Natural Product Reports

ROYAL SOCIETY OF CHEMISTRY

REVIEW

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Cite this: *Nat. Prod. Rep.*, 2024, **41**, 649

Nitrile biosynthesis in nature: how and why?

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Covering: up to the end of 2023

Natural nitriles comprise a small set of secondary metabolites which however show intriguing chemical and functional diversity. Various patterns of nitrile biosynthesis can be seen in animals, plants, and microorganisms with the characteristics of both evolutionary divergence and convergence. These specialized compounds play important roles in nitrogen metabolism, chemical defense against herbivores, predators and pathogens, and inter- and/or intraspecies communications. Here we review the naturally occurring nitrile-forming pathways from a biochemical perspective and discuss the biological and ecological functions conferred by diversified nitrile biosyntheses in different organisms. Elucidation of the mechanisms and evolutionary trajectories of nitrile biosynthesis underpins better understandings of nitrile-related biology, chemistry, and ecology and will ultimately benefit the development of desirable nitrile-forming biocatalysts for practical applications.

Received 23rd June 2023

DOI: 10.1039/d3np00028a

rsc.li/npr

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1 Introduction

Nitriles (RC≡N, *i.e.*, organic cyanides) are a family of molecules containing one or more cyano groups consisting of a carbon atom joined to a nitrogen atom *via* a triple bond. The notorious use of cyanides as chemical warfare agents dates back to the Franco-Prussian War and was also seen in the World Wars I and II.[1] Despite the horrific toxicity, when casting our mind back to four billion years ago, atmospheric cyanides might have enabled the biological carbon metabolism from carbon dioxide to carbon-based compounds necessary for life.[2] Today, more than 400 natural nitrile compounds have hitherto been discovered from various origins across the plant, animal, and microbial worlds in both terrestrial and marine environments.[3–5]

According to the hybridization of the carbon atom in the nitrile group, natural nitrile compounds can be classified into four categories: alkyl nitriles, aromatic nitriles, α,β-unsaturated nitriles, and miscellaneous nitriles (Fig. 1). Alkyl nitriles are characterized by a cyano group (–CN) attached to an alkyl chain. These compounds can vary in terms of the size, structure, and modifications of the alkyl chain. Three review articles published in 1999, 2021 and 2022 have summarized the nitrile compounds derived from natural sources by focusing on their structures, producing organisms, bioactivities, *etc*[3–5]. Examples include 1-cyano-4,5-epithiopentane (**1**),[6] long-chain aliphatic compounds (**2** and **3**),[7–9] albanitrile A (**4**),[10] and amino nitriles such as lahadinine B (**5**),[11,12] 6″-cyano-6″-deoxy-TAN-1120 (**6**),[13] renieramycin M (**7**),[14,15] and ecteinascidin 770 (**8**).[16,17] Aromatic nitriles contain a cyano group attached to an aromatic ring.

Examples of aromatic nitriles include dicyanoazulene (**9**),[18] auranthine (**10**),[19] cyanosporaside A (**11**),[20] and hamigeran R (**12**).[21] α,β-Unsaturated nitriles feature a cyano group connected to a carbon–carbon double bond (C=C) system at the α,β-position. Some examples are compound **13**,[22] nitriloside compounds such as menisdaurin D (**14**),[23] campyloside C (**15**),[24] as well as simmonoside A (**16**),[25] ambiguinine G (**17**),[26] benthocyanin C (**18**),[27] borrelidin (**19**),[28,29] and calyculin A (**20**).[30,31] Miscellaneous nitriles encompass a diverse range of compounds that do not fall neatly into the previous categories, such as alkaloid derivative (**21**),[32] axinynitrile A (**22**),[33] 12-*epi*-ambiguine B (**23**),[34] and cyanoformamide (**24**).[35,36] Natural nitriles can be found across various natural product classes, including alkaloids, terpenes, macrolides, and glycosides. The most common naturally occurring nitriles are cyanogenic glycoside toxins, which are found pervasively in thousands of plant species as well as in cyanogenic bacteria, fungi, and arthropods.[37] Biological nitriles as specialized metabolites perform diverse physiological and ecological roles[38] by participating in chemical defense against predators/attackers, nitrogen supply in primary metabolism, bride price for mating, regulation of the biotic community, *etc*[39] (Fig. 2).

Nitriles have also become an indispensable part of human life as versatile molecules broadly applied in pharmaceutical and chemical industries.[40,41] More than 70 nitrile-containing pharmaceuticals have hitherto been clinically approved for treatment of a wide array of diseases, such as saxagliptin for the type 2 diabetes mellitus, anastrozole for breast cancer, glasdegib for acute myeloid leukemia, and verapamil for arrhythmia, hypertension, and angina.[42] Cyano groups in these drugs contribute to improving the molecules' pharmacokinetic and pharmacodynamic profiles, including improved water solubility, increased system exposure, prolonged half-life time, and enhanced bioavailability, benefiting from different types of interactions of nitriles (noncovalent, hydrogen bonding, hydrophobic interactions, covalent contacts, *etc.*) with macromolecular targets.[40] Introduction of the cyano group in a compound has become an attractive approach for designing lead compounds. Moreover, the cyano group, existing in many synthetic intermediates or precursors in the chemical industry, is a valuable and readily available functional group for preparation of amines, ketones, and carboxylic acids.[41] Thus, deeper understanding of nitrile natural products and their natural origins will provide significant insights into efficient (bio) synthetic patterns of valuable nitriles and reasonable usage of the cyano moiety in commodity chemicals and medicines.[43–45]

Nitrile biosynthesis in nature mainly follows the aldoxime-nitrile pathway, in which aldoxime dehydratases play a central role (Fig. 3).[46] Aldoxime dehydratases catalyze the dehydration of aldoximes (RCH=N–OH) to the corresponding nitriles (RC≡N). In plants and animals, this process is normally mediated by cytochrome P450 (CYP) enzymes; while in bacteria and fungi, it is achieved by heme-containing aldoxime dehydratases, which are usually referred to as Oxds. Another important route of nitrile biosynthesis is conducted by hydroxynitrile lyases (HNLs) in plants, microorganisms, and arthropods.[47] HNLs catalyze the reversible addition of hydrogen cyanide (HC≡N) to aldehydes or ketones to produce cyanohydrins.[48] Besides, simple nitriles and epithionitriles can be generated during glucosinolate (GSL) breakdown with the aid of myrosinases and specifier proteins.[49] Other unusual pathways, including carboxylic acid-nitrile and type I nitroreductase-

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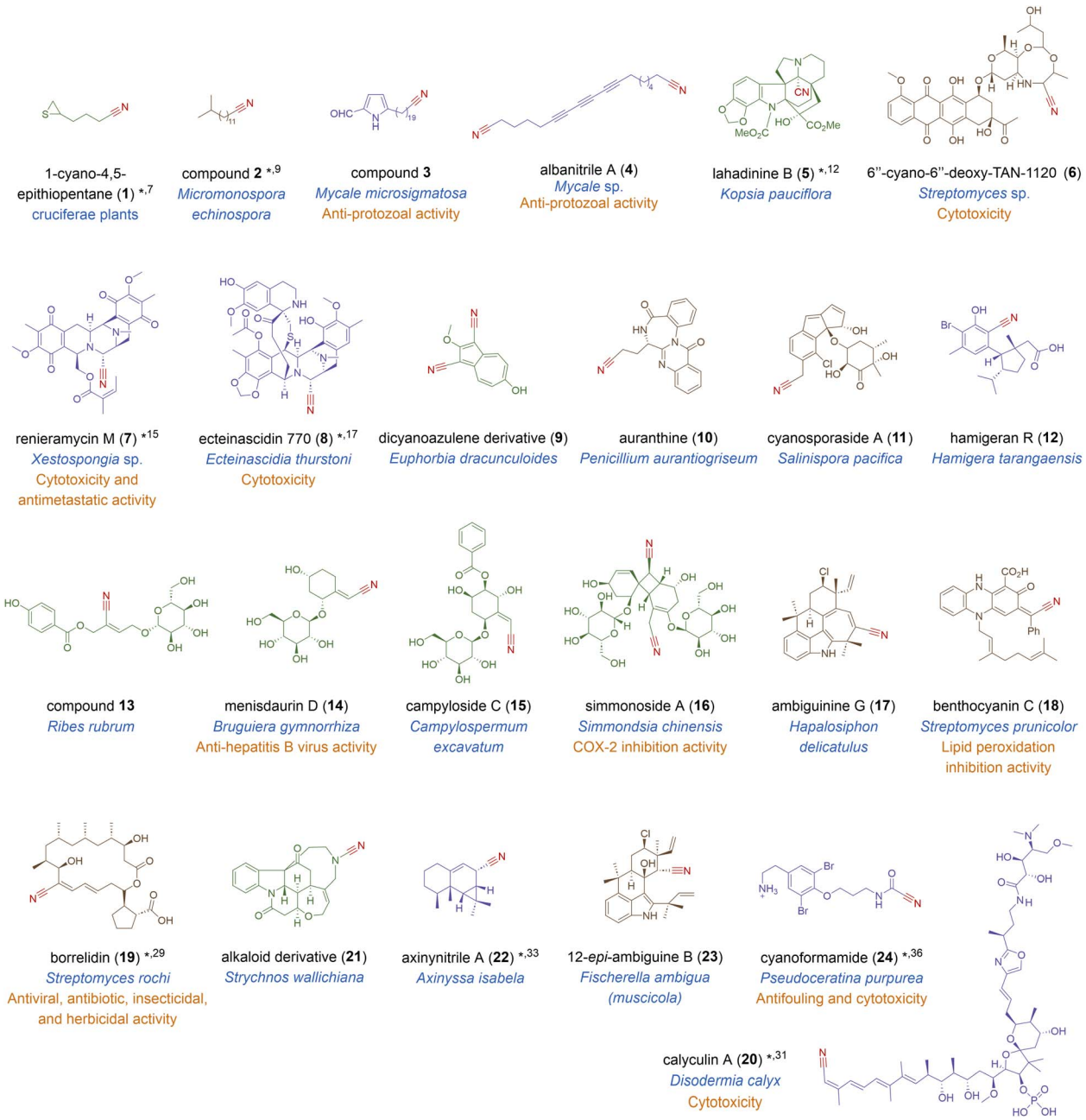

Fig. 1 Representative natural nitrile compounds. The nitrile groups are highlighted in red. The different backbone colours indicate the compound origin: green for plant, brown for microorganism, and purple for animal. Producing organisms (in blue), reported activities (in orange), and available total syntheses (noted with asterisks and references) of the nitrile compounds are listed.

mediated pathways, sporadically exist in different species.[50,51] These characterized nitrile-forming enzymes have been unveiling the rules of nitrile biosynthesis in nature and showing increasing potential in industrial syntheses of valuable nitrile compounds.[52]

