynthesis of isothiocyanates in *Moringa oleifera* Lam. and Brassicaceae: an update. *Food & Medicine Homology*, 2025, 2: 9420060. https://doi.org/10.26599/FMH.2025.9420060

1 Introduction

Moringa oleifera Lam. is a perennial tropical deciduous tree of the genus *Moringa Adans*, native to arid regions such as India and Africa, and is a multi-purpose fast-growing tree species[1]. *M. oleifera* Lam., a tropical deciduous tree native to regions like India and Africa, is renowned for its nutritional richness and medicinal properties. It is rich in plant protein, vitamin A, vitamin C, folic acid, pantothenic acid, calcium, iron, zinc, selenium, potassium and other nutrients, as well as isothiocyanates (ITCs), flavonoids and other functional active substances, and is known as a "super nutrient bank"[2]. ITCs in moringa has high medicinal value and can be used to intervene in the treatment of pain, ulcers, hypertension, cancer, diabetes, skin infections, inflammation, and other diseases[3]. With the further understanding of the nutritional value and medicinal value of moringa, moringa is becoming more and more important. In 2012, the Ministry of Health of China approved moringa leaves as a new resource food raw material in Announcement No. 19, and in the same year, moringa was recognized as the "first green food recommended by the country" by the China Green Food Development Center, and moringa was also listed as a medicinal and edible homologous plant[4].

In fact, ITCs have the general formula of R–N=C=S structure, which is isomeric with thiocyanate, and belongs to heteronuclear cumulative diolefins, which are a class of sulfur-containing and nitrogen-containing compounds. Natural ITCs are mainly derived from cruciferous plants, and are a class of secondary metabolites produced by direct contact with myrosinase (EC 3.2.1.147) in cells and catalyzing the hydrolysis of glucosinolates in vacuoles when plants are subjected to biological stress[5], mainly including sulforaphane (SFN), allyl isothiocyanate (AITC), and benzyl isothiocyanate isosulfocyanide (BITC) and phenethyl isothiocyanate (PEITC), among others[6]. ITCs not only give food its unique flavor, but also exhibit multifaceted biological activity,

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including tumor inhibition, diabetes symptom relief, nerve tissue protection, bacterial growth, and powerful antioxidant effects. These characteristics make ITCs have a wide range of practical potential and applications in the food industry, human healthcare, and agricultural production[5,7,8]. They are formed when glucosinolates are hydrolyzed by myrosinase under biological stress. Despite their beneficial effects, natural sources of ITCs are limited, and extraction is challenging due to their low abundance in plants. Traditionally, chemical synthesis of ITCs involves toxic reagents and is environmentally hazardous. This method suffers from long production cycles, low yields, and high costs, contributing to environmental pollution. Therefore, there is a growing interest in developing sustainable and efficient methods for ITC synthesis.

The content of ITCs from natural sources is limited, and extraction is difficult. The chemical synthesis of ITCs generally relies on the reaction of thiophosphine or CS_2 with amines, which requires the use of highly toxic reagents with narrow functional group compatibility[9-12], but the use of highly hazardous chemicals is not conducive to safe operation and production[13]. In addition, there are several technical bottlenecks in the traditional ITCs synthesis process, such as long preparation cycles, low product yields, high overall production costs, large number of by-products, and the use of toxic reagents to cause greater potential pollution to the environment, which is undoubtedly contrary to the requirements of modern society for sustainable development and environmental protection[14]. Therefore, researchers are eagerly seeking and developing a new preparation strategy that is more environmentally friendly, efficient and stable to replace the existing ITCs synthesis process, which has attracted much attention in the current research field.

The relationship between ITCs and moringa highlights the plant's potential as a source of these beneficial compounds. The challenges in traditional extraction methods underscore the need for innovative approaches, such as synthetic biology, to produce ITCs more efficiently and sustainably. This review emphasizes the importance of advancing environmentally friendly strategies in line with modern societal demands for sustainable development and environmental protection. In recent years, the method of designing and modifying microbial strains to ferment and produce natural medicines based on synthetic biology has been widely recognized by discovering the key enzyme genes for the biosynthesis of pharmaceutical active ingredients, so synthetic biology is expected to become a more convenient and effective way to obtain ITCs. In this paper, the classification, biological activity, bioavailability and biosynthesis pathways of ITCs and the molecular biology methods to increase the content of ITCs are described, aiming to provide ideas for high-yield ITCs and their modern industrial applications.

2 Major classifications of ITCs in *M. oleifera* Lam. and Brassicaceae

The presence of ITCs has also been detected in *Moringa*, and has attracted much attention due to its high isothiocyanate content and strong activity in moringa. ITCs are typically stored in cell vacuoles as glucosinolates[15]. To date, more than 154 different glucosinolates have been identified[15,16], which can be divided into four categories: aliphatic, aromatic, indole, and glycosylated[17,18]. Glucosinolates hydrolysates include a variety of nitrogenous compounds, such as ITCs, thiocyanates, nitriles, and thionitriles, depending on the reaction conditions, such as pH, temperature, and reducing agents such as ferrous ions, ascorbic acid[19,20]. For example, at neutral pH, thiohydroxyate-*O*-sulfate is rearranged into ITCs, which are catalyzed by myrosinase to form ITCs with different structures and functions[21]. Depending on the type of precursor amino acids, that is glucosinolates, the R-side chain of ITCs can be divided into aliphatic, aromatic, and indole ITCs[22].

3 Bioavailability and stability of ITCs

3.1 Bioavailability

In fact, the study of the bioavailability of ITCs is also a very important part of their practical application. The bioavailability of ITCs depends on the interaction between plant myrosinase and gut microbiota during glucosinolate hydrolysis after consumption of cruciferous vegetables[23]. Due to the lipophilic nature of ITCs and the degradation and conversion of glucosinolates by glucosinolates by direct intake, direct intake of ITCs ensures higher absorption efficiency in the body[24], and the bioavailability of vegetables *in vivo* is also affected by differences in the way vegetables are cooked[25].

An *in vitro* study found that SFN is transported primarily through the intestinal tight junction controlled paracellular pathway and the transcellular pathway, suggesting that SFN is easily absorbed[26], and that absorbed SFN and other ITCs will be metabolized through the thiol acid pathway. Several studies in rodents and humans have shown that peak concentrations of SFN and its metabolites in plasma occur within 1–3 h after administration. Gasper *et al.*[27] injected mice with SFN and found that metabolites were detected in all organs at 2 and 6 h post-dose, and the highest concentrations of SFN were found in the small intestine, prostate, kidney, and lung, suggesting that SFN is bioavailable. At the same time, it also proves that ITCs may be an effective anticancer agent or other bioactive agent in organs and tissues. Due to its clinically proven biological efficacy[28,29], it is also an emerging compound in the nutritional supplement market, with its precursors sometimes used in combination with myrosinase or its isomers. In conclusion, the bioactive compound isothiocyanate is an essential or non-essential molecule found in cruciferous fruits and vegetables, which is able to regulate metabolic processes and thus improve human health.