It is worth noting that isonitriles, a distinct group of compounds containing a nitrogen-carbon triple bond (–N≡C), are also significant in the realm of natural products. Notable examples include xanthocillin (**25**), which is a terpenoid isolated from the fungus *Penicillium notatum* and represents the first characterized natural isonitrile,[53] and axisonitrile-1 (**26**) derived from the marine sponge *Axinella cannabina*.[54] Additionally, isocyalexin A (**27**), the first and only isocyanide of plant origin, was identified from UV-irradiated rutabaga roots (Fig. 4).[55] This review specifically concentrates on the biosynthesis of nitriles, as the biosynthetic pathways of isonitriles and nitriles are distinct from each other. Furthermore, there have been several comprehensive reviews on isonitrile biosynthesis.[56,57]

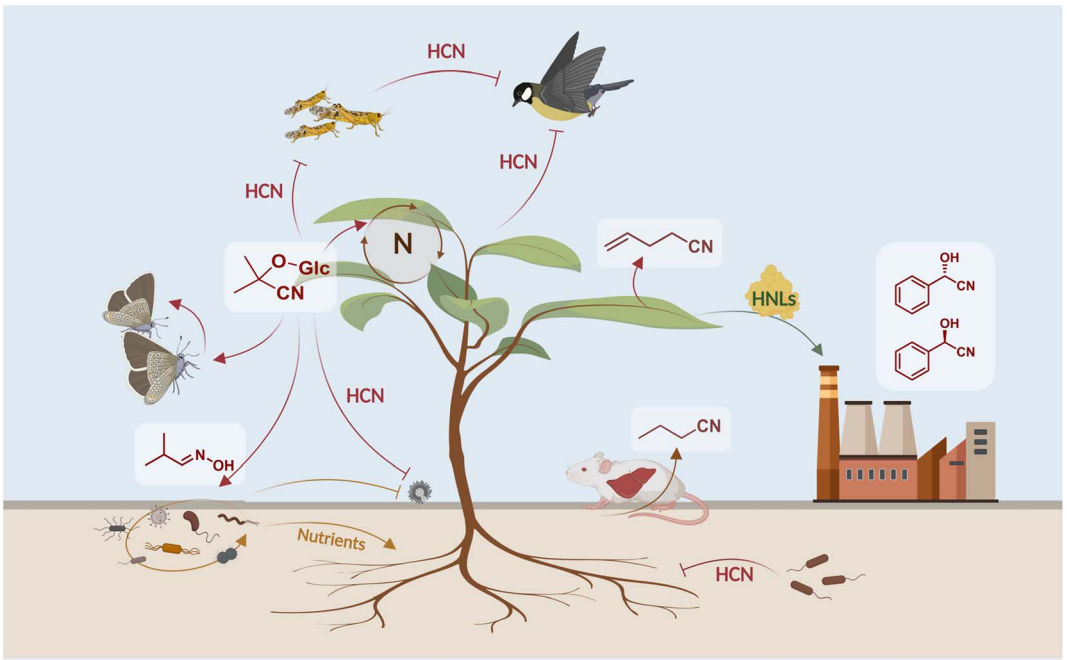

Fig. 2 The nitrile cycle in nature.

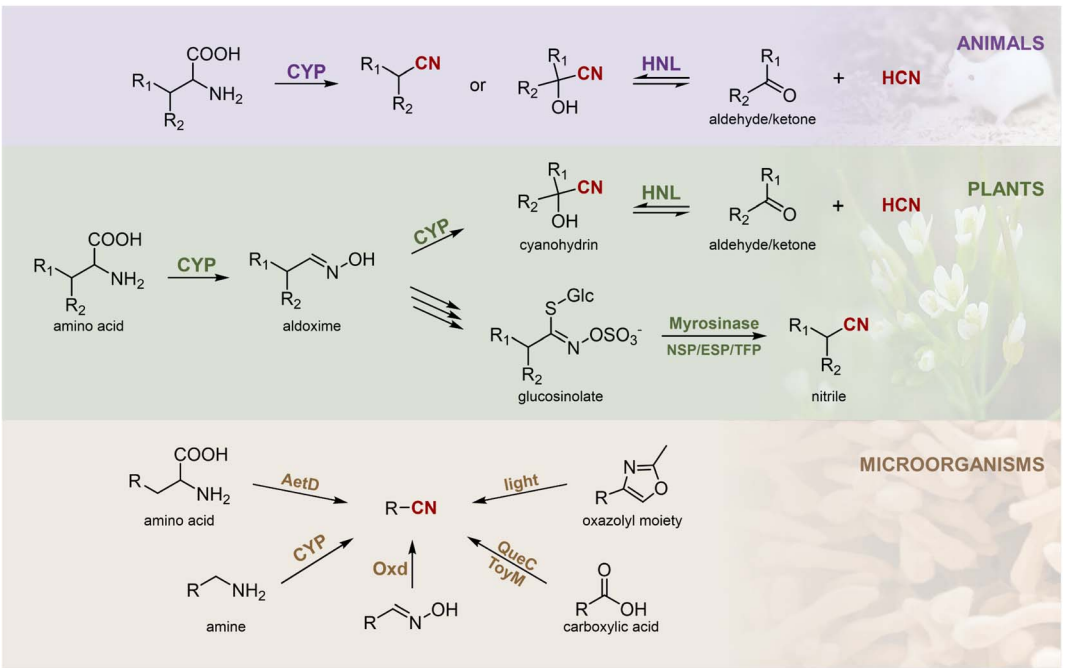

Fig. 3 Biogenesis of nitriles in plants, microorganisms, and animals. CYP, cytochrome P450 enzyme; Oxd, aldoxime dehydratase; HNL, hydroxynitrile lyase; NSP, nitrile-specifier protein; ESP, epithio-specifier protein; TFP, thiocyanate-forming protein.

2 Nitrile biosynthesis in plants

Plants have evolved a complex set of chemical defense strategies, producing toxins and feeding deterrents that act as direct defenses to herbivores or attractants to induce herbivore enemies as indirect defenses, such as cyanogenic glycosides (CNglcs) and glucosinolates (GSLs).[58–60] Nitrile biosynthesis in plants is tightly tied to the metabolism of these molecules, and the major types of involved enzymes are oxime-metabolizing CYPs, myrosinases and specifier proteins, and HNLs (Fig. 3).

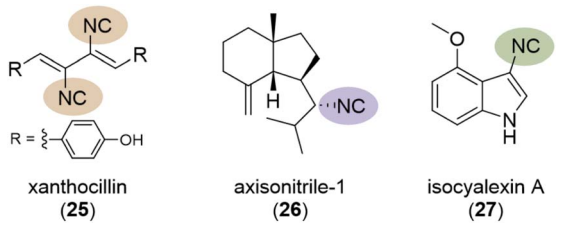

Fig. 4 Representative natural isonitrile compounds.

2.1 Oxime-metabolizing CYPs

2.1.1 Functions and properties of CYPs in nitrile biosynthesis. CNglcs are widely dispersed in more than 3000 plant species, spanning over 130 different plant families of monocotyledones, eudicotyledones, and ferns, as well as some cyanogenic arthropods. More than 112 CNglcs have hitherto been found, serving as one of the most important naturally occurring defensive secondary metabolites consisting of an α-hydroxynitrile aglycone linked to a sugar moiety; for example, dhurrin (**28**), taxiphyllin (**29**), prunasin (**30**), and lotaustralin (**31**), and linamarin (**32**)[5] (Fig. 5A). The content of CNglcs varies significantly among plant species, and even within the same species; temporal and spatial variations occur due to factors such as growth patterns, domestication level, and environmental conditions.[61]

The first and best understood natural nitrile biosynthetic pathway is that of CNglcs in plants. The overall pathway usually requires three genes, encoding two membrane-bound multifunctional CYPs and a soluble UDP-glycosyltransferase (UGT). Despite the wide existence and structural diversity of CNglcs, the pathway merely begins with five hydrophobic proteinogenic amino acids (L-isoleucine, L-leucine, L-phenylalanine, L-tyrosine, and L-valine) and a nonproteinogenic amino acid cyclopentenyl glycine.[60,62,63] In the first step, the amino acid-metabolizing CYP, which typically belongs to the CYP79 family, produces an aldoxime by *N*-hydroxylation of the amino group of the amino acid precursor, oxidative decarboxylation, and dehydration in a rate-limiting way. CYP79s are evolutionarily conserved and can be easily differentiated from other CYPs by distinct substitutions in the conserved heme-binding domain and the "PERF" motif.[64] The other CYP, which usually comes from the CYP71 clan, functions in the second oxime-metabolizing step. In most cases, CYP71s catalyze (1) the geometrical transformation of oxime from *E* to *Z* configuration, (2) the dehydration of *Z*-oxime into nitrile, and (3) the α-hydroxylation of nitrile. Interestingly, CYP71s that only mediate the first two steps have also been identified, such as CYP71B40v3, CYP71B41v2, and CYP71AT96.[65,66] In other words, the formed nitriles could serve as either end products or intermediates which undergo further conversions into acids, amides, alcohols, aldehydes, and/or esters (Fig. 5A). The whole CYP71 family forms the largest CYP family in plants, whereas the members able to convert aldoximes into nitriles are in a quite tiny minority.[67] Interestingly, nature evolutionarily sets a constraint of substrate specificity only on CYP79s. Unlike CYP79s focusing on one or two homogeneous amino acids, CYP71s are usually tolerant towards diverse oximes with aliphatic and aromatic side chains.

The subsequent glycosylation of cyanohydrin is catalyzed by UGTs, resulting in stable CNglcs as a storage form. When organisms are attacked, CNglcs, as the substrates of HNLs, are

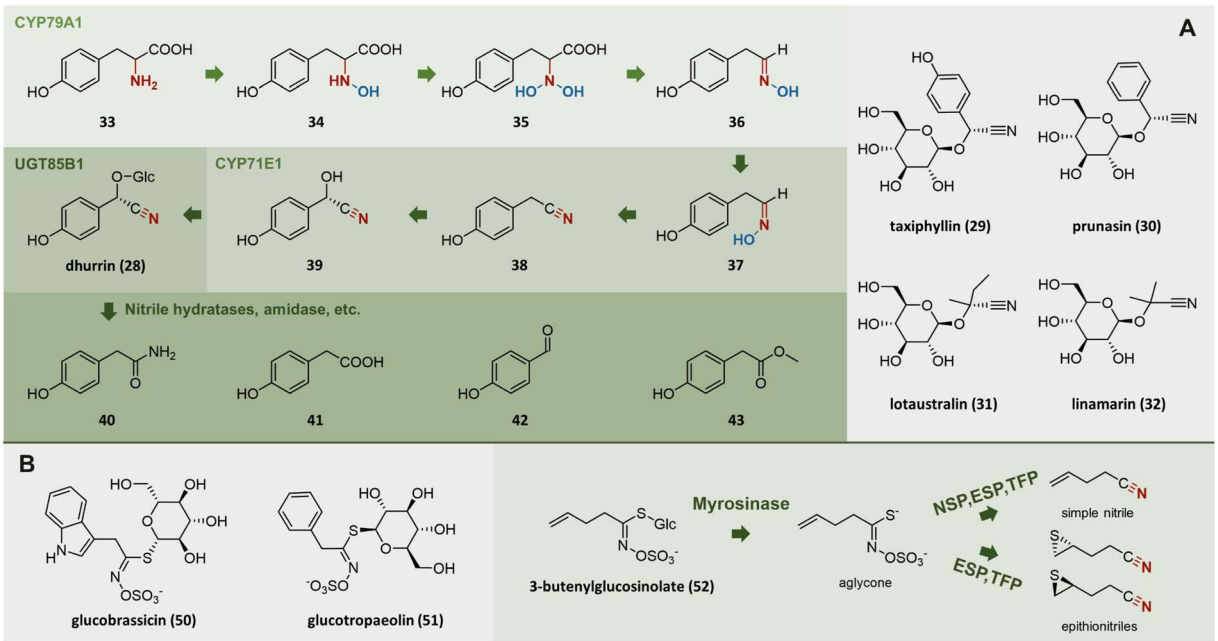

Fig. 5 Examples of plant-derived nitriles and representative biosynthetic pathways. (A) Examples of cyanogenic glycosides and the representative biosynthetic pathway of dhurrin in *Sorghum bicolor*. (B) Examples of glucosinolates and the breakdown process of 3-butenylglucosinolate in *Arabidopsis thaliana*.

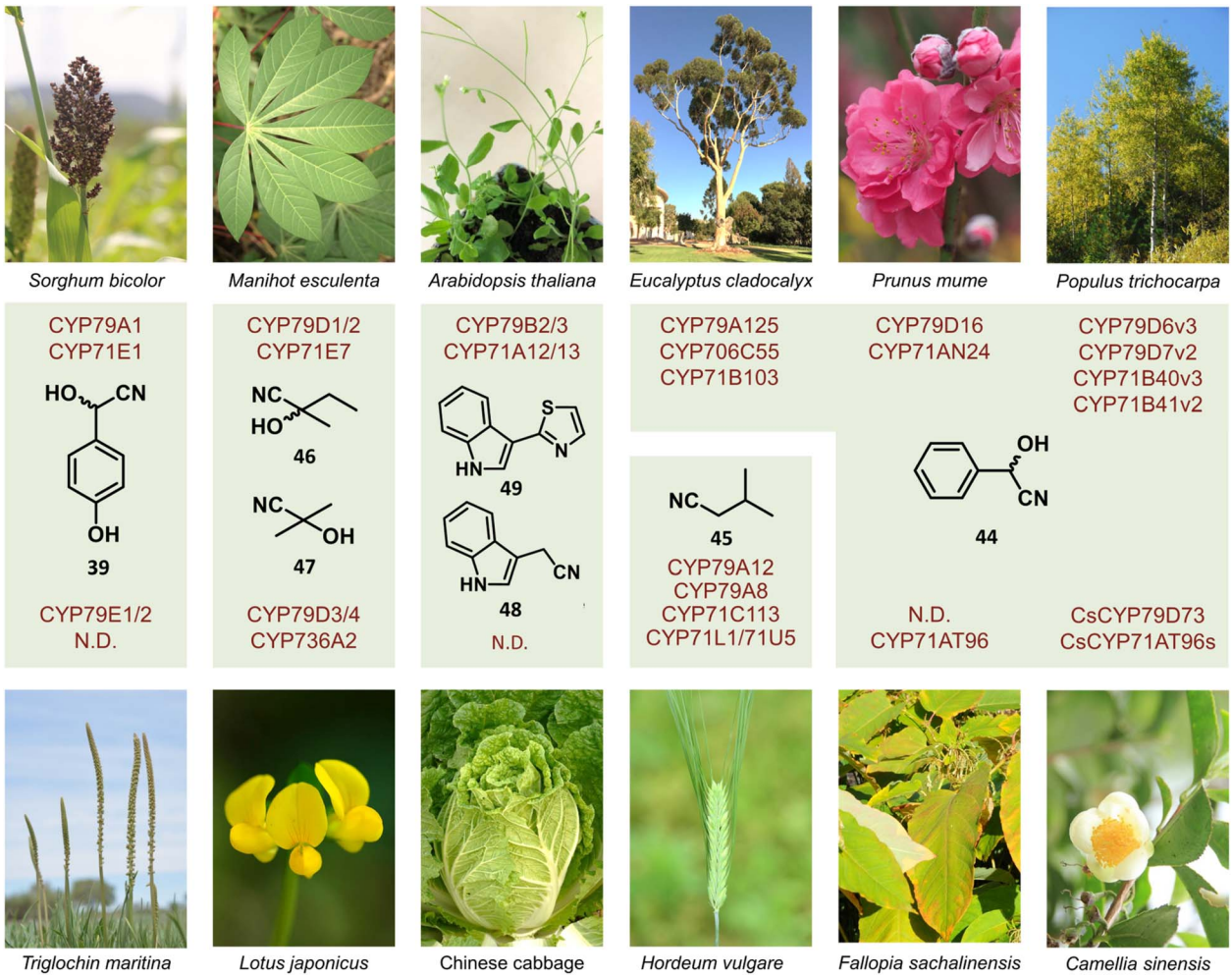

Fig. 6 Overview of CYPs involved in the plant nitrile biosynthesis from six amino acid precursors. N.D., enzyme not determined.

dissociated into the corresponding aldehyde/ketone and defensive hydrogen cyanide.[68] The organization of the CNglc biosynthetic pathway within a metabolon (assembly of the biosynthetic enzymes) improves the catalytic efficiency by bringing cooperative active sites spatially closer, thus avoiding undesirable leakage of the toxic intermediates.[69,70] Rather than being a constitutively expressed defense system, cyanogenesis in plants is immediately induced upon tissue disruption by attackers, as a result of de-compartmentalization. The compartmentalization of CNglcs and the metabolizing enzymes at the tissue or subcellular levels have been demonstrated in many species, which is essential to avoid autotoxicity and to serve as a precise return fire to attackers.[71] In the leaves, CNglcs are usually located in isolated vacuoles, while within the stems and petioles, CNglcs are confined to vesicle-like structures in the latex (*e.g.*, linamarin from cassava).[72] The locations of the hydrolytic β-glycosidases vary among different species, including the apoplastic space, cytoplasm, chloroplast, small vesicles, and cell wall.[73,74] The two components come into contact upon tissue disruption. Therefore, the defensive role of CNglcs in plants is spatially and developmentally regulated.

2.1.2 Examples of oxime-metabolizing CYPs. The enzymes for plant nitrile biosynthesis were firstly identified in the great millet *Sorghum bicolor*[75,76] (Fig. 6). The biosynthesis of dhurrin begins with the conversion of tyrosine (**33**), through *N*-hydroxytyrosine (**34**) and *N*,*N*-dihydroxytyrosine (**35**) into (*E*)-*p*-hydroxyphenyl acetaldoxime (**36**) by CYP79A1 (EC 1.14.14.36), and CYP71E1 (EC 1.14.14.37) catalyzes the following isomerization, dehydration, and hydroxylation to produce (*Z*)-*p*-hydroxyphenyl acetaldoxime (**37**), *p*-hydroxyphenylacetonitrile (**38**), and *p*-hydroxymandelonitrile (**39**)[77–80] (Fig. 5a). The same process is present in the biosynthesis of taxiphyllin and triglochinin in seaside arrow grass *Triglochin maritina*. CYP79E1 and CYP79E2 are functionally equivalent to *S. bicolor* CYP79A1. Though the dehydratase has yet to be identified, considering the close interaction and high conservation of CYP79s from *T. maritima* and *S. bicolor*, the presence of a CYP71E1 counterpart in *T. maritima* can be promisingly predicted.[81] Compound **39** can further be converted into *p*-hydroxyphenylacetamide (**40**), *p*-hydroxyphenylacetic acid (**41**), *p*-hydroxybenzaldehyde (**42**), and benzoic acid ester (**43**) for other metabolisms *in vivo* by nitrile hydratases, amidase, *etc.*

CYP71AN24 (EC 1.14.14.44), identified in Japanese apricot *Prunus mume*, catalyzes the conversion of phenylacetaldoxime (PAOx) into 2-hydroxy-2-phenylacetonitrile (mandelonitrile, MAN, **44**), after CYP79D16 (EC 1.14.14.40) converts phenylalanine into PAOx.[82] CYP71AT96 in the giant knotweed *Fallopia sachalinensis* was identified to catalyze the conversion from *E*/*Z*-PAOx to phenylacetonitrile (PAN).[66] In eucalypt tree *Eucalyptus cladocalyx*, CYP79A125 mediates the initial transformation of phenylalanine into PAOx. However, interestingly, the conversion of aldoxime into cyanohydrin requires two distinct CYPs (*viz.* CYP706C55 and CYP71B103) instead of a single multifunctional CYP71 used by other higher plants. CYP706C55 catalyzes the dehydration of PAOx to PAN, while the subsequent hydroxylation to form MAN is achieved by CYP71B103. CYP706s were previously characterized to catalyze C-hydroxylation reactions. For example, CYP706B1 from cotton *Gossypium arboruem* catalyzing the hydroxylation of δ-cadinene at C_8.[83–85] In *E. cladocalyx,* CYP706 demonstrates a new function of catalyzing the cyanidation reaction. This phenomenon supports the independent evolution hypothesis for CNglc biosynthesis in several plant lineages.[86,87] CsCYP71AT96s in oolong tea *Camellia sinensis* converts PAOx, which originates from phenylalanine by CsCYP79D73, into PAN.[88] In balsam poplar *Populus trichocarpa*, CYP79D6v3 and CYP79D7v2 unusually participate in the aldoxime formation from six different amino acid precursors.[89] CYP71B40v3 and CYP71B41v2 convert *E*/*Z*-PAOx, *E*/*Z*-2-methylbutyraldoxime, and *E*/*Z*-3-methylbutyraldoxime into PAN, 2-methylbutyronitrile, and 3-methylbutyronitrile, respectively.[65] In loquat (*Rhaphiolepis bibas*) flowers, PAN formation from (*E*/*Z*)-PAOx is catalyzed by CYP77A59,[90] which reveals a new function of CYP77 family other than fatty acid in-chain hydroxylation and epoxidation.[91,92]