3.2 Stability

The results show that the stability of ITCs can be effectively improved by embedding technology. When the microcapsule products composed of AITC and β-cyclodextrin were prepared by suspension method, the mass concentration of AITC in the microcapsules was measured by photometry to be 43.8 mg/g. After embedding, the retention rate of AITC in the microcapsules was 17.0% after 48 h at 85 °C due to the space obstruction of the β-cyclodextrin shell, while the control group had complete volatilization after 3 h. There was little change in the amount of AITC in the microcapsules after 14 days of storage at room temperature[30]. The use of ultrasound methods to encapsulate BITC in cyclodextrins also enhances its stability in biological applications[31]. The polymer particles composed of chitosan-olive oil-PEITC showed that the encapsulation of PEITC improved its stability and bioavailability. The optimal compounding conditions were olive oil with chitosan and PEITC with chitosan ratios of 1.46 and 0.25, respectively. The size of the composite particle is 629 nm, the zeta potential is 32.3 mV, the polydispersity index is 0.329, and the rejection efficiency is 98.49%[32]. In addition, in

addition to molecular embedding, membrane emulsification is also an effective way to prepare microcapsules. A study of microcapsules prepared by sodium alginate composite and membrane emulsification showed that the microcapsules prepared by membrane emulsification had better protective effect on SFN, increasing its thermal stability and antioxidant stability. The study of SFN and the corresponding microcapsules under the conditions of heating and the presence of H_2O_2 confirmed that when the temperature was greater than 60 °C, the SFN became extremely unstable, and the microcapsules showed good protective effect. At H_2O_2 concentrations greater than 20%, SFN is degraded to a certain extent due to external oxidants, with a retention rate of only 45.7%, while the retention rate of SFN in microcapsules is 81%[33]. In other studies, fish skin gelatin was used as an emulsifier, Tween-based emulsion was used as a control, and BITC was used as a nutrient delivery model to construct a stable emulsion. After the lipids were digested in the stomach, electron microscopy scans showed significant aggregation of emulsifiers. During the digestion process of small intestine, the free fatty acid release of fish skin gelatin emulsion reached (87.5 ± 1.45)%. The bioavailability of BITC in the fish skin gelatin-based emulsifier was 80.77%, which was nearly 27% higher than that of the control group. The results showed that the emulsion delivery system based on fish skin gelatin improved the stability of BITC *in vivo*, and had a slow-release and protective effect on it[34].

In addition, ITCs exhibit good stability in the environment of specific organic solvents, including *n*-hexane, acetone, dichloromethane, ethyl acetate, and certain acidic solutions. In the food system, the stability of ITCs can be enhanced by the addition of ingredients such as vegetable oils, specific antioxidants, or acidity modifiers[35]. These findings provide a theoretical basis for in-depth discussion on the stability guarantee of ITCs in the process of research and development (R&D), production and practical application.

4 Biological activities and application of ITCs in *M. oleifera* Lam. and Brassicaceae

Since the 1950s, ITCs have attracted great attention from scholars from all walks of life due to their special odor and biochemical properties, especially in anti-cancer and plant defense[36]. So far, there have been many reports on the intervention of ITCs in various diseases, which has laid an important foundation for the development of the field of human medicine. The biological activity and related mechanisms of ITCs in human medicine are shown in Fig. 1. The biological activity and functional applications of ITCs are mainly reflected in the following three areas.

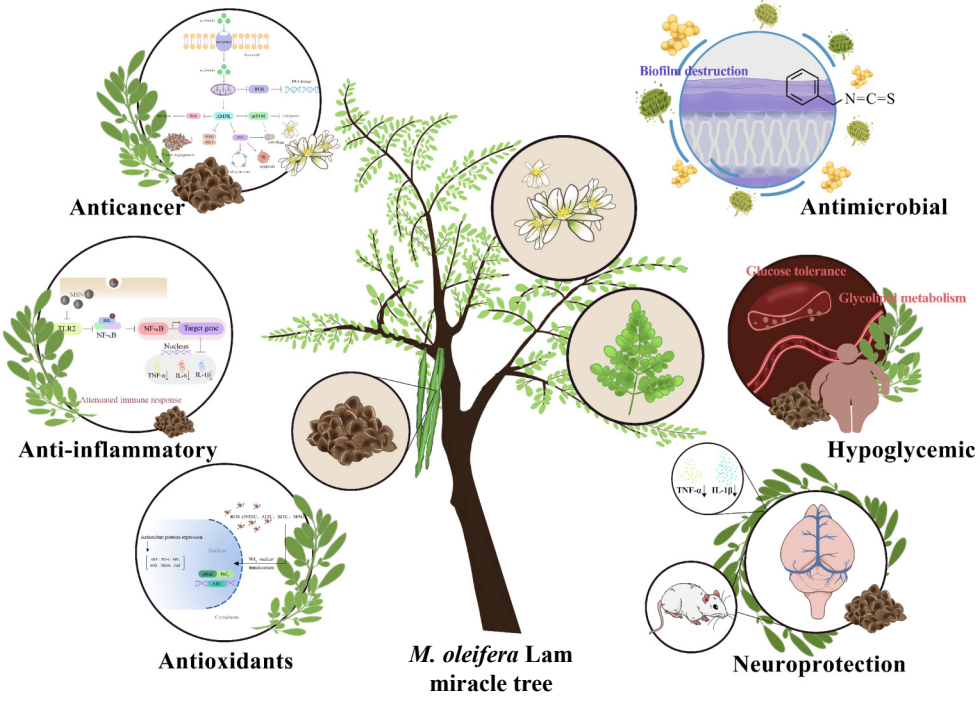

Figure 1 Special biological activities and mechanisms of *M. oleifera* Lam. and Brassicaceae ITCs in human medicine.