In barley *Hordeum vulgare* L., five multifunctional CYPs, including two CYP79s and three CYP71s, have been identified. Either CYP79A12 or CYP79A8 mediates the formation of *E*/*Z*-3-methylbutyraldoxime from leucine. CYP71C113, CYP71L1, and CYP71U5 achieve the production of isovaleronitrile (**45**).[93] Conversions from isoleucine or valine to lotaustralin and linamarin have been identified in cassava *Manihot esculenta* and legume *Lotus japonicus* by different CYPs. In cassava, CYP79D1 and CYP79D2 (EC 1.14.14.38/39) both catalyze the first committed step, turning isoleucine and valine into 2-methylbutanal oxime and 2-methylpropanal oxime, respectively.[94] CYP71E7 (EC 1.14.14.41) then generates butanone cyanohydrin (**46**) and acetone cyanohydrin (**47**). CYP71E7 can also catalyze the conversion of tyrosine and phenylalanine-derived PAOx, albeit with lower efficiency.[95] In *L. japonicus*, the whole process is mediated by CYP79D3/D4 and CYP736A2.[96,97]

In banana leaves, a soluble indolyl-3-acetaldoxime (IAOX, **48**) dehydratase was discovered.[98] The conversion of IAOX to indole-3-acetonitrile (IAN) was also detected *in vivo* in Chinese cabbage seedlings and the indolyl-3-acetaldoxime dehydratase was identified by using [^{14}C]-IAOx as substrate.[99–101] Camalexin (**49**) is an important indole alkaloid phytoalexin produced by *Arabidopsis thaliana* that shows resistance to necrotrophic fungal pathogens.[102] The nitrile-forming part in the biosynthesis of camalexin is similar to that of CNglcs. In *A. thaliana*, the first step from tryptophan to IAOx is catalyzed by CYP79B2 and CYP79B3 (EC 1.14.14.156).[103] Then, CYP71A12 [104] or CYP71A13 (EC 4.8.1.3)[105] mediates the dehydration of IAOx to generate IAN. IAOx constitutes a metabolic branch point among indole glucosinolates, indole-3-carboxylic acid, and camalexin biosynthesis. Whereas, how these pathways are organized and how much IAOx is dedicated to the nitrile-forming pathway remain unclear.[106,107]

2.1.3 Metabolic engineering of CNglcs biosynthesis. With the increasing understanding of CNglcs biosynthesis and advancements in genetic engineering, it is possible to employ metabolic engineering techniques to enhance commercially valuable plants by conferring them resistance against herbivores/pathogens and improving their nutritional value. Manipulation of the CNglcs contents has been achieved in various plant species, including cassava, sorghum, barley, lotus, tobacco, and arabidopsis.[108] For instance, the biosynthetic pathway of dhurrin from sorghum was successfully transferred to *A. thaliana*, a non-cyanogenic model plant, through metabolic engineering. The introduction and accumulation of significant amounts of dhurrin in transgenic *A. thaliana* plants do not exhibit noticeable phenotypic differences or inherent physiological drawbacks. Notably, this metabolic engineering approach empowers *A. thaliana* with resistance against *Phyllotreta nemorum*, a crucifer-specialist flea beetle, thus highlighting the effectiveness of cyanogenic glucosides in pest control.[109]

Cassava (*M. esculenta*), a highly important root crop globally, faces challenges related to low tuber protein content and the presence of toxic cyanogenic glucosides such as linamarin and lotaustralin. To address these issues, RNA interference was employed to suppress the expression of CYP79D1 and CYP79D2, which are involved in the production of linamarin and lotaustralin in cassava. This genetic intervention resulted in transgenic cassava plants with a remarkable 92% reduction in cyanogenic glucoside content in their tubers, along with nearly acyanogenic leaves. Consequently, this engineered process not only lowers the levels of cyanogenic glucosides in cassava tubers for safer consumption, but also avoids the loss of nutritional components such as proteins, vitamins, and minerals that may be caused by complex food processing required to remove cyanogenic glucosides.[110]

2.2 Hydroxynitrile lyase

Hydroxynitrile lyases (HNLs, EC 4.1.2.10/11/46/47/B6), as a key component of the cyanogenic pathway, have been found in plants, bacteria, and arthropods (cyanogenic millipedes).[111–114] HNLs belong to the aldehyde lyases and mediate the cleavage reaction of cyanohydrins, giving rise to the corresponding aldehydes/ketones and defensive agent hydrogen cyanide. Of note, the reverse reactions can also be achieved by HNLs, forming enantiomerically pure cyanohydrins (*i.e.*, α-hydroxynitriles).[115] As cyanogenesis can take place in a variety of cyanogenic plant species through the chemical decomposition of cyanohydrins, this process results in the production of cyanide without the involvement of HNLs, solely through a gradual chemical transformation. Therefore, even though cyanogenic

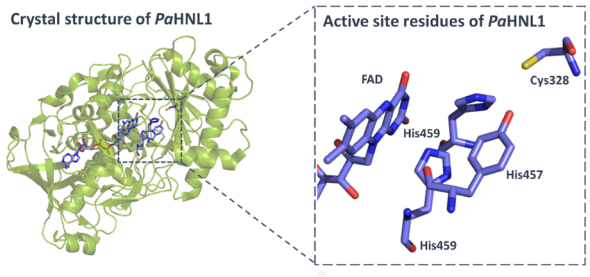

Fig. 7 Crystal structure and active site residues of PaHNL1 (PDB ID code: 1JU2).

defense mechanisms are widely employed by thousands of plant species, the involvements of HNLs are quite limited, and the investigations of novel HNLs and their enzymatic characteristics are still growing.

HNLs from different sources all catalyze the nitrile-forming reaction, but the substrate preferred, enantioselectivity to cyanohydrins, and catalytic mechanisms behind may vary case by case. For example, HbHNL (from *Hevea brasiliensis*) and MeHNL (from *M. esculenta*) catalyze the stereoselective synthesis of (*S*)-cyanohydrins, while AtHNL (from *A. thaliana*) and PeHNL (from *Passiflora edulis*) are dedicated to producing (*R*)-MAN. HNLs are evolved from ancestors of diverse protein classes, therefore comprising a heterogenous group of enzymes with distinct biochemical properties (sequences and topologies, molecular weights, pH optimum conditions, kinetic properties, *etc.*) as a result of convergent evolution. Many HNLs possess diverse primary structures due to the scarcity of homologies among their amino acid sequences, based on which HNLs are classified into seven superfamilies, including α/β-hydrolases fold (AtHNL, BmHNL, HbHNL, MeHNL, and SbHNL), Bet v1 like fold (DtHNL), cupin (AcHNL, BpHNL, GtHNL, and PsmHNL), dimeric α+β barrel (PeHNL), FAD-binding oxidoreductase (EjHNL, PaHNL, PmHNL, and PsHNL), lipocalin-like fold (ChuaHNL and PlamHNL), and Zn^{2+}-containing alcohol dehydrogenase (LuHNL).[116,117]

Cyanohydrins serve as the fundamental units for numerous reactions and also form the basis for various compounds utilized as pharmaceuticals, agrochemicals, and fine chemicals. As an efficient, environmentally friendly, and highly stereoselective biocatalyst, HNLs have received much attention for a long time. Some HNLs have been successfully overexpressed in *Escherichia coli*, *Pichia pastoris*, and *Saccharomyces cerevisiae*. Further endeavors in enzyme engineering and fermentation condition optimization render HNLs well-established for large-scale industrial applications. The asymmetric syntheses of (*R*)-MAN and (*S*)-MAN in preparative scales have been extensively studied. A classical example is the FAD-dependent HNL from bitter almonds (PaHNL), which catalyzes the formation of (*R*)-MAN from HCN and benzaldehyde, representing a model reaction. This reaction has been widely utilized in the chemical industry as one of the few efficient enzyme-mediated chiral nitrile formations, due to its broad applicability and ease of availability.[118] Further modifications of PaHNL and reaction conditions have been carried out for decades. Immobilization of PaHNL on solid supports, *e.g.* cellulose and silica beads, has enabled the continuous synthesis in a methanol–water mixture.[119] Utilization of HNL immobilization in different organic solvents and biphasic buffer-organic solvent systems is a useful strategy to increase PaHNL's catalytic efficiency and enantioselectivity. Applications of ionic liquids (EMIM·BF_4, PMIM·BF_4, and BMIM·BF_4) in biphasic solvent systems with aqueous buffer have achieved higher conversion rates.[120] The crystal structure of PaHNL1 (one isoform of PaHNL) was solved to bring more insights into its catalytic mechanism and key amino acid residues.[121] The overall protein fold and active-site architecture of PaHNL1 suggests that it belongs to the family of glucose–methanol–choline oxidoreductases, characterized by an FAD-binding domain and a substrate-binding domain. The active site of PaHNL1 is composed of specific amino acid residues, namely Cys_{328}, Tyr_{457}, His_{459}, and His_{497} (Fig. 7). These residues play vital roles in the catalytic activity of the enzyme. His_{497} serves as a general base, facilitating the abstraction of a proton from the hydroxyl group of the MAN substrate. Cys_{328} and Tyr_{457} both function as hydrogen bond donors to the MAN hydroxyl group. His_{459} is responsible for the protonation of the cleaved cyanide ion during the catalytic cycle. Moreover, many efforts have been made in the directed evolution and (semi) rational design of PaHNL, such as the Leu1Gln and Ala111Gly mutations of PaHNL5 that improved its productivity and low-pH stability in *P. pastoris*, as well as the Val360Ile and Val317Ala mutations which enhanced its enantioselectivity and conversion rate.[122,123]

2.3 Myrosinases and specifier proteins

Besides the aldoxime-nitrile network, the glucosinolate–myrosinase system is another metabolic pathway that produces nitriles and plays important roles in mediating plant growth and interaction with the biotic environment[124] (Fig. 5B). The glucosinolates (GSLs) form a large and diverse family of defensive metabolites abundant in Brassicaceae family, for example glucobrassicin (**50**), glucotropaeolin (**51**), and 3-butenylglucosinolate (**52**). The common structure of GSLs comprises a β-D-thioglucose group, a sulfonated oxime moiety, and a variable amino acid-derived side chain.[125] The chemical diversity of more than 100 GSLs mainly comes from different precursor amino acids, side-chain elongations, and extensive side-chain modifications.[126] Specifically, aliphatic GSLs originate from alanine, valine, leucine, isoleucine, methionine, or glutamate, aromatic GSLs are made from phenylalanine and tyrosine, and indole GSLs are derived from tryptophan. Further side-chain elongations and modifications lead to the insertion of various side chains such as sulfur-containing side chain, aromatic side chain, indole side chain, ω-hydroxyalkyl side chain, aliphatic straight/branched side chain with C=C double bond(s), hydroxyl group, carbonyl group, and varied glycosylated side chain.