4.1 Food

4.1.1 *Food flavor*

ITCs are one of the main components that form the spicy flavor of foods[37]. For example, the spiciness of wasabi and horseradish, as well as the typical flavor of pickled cabbage, are derived from ITCs, which have a strong spicy and tear-jerking properties, as well as an aromatic flavor[38]. In addition, nearly 100 aroma compounds have been detected and confirmed in the complex aroma composition of Sichuan mustard, among which the main components such as nitriles, ITCs, and methyl trisulfide jointly construct the unique aroma of mustard, which is also produced by glucosinolates catalyzed by myrosinase degradation[39,40]. In addition, it was found that after 3 days of fermentation, the original strong pungency of sour radish kimchi was significantly reduced, mainly because the myrosinase contained in it was inactivated under acidic conditions (pH < 4), and the concentration of 4-methylthio-3-butenylisothiocyanate catalyzed by the enzyme was reduced, so the pungent taste became less[41].

4.1.2 *Preservative and preservation*

It has been found that PEITC and BITC in ITCs mainly inhibit the growth and toxin production of fungi such as *Aspergillus*

parasiticus and aflatoxins by destroying the mycelium structure, cell wall and membrane integrity of fungi, and down-regulating the expression of ochratoxin A synthesis genes[35, 42]. ITCs are often used as food preservatives in beverages such as alcohol and fruit juices, and have shown significant preservation effects, so they have great application prospects in the field of food preservation[43].

4.2 Human medicine

4.2.1 *Anticancer*

Cauliflower and broccoli are internationally recognized anti-cancer and anti-cancer vegetables, and their functions are mainly reflected in the SFN they contain. Epidemiological studies have shown that ITCs with abundant sources have been shown to have an intervention effect on breast cancer[44], prostate cancer[45, 46], colon cancer[47], skin cancer[48-50], and pancreatic cancer[51]. Recent studies have found that BITC and PEITC in Chamagu ITCs can effectively inhibit the migration and uncontrolled proliferation of Hela cells by inducing nuclear DNA condensation in cervical cancer cells, and have anti-cervical cancer activity, which is one of the most effective tumor prevention factors[52].

Moringa glucosinolates are often converted to ITCs for utilization. Rajan *et al.*[53] confirmed that *Moringa* Moringin (4-(α-*L*-rhamnopyranosy-loxy)-benzyl isothiocyanate (MIC-1)) can induce apoptosis and reduce 5S rRNA expression in astrocytoma grade IV CCF-STTG1 cells, and can be used for the treatment of malignant astrocytoma. Tiloke *et al.*[54] synthesized gold nanoparticles from *Moringa* extract can regulate oncogenes, tumor suppressor genes and caspase-9 splice variants in A549 lung cancer cells, and have anti-A549 cell proliferation and apoptotic effects. Cuellar-Núnez *et al.*[55] reported that an isothiocyanate-rich moringa extract can effectively reduce the levels of cytokines tumor necrosis factor-α (TNF-α) and inter leukin-1β (IL-1β) and induce apoptosis in human colon cancer cells. Guevara *et al.*[56] proposed *in vitro* antitumor promoter activity of ITCs from *Moringa* seeds. The results show that *Moringa* ITCs inhibit EBV-EA activation in Raji cells (half maximal inhibitory concentration (IC_{50}): 32.7 μg/mL). Brunelli *et al.*[57] reported a decrease in the NF-κB pathway and an increase in glutathione-S-S-transferase levels after treatment of different cell lines with MIC-1 of *Moringa* leaves at 10–30 μmol/L. Michl *et al.*[58] evaluated the chemopreventive effect of MIC-1 in *Moringa* seeds, reporting modulation of JAK/STAT signaling by inhibition of STAT5 signaling in 0.4–10 μmol/L MIC-1 Ba/F3 cells. Inhibition of NF-κB signaling due to reduced expression of *IL-6* and *IL-8* genes has also been reported in 2 μmol/L ITCs-treated HeLa cells. In addition, Maiyo *et al.*[59] extracted 20 μg/mL of MIC-1 from different moringa tissues and incubated them with Caco2 and HepG2 cells for 48 h, indicating that the compound has chemopreventive effects. The authors reported that Caco2 cells had a higher rate of apoptosis induction compared to the control group. In addition, Förster *et al.*[60] observed a decrease in cell viability after treatment of HepG2 cells with more than 15 μmol/L of *Moringa* leaves, suggesting that the Nrf2 pathway induces a protective effect. This is confirmed by the expression of detoxification enzyme genes such as NAD(P)H quinone oxidoreductase 1 (*NQO1*). Fahey *et al.*[61] also reported studies on the use of moringa leaves to induce the synthesis of NQO1 and glutathione (GSH) in different cell lines (e.g., Hepa1c1c7 hepatocellular carcinoma cells, RAW 264.7 macrophages, PE keratinocytes, and APRE-19 human epithelial cells). *Moringa* ITCs-induced expression of NQO1 and GSH promotes cytoprotective activity, thereby reducing the risk of tumor development. In addition, Jaafaru *et al.*[62] reported the inhibitory effect of MIC-1-rich *Moringa* seed extract on human prostate cancer cell proliferation. The authors believe that treatment of cells with 2.5 μg/mL of moringa seed extract for 24, 48, and 72 h has time-dependent antitumor effects, although the molecular mechanisms by which these effects occur have not been reported. However, some of the above studies did not indicate which ITCs in *Moringa* exert anti-tumor effects, and it is still necessary to clarify the types of ITCs that play a role through different *in vivo* inducible cancer models.

4.2.2 *Hypoglycemic*

ITCs have been found to improve insulin resistance and triglyceride levels and reduce the risk of cardiovascular disease in patients with type 2 diabetes[63]. Studies have shown that BITC in *Moringa* can regulate fat synthesis and storage by activating the adenylate-activated protein kinase pathway and thereby inhibiting the mechanism of peroxisome proliferator-activated receptor γ expression, and it has been found that BITC of *M. oleifera* has a significant inhibitory effect on lipid accumulation in 3T3-L1 adipocytes derived from mouse embryos[64].

The main ingredient of moringa seeds, isothiocyanate, has the effect of improving diabetes. Jaja-Chimedza *et al.*[65] reported that moringa extract containing ITCs can reduce body weight, reduce fat, improve glucose tolerance, reduce inflammatory gene expression, increase antioxidant gene expression, and can cause anti-inflammatory and antioxidant responses in cells and antibiotic-like responses in intestinal microbiota to improve body health. Parameters related to the insulin pathway were evaluated in liver and muscle tissues at the same time, and improvements in glucose tolerance in C57BL/6J mice were found after supplementation with *Moringa* seed extract enriched with ITCs. The mice used in both 9 experiments were not diabetic, but obese. Therefore, it is also appropriate to use a diabetes-induced adipose tissue model to assess the results. Gopalakrishnan *et al.*[66] demonstrated that the main component extract of moringa can inhibit the occurrence of atherosclerosis caused by diabetes by scavenging reactive oxygen species (ROS) and preventing the formation of advanced glycation end products (AGE) and low-density lipoprotein, the end products of higher glycation. Waterman *et al.*[67] reported that MIC-1 and its isomer IV present in *Moringa* leaves had a hypoglycemic effect after treatment of male C57BL/6J mice with *Moringa* concentrate enriched with 5% ITC. Metabolic, biochemical, and molecular parameters were evaluated during the study, and a decrease in weight gain and glucose-regulating hormones (insulin, leptin, and resistin) in mice treated with moringa extract was reported, as well as a decrease in inflammatory cytokines (IL-1β, TNF-α). Increased expression of insulin signaling protein in muscle tissue of mice treated with *Moringa* ITCs.

4.2.3 *Neuroprotection*

Tumer *et al.*[68] reported induction of NQO1 activity after treatment of Hepa1c1c7 cells with MIC-1 in moringa leaves. It was found that *Moringa* MIC-1 demonstrated the neuroprotective effect of moringa MIC-1 on CD1 male mice by inhibiting the degradation of human nuclear factor Iκβ-α and reducing the expression of inducible nitric oxide synthase 7 days before induction of spinal cord injury[69]. These studies show that the presence of ITC in genetically modified seeds appears to have a

positive effect on neurological-related problems. Male C5TBL/6 mice with subacute Parkinson's disease were also given the same compound MIC-1. The levels of pro-inflammatory cytokines TNF-α and IL-1β in the brains of mice were reduced after pretreatment with ITCs, suggesting that MIC-1 has a protective effect against neuronal damage[70]. Notably, these studies have demonstrated the protective effect of ITC on neurological related diseases. This suggests that ITC may be useful in the treatment of non-communicable chronic diseases (NCDs) in older adults. The neuroprotective effect of SFN is to activate the nuclear factor-erythroid 2 related factor 2 (Nrf2) antioxidant pathway, upregulate phase I and phase II enzymes, and activate the mechanism of extracellular regulated protein kinases 1/2 (ERK1/2) and Akt kinase cascade signaling pathways[71] to achieve neuroprotective effects in acute brain injury such as ischemic brain injury[72], cerebral hemorrhage injury[73], and traumatic brain injury[74], and chronic brain injury such as Alzheimer's disease[75] and Parkinson's disease[76]. In addition, studies have shown that SFN metabolites have also been shown to have a good intervention effect in alleviating autism by participating in pathways such as oxidative stress, amino acids/gut microbiota, neurotransmitters, hormones, and sphingomyelin metabolism[77].

4.2.4 *Antioxidants*

Oxidative stress is implicated in the pathogenesis of various diseases. ITCs, such as PEITC, SFN, AITC, and BITC found in moringa and cruciferous plants, exhibit notable antioxidant potential by effectively neutralizing ROS like organic hydroperoxides and hydrogen peroxide[78]. ROS contribute significantly to oxidative stress, which arises from an imbalance between ROS production and cellular antioxidant defenses, impacting cellular function positively or negatively.

ITCs play a dual role as both antioxidants and pro-oxidants[79]. For instance, Cheng *et al.*[80] demonstrated that MIC-1, an ITC compound, activates the Nrf2 pathway in HepG2-C8 cells, enhancing the expression of Nrf2-related genes and proteins to exert antioxidant effects. Similarly, Waterman *et al.*[81] observed reduced levels of NO and TNF-α in RAW 264.7 macrophages treated with ITC from moringa leaves, attributed to phenolic compounds associated with ITCs.

While ITCs show promise in combating oxidative stress, further research is essential to comprehensively understand their effects, either alone or in combination with other compounds[70]. Giacoppo *et al.*[70] reported that moringa ITCs protect against secondary injury following spinal cord injury through antioxidant mechanisms. Studies indicate that SFN, for example, protects cells from oxidative damage by inducing phase II detoxification enzymes as indirect antioxidants. These findings suggest potential preventive and therapeutic applications of ITCs in chronic noncommunicable diseases like certain cancers[82-84], neurodegenerative diseases[85], gastric ulcers[86], and heart disease[87]. ITCs derived from moringa and cruciferous plants exhibit robust antioxidant properties, potentially mitigating oxidative stress-related diseases. Continued research will shed light on their mechanisms and expand their therapeutic applications in various health conditions.

4.2.5 *Antimicrobial*

ITCs have attracted much attention in biomedical research due to their potential for biological activity against bacterial pathogens and fungi[88]. PEITC, AITC, and BITC have demonstrated effective antimicrobial effects against a range of gram-negative and gram-positive bacteria, including major pathogens such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Klebsiella pneumoniae*, and *Escherichia coli*[89-91]. These effects are facilitated by a variety of mechanisms, including disruption of bacterial membranes, induction of stress responses, and metabolic alterations through the activation of tightly controlled pathways[92]. PEITC effectively inhibits the growth of *Vibrio cholerae* and the formation of biofilms, reducing the production of toxins, thereby protecting mammalian cells from its toxicity[93]. Under different physiological conditions, SFN may have specific antimicrobial effects on bacteria *in vitro* or *in vivo*. Recent studies have shown that SFN can inhibit the virulence of bacteria and thus resist the invasion of pathogenic bacteria in plants, and play an important role in plant defense mechanisms[94]. In addition, ITCs produced by the "glucosinolin-myrosinase" defense system in the cruciferous family also have broad antagonistic activity against various fungal pathogens[6, 95]. One study investigated the inhibitory effects of PEITC, AITC, and BITC on *A. parasiticus*[35]. It was found that fungal growth and aflatoxin production correlated with ITC concentrations: antimicrobial activity decreased with increasing concentrations of ITCs. When used at concentrations of 0.5 mg/L or higher, the inhibitory activity of AITC, BITC, or PEITC decreases to less than 50%. Among them, AITC and BITC had the highest antibacterial activity, and the lowest inhibitory concentration (MIC) was the lowest, which was 20 mg/L, while the MIC of PEITC was 50 mg/L. In contrast, BITC was the most potent compound to achieve a minimum fungicidal concentration (MFC) of 20 mg/L, compared to 50 mg/L for both AITC and PEITC[35]. The antimicrobial efficacy of ITCs has been demonstrated *in vitro* and in various clinical trials.