The intact GSL itself is considered to be biologically inactive, and its activation occurs upon tissue damage. Myrosinases (thioglucoside glucohydrolases, EC 3.2.1.147) generally catalyze

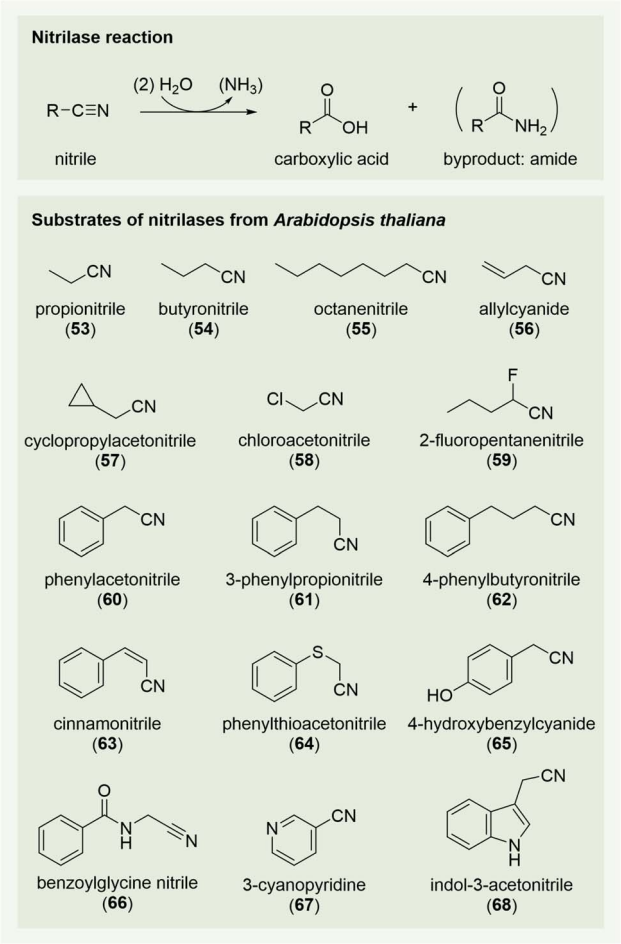

Fig. 8 (A) Nitrilase reaction. (B) The substrates of nitrilases from *Arabidopsis thaliana*.

the initial step of the bioactivation by hydrolyzing GSL into an unstable aglycone (thiohydroximate-*O*-sulfate) intermediate, which spontaneously turns into the toxic isothiocyanate.[127] Hydrolysis can also be rechanneled towards alternative breakdown products (simple nitriles, epithionitriles, and thiocyanates) from the default route under certain conditions: (1) the presence of nitrile-specifier protein (NSP, EC 3.2.1.147), epithiospecifier protein (ESP, EC 3.2.1.147), and/or thiocyanate-forming protein (TFP) leads to the formation of corresponding simple nitriles in the presence of ferrous ion at pH 2–5; and (2) epithionitriles are generated by an ESP or TFP from alkenyl GSL with a Fe^{2+}-dependent mechanism at pH < 6.5.[128] Specifically, NSPs facilitate the generation of simple nitriles regardless of the diversified GSL side-chains.[129] ESPs enable the epithionitrile formation when the GSL side chain contains a terminal double bond, and otherwise promotes the nitrile formation.[130] TFPs share a similar reactivity as the aforesaid proteins except for the additional ability to form organic thiocyanates.[131]

The GSL hydrolysis products, especially isothiocyanates, show defensive and deterrent activities against a rather wide range of herbivores, such as birds, rabbits, plant pathogens, and non-adapted insects.[132–134] However, some herbivores, particularly crucifer-feeding Pierinae, are observed to feed on chemically defended plants without apparent negative effects by diverting the glucosinolate–myrosinase system as evolved counteradaptations.[135,136] For example, in the gut of *Pieris rapae* larvae, the presence of an NSP circumvents the release of the glucosinolate hydrolysis products from noxious isothiocyanates to less toxic nitriles, which are excreted in the feces, either unchanged or after further metabolism.[137] In human gut microbiota, evidence of myrosinase-like activity and glucoraphanin hydrolysis to bioactive metabolites, including sulforaphane nitrile and erucin nitrile, has been reported both *in vitro* and *in vivo*. However, the knowledge of specific bacterial myrosinases is still limited.[138,139]

Following the NSP-mediated formation of simple nitriles, a subsequent hydrolysis reaction catalyzed by nitrilases is proposed in the glucosinolate catabolism pathway. This enzymatic process provides a means to mobilize both sulfur and nitrogen atoms into primary metabolism, without exposing to toxic isothiocyanates and necessitating the consumption of glutathione.[140,141] In *A. thaliana*, the nitrilases NIT1-4 have been demonstrated to play a pivotal role in catalyzing the conversion of various aliphatic or aromatic glucosinolate-derived nitriles (**53–68**) as shown in Fig. 8. Moreover, these enzymes exhibit the capability to generate the plant hormone auxin, specifically indole-3-acetic acid from indole-3-acetonitrile, a product resulting from the breakdown of indole-3-ylmethylglucosinolate.

3 Nitrile biosynthesis in microorganisms

Nitriles in microorganisms are mainly biosynthesized through the "aldoxime–nitrile pathway" that exists as a cascade process as follow: aldoxime → nitrile → amide → acid → acyl-CoA.[142–146] Aldoxime biosynthesis in microorganisms is rarely reported. Unlike the CYP79s in plants which exclusively convert amino acids into aldoximes, a few pieces of evidences indicate that two enzymes, namely *N*-hydroxyamino acid-forming enzyme and aldoxime-forming enzyme, are involved in the two-step microbial aldoxime biosynthesis.[147] Besides, the co-occurrence of the downstream nitrile-degrading enzymes suggests that in microorganisms the oximes possibly come from the CNglc pathways in their host plants, and the "aldoxime–nitrile pathway" is for detoxification and supply of carbon and/or nitrogen. It is hypothesized that the aldoxime-degrading microorganisms detoxify the toxic aldoximes for their own use and concomitantly provide valuable metabolites to their microbiota communities.[148] The ability to convert aldoximes into nitriles is widespread in the microbial world. The enzymes responsible for nitrile formation are aldoxime dehydratases, which are termed Oxds. Other intriguing nitrile biosynthetic pathways, including the CYP-mediated reactions and carboxylic acid–nitrile pathway, have also been discovered.

3.1 Aldoxime dehydratase

Aldoxime dehydratase (Oxd), a class of heme-containing enzymes, catalyzes the dehydration of aldoximes to the

corresponding nitriles. Oxds are widely distributed in microorganisms. Among 975 microorganisms screened, Oxds were detected in 70 genera (198 strains), including 37 genera (133 strains) of bacteria, 31 genera (63 strains) of filamentous fungi, and 2 genera (2 strains) of yeasts.[149] Oxds show a broad substrate scope and are classified according to their substrate preferences as aliphatic aldoxime dehydratase (EC 4.8.1.2), indoleacetaldoxime dehydratase (EC 4.8.1.3), and phenylacetaldoxime dehydratase (EC 4.8.1.4).

3.1.1 Oxds from bacteria. Four phenylacetaldoxime dehydratases have hitherto been discovered, including OxdB from *Bacillus* strain OxB-1,[150,151] OxdYH3-3 from *Rhodococcus* sp. YH3-3,[152] OxdBr1 from *Bradyrhizobium* sp. LTSPM299,[153] and OxdF1 from *Pseudomonas putida* F1.[154] A whole-cell OxdB catalyst showed an excitingly high catalytic activity, achieving a 100% yield of PAN from 0.5 M *Z*-PAOx.[155] Besides aldoxime, OxdB was otherwise capable of taking an array of dihydroisoxazoles as substrates into Kemp elimination, forming the corresponding β-hydroxy nitriles.[156] The same reactions could also be achieved by OxdA and OxdRE.[157,158] OxdYH3-3 exhibited a prospect for production of aromatic nitriles in industry. In the first investigation in 1999, though the functional OxdYH3-3 was not

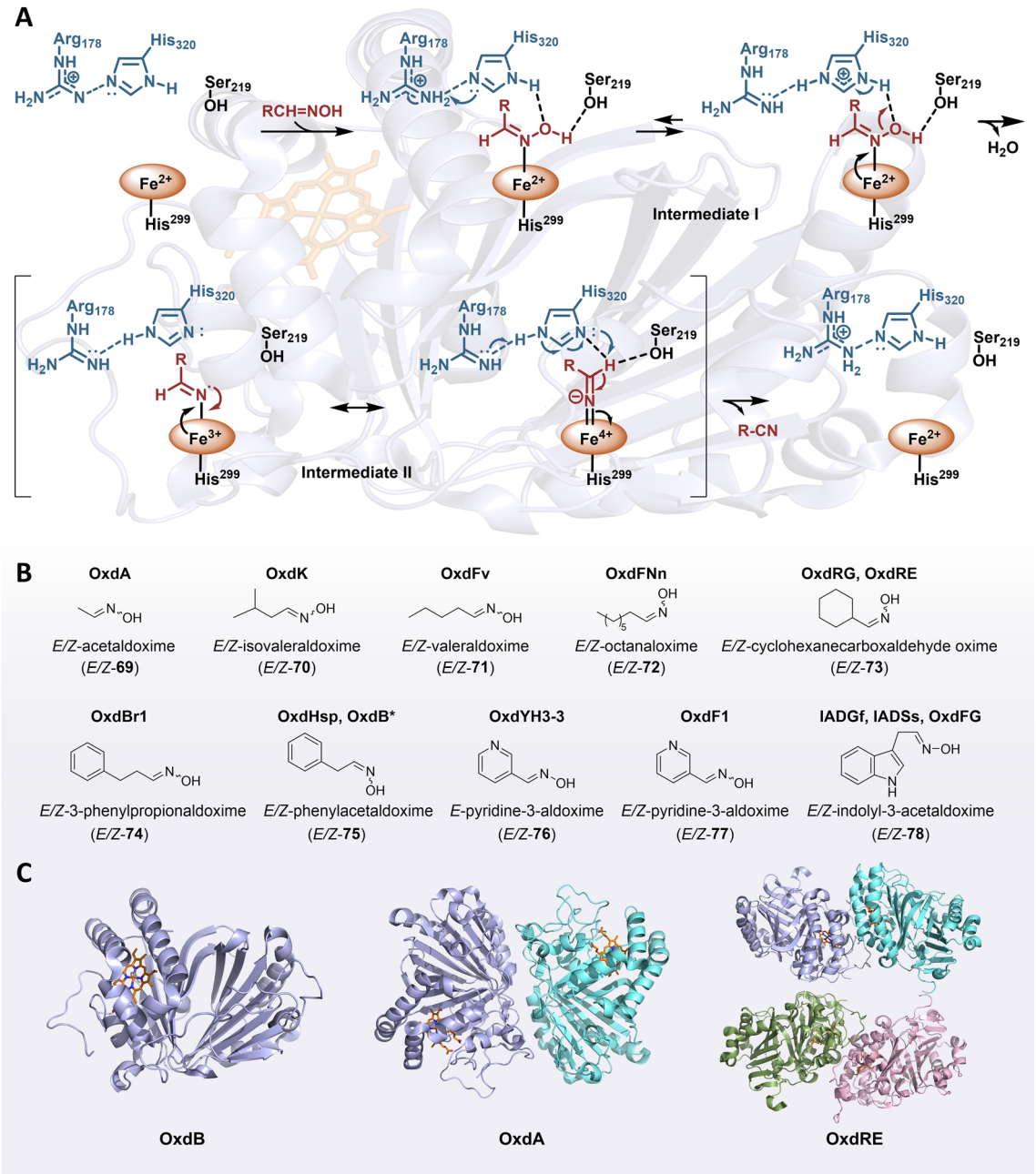

Fig. 9 (A) A putative mechanism for nitrile biosynthesis by aldoxime dehydratases (*e.g.*, OxdA). (B) The substrate preference of fourteen aldoxime dehydratases. *For OxdB, *Z*-phenylacetaldoxime is preferred. (C) The crystal structures of OxdB (PDB ID code: 7F2Y), OxdA (PDB ID code: 3W08), and OxdRE (PDB ID code: 3A15).

purified, its substrate specificity was examined thoroughly using whole cell catalyst, where *E*-pyridine-3-aldoxime acted as the most preferential substrate. Upon further improvement of reaction conditions, strain YH3-3 achieved the microbial synthesis of nitriles in preparative scales.[147] Overexpression of OxdYH3-3 and the directed evolution to improve its catalytic properties were achieved in recent years, and OxdYH3-3 has been used for the full conversion from *E*-2-furfurylaldoxime to the pharmaceutical intermediate 2-furonitrile.[159,160] Nearly one hundred hypothetical Oxd-encoding genes have been detected in the genome sequences of rhizobium *Bradyrhizobium* species, in which OxdBr1 is the only protein characterized. OxdBr1 acts on a variety of (aryl)aliphatic aldoximes, such as *E*/*Z*-PAOx, phenylacetaldehyde oxime, and hexanal oxime. A chemoenzymatic "carboxylic acid–aldehydes–oxime–nitrile" cascade has been established in a two-phase mode by using carboxylic acid reductase, NH_2OH and OxdBr1, achieving the full conversions of phenylacetic acid and hexanoic acid to their corresponding nitriles.[161] OxdF1 exhibits excellent capacities to accept various aromatic and heterocyclic aldoximes as substrates. The semi-rational protein design of OxdF1 greatly enhanced its catalytic activity toward benzaldehyde oximes, based on which an efficient chemoenzymatic strategy to prepare nitriles from benzyl amines has been achieved.[162]

To date, four aliphatic aldoxime dehydratases have been characterized, including OxdA from *Pseudomonas chlororaphis* B23,[163] OxdK from *Pseudomonas* sp. K-9,[143] OxdRG from *Rhodococcus globerulus* A-4,[164] and OxdRE from *Rhodococcus* sp. N-771.[165,166] These Oxds are more active towards alkyl- and arylalkyl-aldoximes than towards aryl-aldoximes. OxdK accepts both *E*/*Z*-PAOx and *E*/*Z*-*n*-valeraldoxime as substrates and consumes isomers in a time-dependent manner at almost the same rates, as OxdB does. These observations could be explained by the acceptance of both *E*- and *Z*-isomers or isomerization of the geometry of the oxime group binding to the enzyme. The crystal structures of OxdA, OxdRE, and OxdB in the substrate-free and substrate-bound forms have been determined, which provide solid insights into the catalytic mechanism[166–168] (Fig. 9C). OxdA conducts nitrile synthesis not only by dehydration of aliphatic aldoximes but also by enantioselective N–O bond cleavage of isoxazole derivatives as mentioned earlier. Enzyme engineering of OxdA resulted in the OxdA-L318I mutant, which catalyzes the conversion from (±)-5-(chloromethyl)-4,5-dihydroisoxazole to (*R*)-4-chloro-3-hydroxybutanenitrile.[158] Furthermore, the versatile OxdA also functions as catalase, peroxidase, and peroxygenase, revealing its catalytic promiscuity.[169]

Six new bacterial aldoxime dehydratases were discovered recently by using 3DM system (a software suite for protein superfamily analysis) based on the well-studied OxdB, namely OxdVP from *Variovorax paradoxus*, OxdHsp from *Hydrogenophaga* sp. RAC07, OxdPsp from *Pseudomonas* sp. RIT-PI-q, OxdHR from *Herbaspirillum rubrisubalbicans* M1, OxdMR from *Methylobacillus rhizosphaerae*, and OxdFNn from *Fusobacterium nucleatum* subsp. nucleatum ATCC 23726.[170] These Oxds showed diverse substrate scope and catalytic activity. For example, OxdPsp was found to be the most active enzyme, achieving full conversion for nearly all tested substrates ranging from aliphatic aldoximes to aromatic aldoximes. In particular, OxdPsp accepted substituted benzaldoximes and gave high conversions of 2-methylbenzaldoxime (>99% conversion) and 4-methylbenzaldoxime (56% conversion). OxdHR exhibited a three-fold higher catalytic activity towards *E*/*Z*-*n*-octanaloxime in comparison to OxdRE. The applicability of OxdHR as a biocatalyst in preparative synthesis of *n*-octanenitrile was proved in whole-cell biotransformation at a 10 mL-scale. To better utilize these enzymes for real industrial application, more investigations of their enzymatic properties are demanded.

3.1.2 Oxds from fungi. The first attempt to isolate an Oxd from fungi dates back to 1963 by Shukla and Mahadevan. The first fungal Oxd indolyl-3-acetaldoxime dehydratase (IADGf) was only partially purified from the phytopathogenic fungus *Gibberella fujikuroi* and displayed high substrate specificity towards IAOx.[171–173] In the plant pathogen *Sclerotinia sclerotiorum*, the fungal Oxd IADSs was discovered, which has a new primary structure and shows no substantial amino acid sequence similarity to other characterized Oxds. IADSs tends to catalyze the transformation from *E*/*Z*-IAOX to IAN. The significant differences in substrate specificity of IADSs displayed by various indolyl, naphthyl, and phenyl oximes suggest that there are strict size and shape requirements in the substrate binding pocket of IADSs for substrate recognition.[174] OxdFG was characterized from another fungal pathogen *Fusarium graminearum* which causes *Fusarium* head blight of wheat and barley.[175] As a phenylacetaldoxime dehydratase, OxdFG acts on various arylaldoximes. However, OxdFG distinguishes itself from other Oxds by displaying the unique substrate specificity and excellent enantioselectivity for *E*-aralkyl-aldoxime as substrate. An enantiomeric excess (ee) value of 83% for the (*S*)-nitrile from *E*-aldoxime was observed, while consumption of the *Z*-enriched substrate resulted in an ee value of only 8% for the (*R*)-nitrile.[176] Recently, OxdFv from the ascomycetous fungus *Fusarium vanettenii* 77–13–4 was identified, showing preference on *n*-alkyl aldoximes of medium chain length (C5–C6) over aromatic aldoximes. In OxdFv, the canonical Oxd catalytic triad RSH is replaced by R141-E187-E303, in which R141 and E187 are essential for the dehydration activity, while E303 exhibits little necessity.[177]

3.1.3 Catalytic mechanism and properties of Oxds. The general molecular mass of Oxds in the monomeric form is approximately 40 kDa, and some Oxds are present in nature as homodimers. All Oxds contain heme b (protoheme IX) as a prosthetic group, and the heme content is in proportion to the activity. Two histidine residues are highly conserved among Oxds in both bacteria and fungi. Spectroscopic analysis and alanine-scanning mutagenesis of the heme environments of OxdB and OxdA confirmed the involvement of the proximal (His_{282} for OxdB and His_{299} for OxdA) and distal histidine (His_{306} for OxdB and His_{320} for OxdA) in catalysis.[167,178,179] The proximal histidine plays a role in heme-binding, and the distal histidine serves as an acid-base catalyst for the dehydration reaction.

The catalytic mechanism of aldoxime dehydratases has been proposed to include three steps: the formation of a Michaelis-

complex by binding aldoxime to Oxd, elimination of the aldoxime hydroxyl group, and deprotonation to generate nitrile (Fig. 9A). The first step consists of direct binding of the substrate to the heme-iron in the ferrous form *via* the N-coordination of the aldoxime moiety, forming an enzyme–substrate complex (intermediate I). In contrast, Oxds in the ferric state bind to the substrates *via* oxime oxygen atom (*O*-coordination), resulting in the dead-end complex which is inactive for catalysis.[166,167] The second step involves the transfer of a proton from the distal histidine to the O-atom of the aldoxime group, thereby facilitating the hydroxyl group elimination.[179] Crystal structures of substrate-free and substrate-bound OxdRE revealed that the proper orientation of the heme-bound substrate is fixed by two crucial hydrogen bonds. Then, His_{320}, as a general base, abstracts the β-proton of substrate, resulting in the formation of intermediate II. A 180° rotation of the substrate around the Fe–N bond leaves room for the abstraction of the β-hydrogen in intermediate II by the ε-nitrogen atom of His_{320}, forming the nitrile. The nitrile product is released when the channel that connects the substrate-binding cavity to the protein surface is set opened by the Phe_{306} residue.[166]

Oxds show narrow to wide activities towards aliphatic, aromatic, and arylaliphatic aldoximes. The substrate preference of fourteen aldoxime dehydratases has been established (Fig. 9B),[170] and the detailed substrate ranges of different Oxds have been reviewed elsewhere.[176,180] Oxds from microorganisms are either inducible or constitutive enzymes. For example. the production of OxdRE in *Rhodococcus* sp. N-771 could be induced by *E*-PyOx;[164] by contrast, IADSs from *S. sclerotiorum* was constitutively produced in the fungal cultures.[174] Aldoxime dehydratase activities were reported to decrease in varying degrees during purification. The loss of activity could be attributed to either the loss of cofactors (*e.g.*, Fe^{2+}, PLP, FMN, or protoheme IX) or the oxidation of hemoproteins at the ferrous state. The effects of different activators or inhibitors on Oxd activities have been observed in all the purified Oxds. Commonly, aldoxime dehydratases could be inhibited by thiol, carbonyl, and metal chelating reagents. Conversely, the activities could be enhanced by electron acceptors (*e.g.*, FMN, duraquinone, and vitamin K3) and reducing agents (*e.g.*, $Na_2S_2O_4$, glutathione, and ascorbic acid). For instance, OxdA and OxdRG showed a much higher dehydration activity with the addition of reducing reagents $Na_2S_2O_4$ or Na_2S under anaerobic conditions, suggesting that Oxds might be difficult to reduce.[163,164]

Oxds in microorganisms and CYP71s in plants catalyze the same type of aldoxime dehydration reactions. Interestingly, these two enzyme families both contain heme b as a prosthetic group to which aldoxime substrates bind. Furthermore, they can accept both alkyl- and aryl-aldoximes as substrates, although with distinct preferences for different enzymes. However, there are notable differences between oxime-metabolizing CYP71s and Oxds. In the case of CYP71s, the dehydration of aldoximes relies on NADPH for reducing the heme-iron to the ferrous state and the usage of O_2 or H_2O_2 as catalytic mediators, while the electron donor for Oxds is currently unclear. Regarding substrate preferences, oxime-metabolizing CYP71s primarily accept *Z*-aldoximes as substrates, which can also convert *E*-aldoximes into the corresponding *Z*-isomers before initiating dehydration. In contrast, Oxds generally accept both geometrical isomers of aldoximes. For instance, in the case of OxdB, both *E*- and *Z*-PAOx substrates can be consumed in a time-dependent manner, with the catalytic rate of *Z*-PAOx being higher than that of *E*-isomer.[151] Furthermore, there is a clear distribution difference between these two types of enzymes. None of the characterized microbial Oxds belong to the cytochrome P450 superfamily, which includes CYP71s; and homologous gene sequences of Oxds have not been discovered in the plant kingdom so far. Considering that both Oxds and CYP71s consume aldoximes of plant origin, this distribution difference suggests the possibility of convergent evolution between these two enzyme families.