4.2.6 *Anti-inflammatory*

Moringa and Cruciferous ITCs also protect against many inflammatory and chronic diseases such as colitis, arthritis and respiratory-related problems including asthma and chronic obstructive pulmonary disease. PEITC has been found to play a key role in the regulation of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, which play a central role in coordinating inflammatory responses to foreign antigens[96]. In studies related to AITC, researchers observed that treatment with AITC significantly reduced peroxidase activity and the number of macrophages in mice with colitis, thereby reducing inflammation[97]. The anti-inflammatory effects of BITC are achieved by inhibiting the activity of the NF-κB pathway and the expression of the *IL-13* gene in a23187-induced basophilic cells[98]. In different cell lines, ITCs contained in moringa seeds, leaves, and flowers have been shown to exert most of their anti-inflammatory effects. Cheenpracha *et al.*[99] reported a dose-dependent decrease in inducible nitric oxide sythase (iNOS) protein expression after 18 h of culture in RAW 264.7 lipopolysaccharide-activated cells treated with *Moringa* seeds with different concentrations of moringa seeds. Similarly, Park *et al.*[100] reported a decrease in iNOS expression as well as a decrease in cyclooxygenase-2 (COX-2). RAW 264.7 mouse macrophages were used to evaluate the anti-inflammatory activity of moringa flower ITC. In addition, Jaja-Chimedza *et al.*[101] reported a decrease in the expression of genes encoding inflammatory factors such as iNOS, IL-1β, and IL-16 after treatment of the same cell line, but did not affect cell viability, suggesting that MIC-1 from moringa seeds has anti-inflammatory effects. Poor production of inflammatory markers and antioxidant systems in the body is typical of noncommunicable diseases. Moringa seed extract containing

38.9% ITCs was used for carrageenan-induced foot edema in rats to induce the expression of antioxidant genes (*NQO1*, *HO1*, *GSTPI*, *Nrf2*), and its edema reduction effect was significantly better than that of aspirin. It has also been shown that ITC from different moringa tissues can reduce inflammatory markers such as iNOS, IL-1β, and IL-6, as well as the NF-κB that activates B cells. Several other studies have demonstrated the anti-inflammatory potential of glucosinolates and ITCs through *in vivo* models. Galuppo *et al.*[102] reported that moringa seed ITCs could intervene in the reduction of inflammatory markers in male adult C57BL/6 mice with autoimmune encephalospinitis, and suggested that moringa ITCs can significantly eliminate the inflammatory cascade and can be developed as a drug for the treatment of multiple sclerosis. Santos *et al.*[103] reported a reduction in both inflammation and nociception sensation in male Wistar rats with temporomandibular joint disease after 14 days of treatment with moringa flora MIC-1 and other analogues, but no toxic effects were reported. It was found that seven substituent isothiocyanate analogues were obtained, and three of them had good analgesic and anti-inflammatory effects and lower toxicity, which provided ideas for the design and development of analgesic and anti-inflammatory drugs for moringa ITCs. In addition, an anti-infective drug on the market, ANGOCIN® Anti-Infekt N (Angocin; film-coated tablets, Repha GmbH, Langenhagen, Germany)[104], whose main active ingredient is ITCs, is currently authorized for the treatment of inflammatory diseases affecting the respiratory and urinary tract, such as acute sinusitis[105], acute bronchitis[106], acute respiratory infections[107], and urinary tract infections[108].

4.3 Agriculture

In recent years, scientists have done a lot of research on the bactericidal and insecticidal effects of ITCs, and have shown that BITC and PEITC have good bactericidal and insecticidal effects, and can be used as organic synthesis intermediates of fungicides, insecticides, and herbicides[109-111]. ITCs are safe for humans at low concentrations, easily degradable in the environment, and conform to the characteristics of ideal pesticide compounds, and have great potential to be developed into new pesticides.

5 Biosynthesis of ITCs

5.1 Biosynthetic pathways

At present, the biosynthesis research of ITCs mainly focuses on the mining and functional verification of key enzyme genes in the biosynthesis and metabolism pathways of ITCs. According to the biosynthesis and metabolism pathways of glucosinolates in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the biosynthetic pathways of ITCs were summarized are shown in Fig. 2. Based on the different types of precursor amino acids, the enzymes in the biosynthesis of glucosinolates in the early stage were slightly different. The aliphatic ITCs represented by methionine enter the core-forming part after being extended by the side chain, while the aromatic ITCs derived from phenylalanine, tyrosine, and tryptophan directly enter the core-forming part, and the glucosinolates obtained in the core step are re-modified by relevant enzymes to complete the side chains, and then degraded, metabolized and synthesized various ITCs under certain conditions by myrosinase[112].

5.2 Biosynthetic pathways involved in the reactions with enzymes

In several plants, especially *Arabidopsis thaliana*[113], the biosynthetic structural genes of ITCs have been identified, and

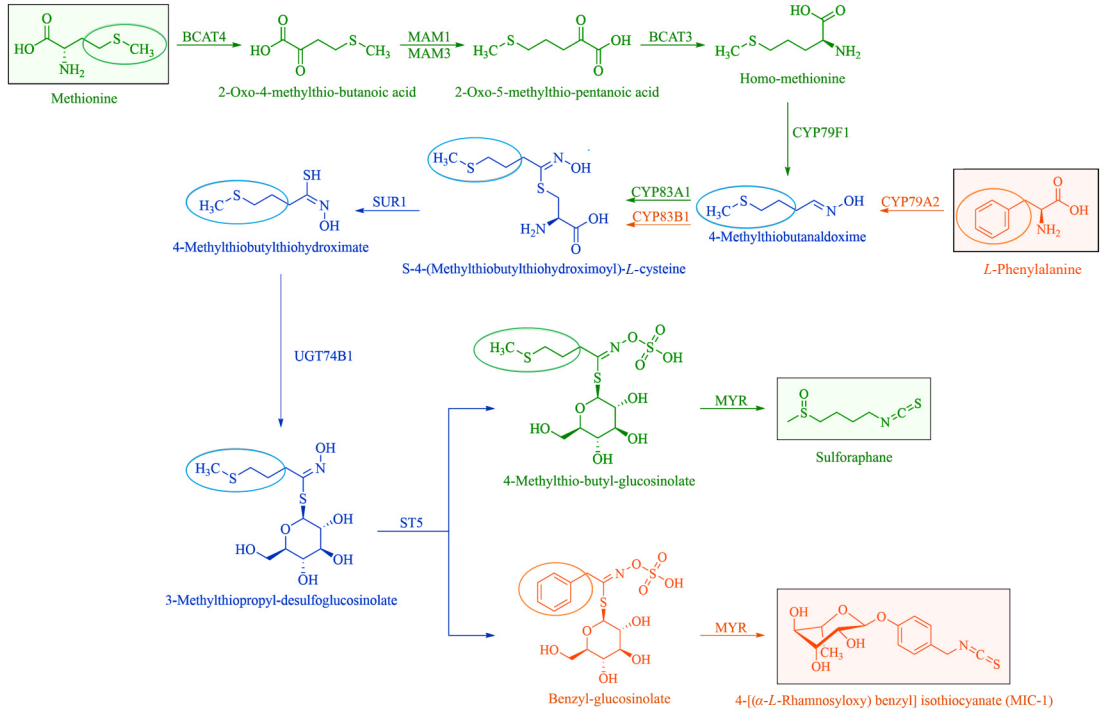

Figure 2 Biosynthesis and metabolism of ITCs in *M. oleifera* Lam. BCAT4/BCAT3, branched-chain amino acid aminotransferase; MAM1/MAM3, methylthioalkylmalate synthase; CYP79F1/CYP79A2/CYP83A1/CYP83B1, cytochrome oxidase; SUR1, *S*-alkyl-thiohydroximate lyase; UGTs, *N*-hydroxythioamide *S*-*β*-glucosyltransfer-ase; ST5, desulfoglucosinolate sulfotransferase; MYR, myrosinase. In addition, green represents SFN biosynthetic pathway, orange represents MIC-1 biosynthetic pathway, and blue represents the shared pathway.

their biosynthesis is mainly accomplished by three synthesis stages[114]. (1) Side chain elongation, catalyzed by five enzymes to generate methylene methionine[115]. The synthesis of ITCs starts with various amino acids as the starting reactants, and is passed through BCAT, MAM, isopropylmalate isomerase (IPMI), isopropylmalate dehydrogenase (IPMDH) undergoes a series of transamination, condensation, isomerization, and oxidative decarboxylation reactions by transaminases to form long-chain amino acids[116,117]. (2) Core generation, with seven enzymes involved in the reaction. The long-chain amino acids derived from methionine are catalyzed to produce the corresponding aldehyde oxime only by cytochrome oxidase (CYP79F1), and then to nitroso compounds by methylthio alkanaldoxime *N*-monooxygenase (CYP83A1). Phenylalanine, tyrosine, and tryptophan are oxidized directly by phenylalanine *N*-monooxygenase (CYP79A2) and tryptophan *N*-monooxygenase (CYP79Bs), respectively, and then all CYP83B1 catalyze the formation of nitroso compounds. In the subsequent steps, the enzymes of the three amino acid reactions are the same, i.e., the nitroso compounds are successively passed through glutathione-*S*-thiohydroximate lyase (GST), C–S lyase, UGT, and sulfotransferase (Aliphatic). ST followed by oxidation reactions such as conjugation, C–S bond breakage, glucosylation, and sulfation to form glucosinolates[118]. (3) Side-chain modification and myrosinase catalysis, in which two enzymes participate in the process. The glucosinolates obtained in the previous step were modified by the sulfur oxidation reaction of flavin-containing monooxygenases (FMO)[119], and then hydrolyzed by myrosinase (MYR) to form unstable glycoaglycans and glucose, which depend on their structure, pH, Fe^{2+} concentration and epidermal-specific thioprotein rearrangement to form different products. Glycoaglycones are usually rearranged by Hoffman to form ITCs at neutral pH[120,121].

6 Molecular biology methods to improve ITCs yield

In order to further improve the bioaccumulation of natural active ingredients in plants, synthetic biology techniques such as overexpression of transgenes and increasing the activity of related enzymes in biosynthetic pathways have become a new trend. Increasing the content of its precursors or discovering sources of myrosinase with high enzymatic activity is an effective way to increase the yield of ITCs[122,123].

6.1 Single gene overexpression

A number of studies have reported that a single genetic modification will have a certain effect on the content of ITCs in plants. Researchers Wu *et al.*[124] introduced the myrosinase gene *MYR* into broccoli plants, and the SFN content of transgenic plants was 1.92 times higher than that of wild-type plants. Huang *et al.*[125] introduced the epithelial sulfotransferase-encoding gene *SMT* into *A. thaliana*, and the SFN mass fraction in experimental plants was 1.52 times that of the wild type. In addition, Wang[126] took broccoli as the research object, focusing on the overexpression of key genes in the biosynthesis of short-chain aliphatic ITCs *CYP79F1* in broccoli, obtaining an Agrobacterium-mediated broccoli genetic transformation system, and using high performance liquid chromatography technology to determine the content of glucosinolates in the synthesis of ITCs in transgenic broccoli plants. The results showed that the content of glucosinolates in transgenic plants was significantly higher than that of the wild type, and the content of ITCs produced by enzymatic hydrolysis of glucosinolates in transgenic plants was also correspondingly increased. However, it was found that the method of increasing the content of ITCs in plants by regulating the expression of single genes in the biosynthesis pathway was not ideal.

6.2 Multi-gene co-expression

Considering the complexity of ITCs biosynthesis, the pathway may involve synergistic effects between multiple key genes, so the multi-gene co-modification strategy may be a more effective approach. Zang *et al.*[127] synergistically expressed three of the key enzyme genes of ITCs biosynthesis in Chinese cabbage, *MAM1*, *CYP79F1*, and *CYP83A1*, which could promote the increase of the total glucosinolate content in Chinese cabbage to a certain extent, which not only achieved the continuous optimization of the flavor quality of Chinese cabbage, but also made it have better anticancer efficacy. Zhang[128] focused on the use of homologous recombination principle to realize the construction of the three-gene tandem co-expression vector XF350-B-C-E (*BCAT4*, *CYP79F1*, *ESM1*), and fully used the Agrobacterium-mediated transformation technology to make the recombinant vector further introduced into broccoli cells, and obtained a transgenic broccoli cell line carrying the co-expression of three genes through antibiotic screening. Compared with the wild-type cell line, the final concentration of SFN in this type of three-gene tandem co-expression cell line even reached 171.4 μg/g, and the mass fraction increased by 119%. Compared with the overexpression of a single *BCAT4-2* gene, the SFN mass fraction in transgenic plants increased by 23%. These results strongly confirm that multi-gene tandem co-expression has greater potential than single gene overexpression in increasing the content of ITCs. In conclusion, compared with the overexpression of a single gene, the strategy of co-overexpression of multiple genes in plants has shown greater possibility and superiority in increasing the content of ITCs in plants, but there are few relevant studies at present.