As no key intermediates or crystal structures of any oxime-metabolizing CYPs have been characterized, the mechanism of dehydration was only reasonably proposed according to spectroscopic analysis of rat liver microsomes and the recombinant human liver CYP3A4 with different oxime substrates[181] and biomimetic reconstruction of iron porphyrin model systems.[182] Though the CYP71Es show no sequence similarity to aldoxime dehydratases in microorganisms, the catalytic mechanism based on the combination of the nitrogen atom and the ferrous state enzyme is believed to be shared.[183]

3.2 Other nitrile biosynthetic pathways

In addition to the classical aldoxime–nitrile pathway, a CYP-dependent nitrile biosynthetic route has been revealed in the biosynthesis of borrelidin (**19**) in *Streptomyces parvulus* (Fig. 10). Borrelidin, an antibiotic with antibacterial and anti-angiogenesic activities, is characterized by an unusual 12-*Z*-configured double bond in the macrolactone skeleton. It has been demonstrated an *E*/*Z* isomerization in post-assembly modification is responsible for the formation of *Z*-configured double bond, but rather a dehydrotase domain-catalyzed dehydration.[184,185] Based on gene inactivation studies, an intriguing multifunctional prokaryotic P450 enzyme BorI was implicated to be involved in the formation of the *Z*-configured double bond and crucial nitrile moiety in borrelidin. Putatively, BorI catalyzes the *E* to *Z* isomerization of the 12, 13-double bond, as well as the oxidative transformation of the C12 methyl group into an aldehyde group, followed by a BorJ-mediated transamination. Then, BorI further catalyzes the two-step *N*-hydroxylations on the amino group, resembling the function of CYP79 in plants; and the following two-step dehydration in a CYP71-like manner finally gives rise to the nitrile moiety.[186] A similar pathway of nitrile formation was also postulated in cyanosporaside (**11**) biosynthesis, however diverging in the types of oxidative enzymes mediating aldoxime formation. Aminotransferase CynN1 and flavin-dependent oxidoreductase CynN2 were proposed for nitrile functionalization in cyanosporasides.[187]

The natural product 7-cyano-7-deazaguanine ($preQ_0$, **79**) plays an important role in various biological processes, including tRNA and DNA modification, as well as secondary

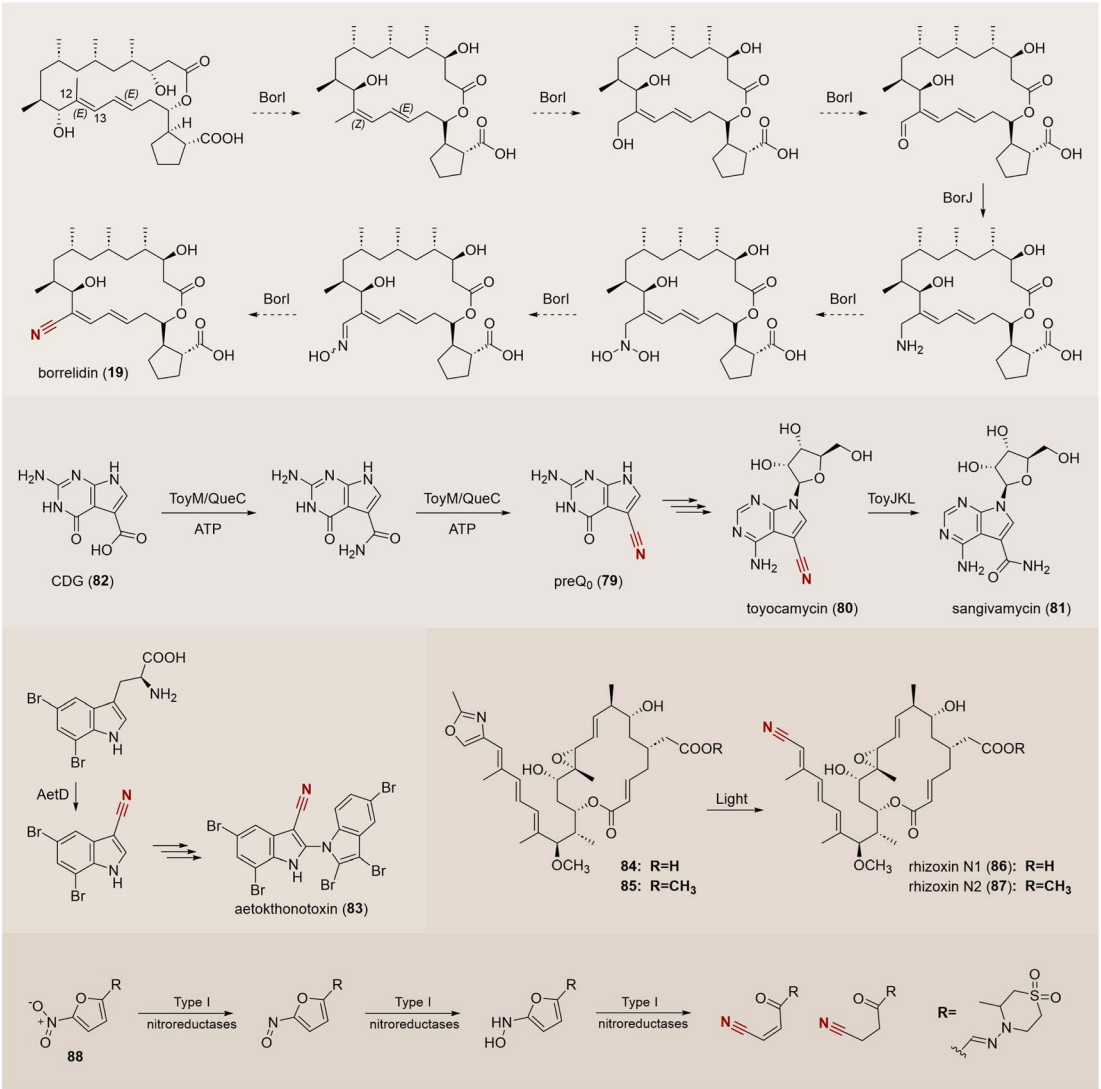

Fig. 10 Other nitrile biosynthetic pathways in microorganisms.

metabolism. Specifically, it serves as a versatile precursor involved in the biosynthesis of queuosine in Eubacteria, archaeosine base in archaeal tRNAs, and the formation of 7-deazapurine derivatives in DNA.[188] Compound **79** is also associated with the production of Streptomycetes-derived secondary metabolites such as toyocamycin (**80**) and sangivamycin (**81**).[189] It was proposed that the conversion from **79** to **80** might be achieved by the successive actions of five proteins (ToyH, E, G, F, and I), which may be "borrowed" from those involved in purine salvage/biosynthesis. The predicted functions of ToyH, E, G, F, and I are phosphoribosyl-pyrophosphate transferase, GMP reductase, adenylosuccinate synthetase, adenylosuccinate lyase, and haloacid dehalogenase, respectively.[190] Then, a three-subunit TNHase ToyJKL (or the α subunit ToyJ alone) was demonstrated to convert the cyano group of **80** into an amide, leading to **81** through hydratation.[191] In addition to its role as a precursor, **79** itself exhibits cytotoxic activity against several cell lines, including HeLa (adenocarcinoma) and HepG2 (hepatocellular carcinoma), suggesting the potential of **79** and its derivatives as lead compounds with antineoplastic properties. QueC (EC 6.3.4.20) from *Bacillus subtilis* and its counterpart ToyM from *Streptomyces rimosus* catalyze an unusual transformation from carboxylic acid into nitrile.[192] Specifically, in the biosynthetic pathway of the hypermodified queuosine-tRNA, the carboxylate moiety on 7-carboxy-7-deazaguanine (CDG, **82**) is converted to a cyano group yielding **79**, by either QueC or ToyM in an ATP-dependent reaction, where ammonia serves as the nitrogen source. Detailed analysis of the catalytic mechanism of ToyM revealed that it starts from the activation of CDG *via* adenylation, followed by the addition of ammonia to generate an amide 7-amido-7-deazaguanine (ADG). Then, collapse of ADG by the second equivalent of ATP yields the nitrile product $preQ_0$. ToyM was characterized to be capable of activating two different substrates, an acid and an amide, to achieve the unprecedented one-enzyme mediated nitrile synthesis. The different rates of the two half reactions suggest that the catalytic ability of ToyM might be derived from an amide synthetase having gained the new function over time.[50]

The attempts to explore a broader spectrum of substrates have been made. However, QueCs from different sources were all proved highly specific for the natural substrate, CDG.[193,194]

The neurotoxin aetokthonotoxin (**83**), derived from the epiphytic cyanobacterium *Aetokthonos hydrillicola*, is implicated in fatal Vacuolar Myelinopathy. This toxin has the capability to spread through a trophic cascade, ultimately leading to the deaths of birds of prey such as the bald eagle. In the biosynthesis of aetokthonotoxin, an iron-dependent enzyme AetD was recently found to catalyze the conversion from 5,7-dibromo-L-tryptophan to 5,7-dibromo-indole-3-carbonitrile. *In vitro* enzymatic reactions and isotope labeling experiments demonstrated the transformation process, where the α-carboxyl group is eliminated from 5,7-dibromo-L-tryptophan and its α-amino group was determined to be the source of the nitrogen atom in the nitrile. AetD lacks conserved domains and does not show significant homology with any characterized proteins, rendering it a new class of nitrile synthase. Unlike CYP-mediated cyano-group formation from amino acids in CNglcs biosynthesis, AetD was proposed to catalyze the nitrile formation from modified tryptophan *via* an unknown but intriguing carbon loss in addition to a decarboxylation reaction. The amino group of 5,7-dibromo-L-tryptophan has been experimentally demonstrated to serve as the source of the nitrogen atom in the resulting cyano group. Therefore, it is likely that a very unusual intramolecular rearrangement is involved in the nitrile formation.[195]