6.3 Heterologous expression of myrosinase

In general, the level of myrosinase activity within a species directly affects the total amount of ITCs catalyzed. However, it is not practical to extract sufficient amounts of highly active myrosinase directly from natural materials. Due to the rapid development of synthetic biology, heterologous expression technology can be used to heterologous the production of myrosinase with high activity from different species in other host systems suitable for expression. In view of the significant differences in myrosinase activity in different species, it is necessary to perform multiple optimization experiments for the enzymes of each species under specific reaction conditions to determine the optimal enzyme activity status. Because myrosinase can catalyze glucosinolates of different side chain groups, the selectivity of myrosinase for substrates varies among various species, and then ITCs with different side chain configurations are generated. This means that researchers need to carefully explore the interaction properties of myrosinase from different sources with substrates in order to effectively achieve high-yield heterologous biosynthesis of target ITCs.

In 1999, Chen and Halkier[129] achieved the first heterologous expression of the myrosinase gene *MYR1* from *Brassica napus* in the host of *Saccharomyces cerevisiae* I. NVSc1, which was the first example of myrosinase that does not bind to a natural lectin-like

protein. However, research analyses have shown that myrosinase expression can have some toxic effects on the host. In order to solve this problem, researchers Härtel and Brabdt[130] secreted and expressed the same myrosinase gene in *Pichia pastoris* GS115, which effectively solved the pre-existing host toxicity problem, and the enzyme activity after purification even increased to 175 U/mg; Xie's research[131] has successfully expressed the myrosinase gene derived from kale in *E. coli*, which to a certain extent reflects that the myrosinase derived from this source can be expressed in *E. coli*, but the enzyme activity has not been measured; Xiao[132] tried to express the myrosinase gene *MYR1* from rape in *E. coli*, and found that there was protein expression but no enzyme activity. Subsequently, by optimizing the induction culture conditions, the enzyme activity of 3.1×10^{-3} U/mg was found after the concentration of the cell disruption solution Zhang[133] used the *A. thaliana*-derived myrosinase *TGG1* gene to complete the intracellular expression in *P. pastoris*.

Studies have shown that enzyme activity with a mass fraction of about 25% can be detected in yeast media, which the researchers attribute to the presence of signal peptides in the protein encoded by the *TGG1* gene in *A. thaliana*; Rosenbergová *et al.*[134] modified the *TGG1* gene in *A. thaliana*, deleted its 57 bp native signal peptide and expressed it in *P. pastoris*, and the results showed that the enzyme activity and productivity were significantly improved, and the production efficiency was increased by as much as 40 times, and the myrosinase activity reached 27 U/mL, which also indicated that the increase in the heterologous expression activity of plant-derived myrosinase is of great significance for obtaining higher yields of ITCs.

Curiqueo *et al.*[135] cloned broccoli-derived myrosinase from *E. coli* and *S. cerevisiae*. The results showed that the thermostability of myrosinase produced in *S. cerevisiae* was about 10 °C higher than that produced in *E. coli*. The kinetic behavior of both enzymes is a Michaelis-Menten model. Compared with myrosinase produced in *E. coli*, the catalytic efficiency of myrosinase in *S. cerevisiae* is more than 4 times higher. Recently, Huang *et al.*[136] used eukaryotic and prokaryotic organisms to heterobiosynthesize SFN and achieved high-yield preparation. The myrosinase gene encoding *Brassica oleracea* (*BMYR*) and broccoli-derived myrosinase gene (*BoTGG1*) were cloned, and then heterologous expression was carried out in *P. pastoris* and *E. coli* respectively and catalyzed by recombinant enzyme degradation, and the final synthesis of SFN could reach a mass concentration of 1.21 g/L, and the molar conversion rate of SFN mass fraction was 99.6%, which was the highest level of ITCs preparation at present. The current research on the heterologous expression of myrosinase is shown in Table 1.

At present, plants are the main source of myrosinase expression research, and in many plants, myrosinase from broccoli, Arabidopsis, cabbage, kale, rape, papaya and other sources have been successfully scientifically expressed in *E. coli* or yeast, and most of them are expressed intracellularly. However, in industrial applications, the use of protein secretion and expression is preferred, as it simplifies downstream processing and makes purification easier. A problem with prokaryotic expression has been found in several studies that plant-derived myrosinase exhibits relatively low enzyme activity in *E. coli*, while enzymes from cabbage aphids and citrobacterium exhibit good myrosinase activity. The most fundamental reason for the expression difference caused by the existing plant-derived myrosinase is that there is no glycosylation modification in the prokaryotic expression system. At present, for the study of the heterologous expression of myrosinase, most of the aliphatic glucosinolates Sinigrin and glucophane are used as substrates to determine the enzyme activity, which is relatively simple, and more studies can be carried out on aliphatic glucoraphenin (GRE) and aromatic and indole glucosinolates as substrates in the future, so as to show the catalytic diversity of myrosinase and obtain ITCs with richer structure.

Table 1 Heterologous recombinant expression of myrosinase

Time	Gene source	Gene	Plasmid	Host
1999[130]	*B. napus*	*MYR1*	pYX223	S *cerevisiae* Ⅰ NVSc1
2002[131]	*B. napus*	*MYR1*	pGEM-T	*P. pastoris* GS115
2005[137]	*Brevicoryne brassicae*	*BMYR*	pET-28a(+)	*E. coli* BL21 (DE3)
2007[132]	*Brassica alboglabra* L. H. Bailey	*MY-ORF*	pET-28a(+)	*E. coli* BL21 (DE3)
2008[133]	*B. napus* L.	*MYR1*	pGEX-4T-1	*E. coli* BL21 (DE3)
2009[138]	*Carica papaya* L.	*CpTGG2*	pPIC3.5	*P. pastoris* GS115
2009[139]	*B. oleracea* L.	*BoMyr2*	pGEX-4T-1	*E. coli* BL21 (DE3)
2009[139]	*B. oleracea* L.	*BoMyr2*	pPIC9K	*P. pastoris* KM71
2010[140]	*C. papaya* L.	*CpTGG1*	pPIC3.5	*P. pastoris* GS115
2010[141]	*A. thaliana*	*TGG5*	pPIC3.5	*P. pastoris* GS115
2010[134]	*A. thaliana*	*TGG1*	pPIC3.5	*P. pastoris* GS115
2014[142]	*Armoracia rusticana*	*ArMY2*	pPIC3.5	*P. pastoris* GS115
2016[143]	*B. napus*		pET-28a(+)	*E. coli* BL21 (DE3)
2016[143]	*B. brassicae*	*BMYR*	pET-28a(+)	*E. coli* BL21 (DE3)
2021[84]	*A. thaliana*	*TGG1*	pPICZα A	*P. pastoris* KM71H
2021[134]	*A. thaliana*	*TGG1*	pPICZα A	*P. pastoris* KM71H
2021[122]	*A. thaliana*	*AtTGG4*	PINA1317	*Y. lipolytica* Po1h
2022[144]	*Citrobacter*	*CMYR*	pET-28b	*E. coli* BL21 (DE3)
2022[136]	*B. oleracea* L.	*MYR*	pMG1	*S. cerevisiae* MGY70
2024[145]	*B. brassicae*	*bmyr*	pET-28a(+)	*E. coli* BL21 (DE3)
2024[145]	*B. oleracea* L.	*BoTGG1*	pGEX-4T-1	*P. pastoris* GS115

6.4 Microbial chassis cell modification

Huang *et al.*[136] modified *E. coli* to catalyze the production of SFN from broccoli seed extract. The results showed that the reaction conditions for the catalytic production of SFN in whole cells of recombinant *E. coli* were 4 g/L of glucoraphanin concentration, and when the biomass was 5% at 45 °C and pH 4.5, the concentration of SFN catalyzed was 1.62 g/L, and the conversion rate reached 99.2%, and the substrate was almost completely transformed, but the operation stability was insufficient. Subsequently, carrageenan was used as a vehicle to immobilize cells of the *E. coli* BL21 (DE3)/pET-28a(+)-*bmyr* strain to successfully improve its operational stability.

Ho *et al.*[137] showed that engineered symbiotic microorganisms can produce active small molecule substances to inhibit the proliferation of tumor cells. The study engineered *E. coli* and specifically bound to heparan sulfate proteoglycans on colorectal cancer cells, and secreted myrosinase to successfully convert host-uptaken glucosinolates into small organic molecule SFNs with anticancer activity. *In vitro*, the engineered bacteria in combination with glucosinolates inhibited the proliferation of mouse, human, and colorectal adenocarcinoma cell lines by 95%.

The engineering transformation of microbial chassis, including gene editing, transcriptional regulation, protein expression, post-translational modification, metabolic engineering regulation, etc., makes *E. coli*, *S. cerevisiae* and *P. pastoris* become high-quality chassis hosts or engineering bacteria for protein production and natural product synthesis. However, at present, there are few studies on the modification of ITCs-producing microbial chassis cells, and more in-depth exploration and research are still needed in the future.

6.5 Research progress and shortcomings in the biosynthesis of ITCs

By utilizing genetic engineering technology, the production of ITCs can be increased by transforming plants or microorganisms[138]. These methods can improve the synthesis efficiency of ITCs by regulating the expression of related genes, such as genes involved in the metabolism pathways of myrosinase and glucosinolate. Through metabolic engineering strategies, the metabolic pathways of microorganisms or plants can be optimized to more effectively synthesize target ITCs. This includes regulating substrate supply, optimizing reaction conditions, and improving the selectivity and purity of the target product. Further research on the design and operation of bioreactors to achieve efficient production of ITCs. This includes controlling cultivation conditions such as pH, temperature, and nutrient concentration to maximize production efficiency and product quality[139].

Despite the development of biosynthetic methods, there are still issues with low production and insufficient purity of ITCs[140]. This limits its feasibility and economy in commercial applications. The metabolic pathways for the synthesis of ITCs by plants and microorganisms are usually complex and require precise regulation and optimization. In addition, intermediate products in some metabolic pathways may accumulate or decompose, affecting the stability and yield of the final product. Translating biosynthetic methods in the laboratory into large-scale industrial production still faces challenges[141]. This includes the scalability of the process, cost-effectiveness, and compliance with environmental sustainability requirements.

Although biosynthetic methods provide new prospects for the production of ITCs, further research and technological development are still needed to overcome current technological challenges and achieve their widespread application in industry and medicine.

7 Conclusions and prospects

M. oleifera Lam. and Brassicaceae are rich in nutrients and bioactive compounds, including glucosinolates and their secondary metabolites ITCs, which have been shown to reduce the risk of some noncommunicable diseases. However, the amount of ITCs in these plants is not sufficient for modern needs. Due to the lengthy biosynthetic pathway of ITCs in plants, its total synthesis is challenging. Therefore, the heterologous biosynthesis of ITCs using synthetic biology strategies has great potential to solve their supply problems. Over the years, significant progress has been made in microbial xenogeneous production of bioactive compounds such as carotenoids. The biosynthetic pathways of ITCs have been widely elucidated, and many enzymes have been found in these pathways. BCATs, MAMs, CYP, GST, UGT, SOT, and myrosinase are key molecules in the biosynthesis of ITCs. Many of these enzymes have been identified through genomics, transcriptomics, metabolomics, and biochemical characterization. Some proteins, such as myrosinase, exhibit broad substrate diversity and high catalytic activity. Many of these enzymes have been identified through genomics, transcriptomics, metabolomics, and biochemical characterization. Some proteins, such as myrosinase, exhibit broad substrate diversity and high catalytic activity. Going forward, screening of new sources of enzymes associated with the biosynthesis of moringa ITCs and directed evolution studies of known enzymes could help improve the yield of key products. On the basis of the in-depth analysis of the identification of key enzyme genes and their pathways in the biosynthesis of moringa ITCs, the inherent pathways of plant active ingredients were transferred, reconstructed and engineered. To achieve high yields of moringa ITCs through synthetic biology, we can obtain amino acid or nucleotide sequences with specific functions and construct recombinant plasmids of target genes. These plasmids can then be introduced into chassis cells such as *E. coli* or yeast to create engineered strains capable of producing the desired compounds; subsequent fermentation and process optimization will aim to produce high-yield moringa ITCs. This approach not only paves the way for the complete biosynthesis and scalability of moringa ITCs but also supports their future industrial production.

Conflicts of interest

The authors declare that there are no conflicts of interest in this work. Yang Tian is an editor of Food & Medicine Homology, but was not involved in the processing, peer review or decision making of this article.

Acknowledgement

This work was supported by the "A Study based on the Scientific Researches Fund Project of Yunnan Provincial Education Commission" (No. 2023J0430), Yunnan Province Ten Thousand Plan Industrial Technology Talents Project (YNWR-CYJS-2020-010), "Yunnan International Joint Laboratory of Green Health Food (China and Thailand)" (No. 202203AP140011), "Yunnan Province-City Integration Project" (No. 202302AN360002), and "Special Project for High-level Scientific and Technological Talents and Innovation Teams of

Yunnan Province" (No. 202305AS350025).

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