The 16-membered ring macrolides rhizoxins are antimitotic agents with impressive antitumor activity originally isolated from the apathogenic plant fungus *Rhizopus microspores* which causes rice seedling blight. A non-enzymatic cyanation was discovered from an endofungal bacterium *Burkholderia rhizoxinica*. Oxazolyl- and thiazolyl-substituted rhizoxins (**84** and **85**) can decompose to yield the corresponding nitriles rhizoxin N1 and N2 (**86** and **87**) by photochemical oxidative cleavage. Stable isotope labeling experiments revealed that the nitrile moiety derives from glycine or serine and that the oxazole assembly precedes nitrile formation. For the transformation mechanism, it was proposed that a cycloaddition of singlet oxygen to an oxazole ring would yield a reactive peroxide, which further undergoes rearrangement to generate the nitrile group.[196]

Furthermore, a two-step two-electron reduction of nifurtimox, a prodrug for Chagas disease, yields the corresponding nitrile derivative by *Trypanosoma cruzi* and *Trypanosoma brucei* type I nitroreductase. Type I nitroreductases are NAD(P)H-dependent and FMN-binding proteins rare in most eukaryotes but abundantly expressed in trypanosomes. These enzymes convert the conserved nitro group of nitrofurans (**88**) *via* nitroso intermediate into hydroxylamine, which is further metabolized to form unsaturated and then saturated open-chain nitriles. These nitrile products are toxic to bloodstream-form trypanosomes and show a significant growth inhibitory activity against the cultured mammalian cell line THP-1. Therefore, the expression of type I nitroreductase by trypanosomes may serve as a new explanation for the selective toxicity of nifurtimox.[51,197] By now the mechanism for these nitrile-forming processes is still unclear. Based on the existing observations, the formation of hydroxylamine is probably intermediated *via* a two-step reduction of the nitro moiety by type I nitroreductases, and the nitrile formation is either enzymatically mediated by type I nitroreductases or occurs non-enzymatically due to the reductive environment of type I nitroreductase reactions.

4 Nitrile biosynthesis in animals

Nitrile biosynthesis has also been observed in animals, including mammals, sponges, and arthropods. The phenobarbital-induced rat liver microsomes (CYP3A) serve as the first evidence of CYP-mediated nitrile biosynthesis in animals. Rat liver microsomes displayed flexible substrate specificities towards both aryl- and alkylaldoximes with *Z* configuration (*e.g.*, *Z*-heptanaldoxime, *Z*-PAOx, and *Z*-4-chlorobenzaldehyde oxime).[198] The human liver CYP3A4 (EC 1.14.14.1), which metabolizes more than 50% of prescribed drugs, is also capable of converting aldoximes into nitriles with characteristics similar to those of rat liver microsomal CYPs.[181] The CYP3A4 NF25, when expressed in yeast *S. cerevisiae*, was found to catalyze the dehydration of *Z*-benzaldehyde oxime into its corresponding nitrile.[199] Gregarious locusts biosynthesize PAN both as an olfactory aposematic signal and an indicator of HCN toxicity to fend off their predators such as the Great tits (*Parus major*) and to avoid cannibalism. The nitrile-forming pathway has partially been identified with a cytochrome P450 CYP305M2 (EC 1.14.14.1) catalyzing the transformation of phenylalanine into *Z*-PAOx in a stereospecific way.[200–202] The vulnerability of gregarious locusts to bird predation was demonstrated to be increased after the knockdown of CYP305M2, and more exposure to intraspecific predation was clearly observed in the CYP305M2 gene knockout mutants. The olfactory receptor LmOR70a, which is necessary for aversive behavior, was proved to be the highly specific and sensitive detector of PAN in the migratory locust *Locusta migratoria*. The catalytic mechanism of animal oxime-metabolizing CYPs was proposed to involve the formation of a CYP Fe(II) ← N(OH)=CHR complex as a key intermediate at first and then undergo cleavage of the aldoxime N–O bond by a charge transfer from CYP Fe(II) to the aldoxime C–N bond. The CYP-type aldoxime dehydratases in animals may function as xenobiotic metabolizing enzymes, transforming aldoximes into nitriles and then thiocyanate, which is subsequently excreted in urine.[203]

Sponges are a nonnegligible source of nitrile-containing natural products. Insight into the biogenesis of the cyano-group in 2-(3,5-dibromo-4-hydroxyphenyl)acetonitrile, which is a part of the pathway from phenylalanine/tyrosine to dibromohomogentisamide, was gained through an isotope labeling experiment in the sponge *Aplysina fistularis* (*Verongia aurea*) and the co-isolation of the related bromophenol oximes from *Verongia* species.[204] Putatively, the nitrile biosynthesis is achieved by oxidation of phenylalanine/tyrosine to the corresponding aldoxime, dibromination, dehydration of the aldoxime as in plants and microorganisms, and decarboxylation. However, the involved enzymes have yet to be identified.

CNglcs are mainly found in arthropods, particularly Lepidoptera.[39] Their presence can be attributed to either

sequestration or *de novo* biosynthesis or both. Certain specialized insects, including butterflies and burnet moths from the Limacodidae, Lycaenidae, Nymphalidae, and Zygaenidae families, are capable of performing *de novo* biosynthesis of CNglcs and sequestering these defense compounds through their close interactions with plants.[205] The aliphatic linamarin and lotaustralin are the most common CNglcs observed in Lepidoptera. However, at present the involving enzymes mostly remain unclear. The biosynthetic pathway of linamarin and lotaustralin in burnet moth *Zygaena filipendula* is the only one that has been elucidated, revealing three functional enzymes CYP405A2, CYP332A3, and UGT33A1. This discovery represents an extraordinary example of convergent evolution with the pathways characterized in plants (which will be analyzed in detail in section 5).[206,207] Heliconius butterflies are producers of CNglcs as well; for example, *Heliconius Melpomene* accumulates linamarin and lotaustralin throughout the entire life cycle. Investigations of its transcriptomics and genomics suggest a similar pathway as *Zygaena*, along with several possible candidate enzymes homologous to the *Zygaena* P450s.[208] As *de novo* biosynthesis of CNglcs is more cost-ineffective than direct sequestration from the food plant, it has been proved in *Z. filipendulae* larvae that CNglc biosynthesis predominates only when necessary. Thus, the biosynthetic process is tightly regulated at both the transcription and translation levels to ensure CNglc homeostasis *in vivo*.[209]

Many millipedes are also cyanogenic but different in their nitrile-forming patterns. Unlike in Lepidoptera, nitrile biosynthesis in polydesmid millipede seems to terminate at the cyanohydrin step without downstream glycosylations, as no glycosylated cyanohydrin products were detected in MAN producers *Harpaphe haydeniana* and *Chamberlinius hualienensis*.[210,211] Additionally, two nitrile-related enzymes have been characterized in *C. hualienensis*: a cytochrome P450 CYP3201B1 to facilitate the synthesis of (*R*)-MAN from phenylacetonitrile and the downstream hydroxynitrile lyase ChuaHNL to catalyze the decomposition of (*R*)-MAN into benzaldehyde and HCN.[113,212] The reverse reaction of ChuaHNL to produce (*R*)-MAN from benzaldehyde and potassium cyanide has been demonstrated to be highly enantioselective and five times more efficient than the industrial PaHNL, showing promising potential for industrial applications.[186] Several HNLs have also been found in Paradoxosomatidae and Xystodesmidae families, including NttHNL from *Nedyopus tambanus tambanus* and Pton3HNL from *Parafontaria tonominea*. Whole-cell reaction systems based on Pton3HNL was established in *E. coli*, yielding (*R*)-MAN with up to 97.6% ee value without using organic solvents.[213]

5 Convergent evolution of nitrile biosynthesis in plants and animals

In the cases of CNglcs which are generated by both plants and animals, convergent evolution has been observed or reasonably speculated. From a biochemical point of view, the *de novo* biosynthesis of CNglcs follows a general pattern in most plant species and arthropods, while the genes encoding the functional enzymes have evolved convergently in these two groups.[39,214] Though the researches on CNglc biosynthesis in animals are quite limited, linamarin and lotaustralin synthesized by moth *Z. filipendulae* represent a remarkable example of convergent evolution (Fig. 11).

Zygaena moths and its food plant *Lotus japonicus* share the identical pathway for the biosynthesis of linamarin and lotaustralin with the same amino acid precursors and oxime intermediates. In addition, the nitrile-forming processes are both catalyzed by two multifunctional CYPs, but, from totally different clans. In *Zygaena* moths, CYP405A2 (EC 1.14.14.38/39) catalyzes the conversion of valine and isoleucine to their corresponding oximes, and then CYP332A3 (EC 1.14.14.41) transforms oximes into 2-methylpropanenitrile and 2-methylbutanenitrile. By contrast, the same conversions are achieved by CYP79D3/CYP79D4 and CYP736A2 in *L. japonicus*. This demonstrates that the pathway in *Zygaena* has likely evolved by convergent evolution instead of horizontal gene transfer or divergent evolution.[207] Phylogenetically, CYP332A3, without any connection with plant CYPs, clusters in the CYP3 insect clade, which contains members generally involved in xenobiotic metabolism. It is highly probable that CYP332A3 was evolved from an ancestral CYP332A enzyme in detoxification pathways and independently gained the dehydration function as CYP71s in plants. Genes homologous to CYP332A3 have been found widespread in the genomes of Lepidoptera species; for example, *Bombyx mori, Heliconius melpomene*, and *Spodoptera frugiperda*.[208,215–217] This proves the biosynthesis of CNglcs could be orthologous within this order and rules out the possibility of divergent evolution with plants.

Though the sequence identity of distantly related CYPs is low, the three-dimensional structures of CYPs are usually conserved with five motifs (WXXXR, GXE/DTT/S, KETLR, PERF, and haem-binding domain).[218] As the functional homolog to plant CYP71Es, CYP332A3 shows unique substitutions in the KETLR and PERF motifs. In the KETLR motif, the conserved basic arginine or lysine is replaced by the aromatic phenylalanine; while in the PERF motif, the conserved phenylalanine is substituted by a smaller valine, which has not hitherto been observed in any other CYPs.[197] Therefore, CYP332A3 was believed to follow a different evolutionary pathway compared with plant CYP71Es. In the phylogenetic tree which shows the relatedness among the aldoxime dehydratases (CYPs and Oxds), enzymes conservatively cluster within their own kingdoms of life (Fig. 11A), which further denotes the convergent evolution of nitrile formation in nature. Though till now our understanding of nitrile biosynthesis in animals is much less than in plants, from a phylogenetic perspective, CNglc biosynthesis in arthropods appears to happen multiple times at very different time points. It was suggested that the ability to synthesize aliphatic CNglcs as a defense substance might emerge earlier in animals, at least in Lepidoptera, than in plants.[219] For instance, considering *Z. filipendulae*, the capacity for *de novo* CNglc biosynthesis likely predates CNglc sequestration from plants, which might have therefore aided in the transition of food plants to cyanogenic ones.

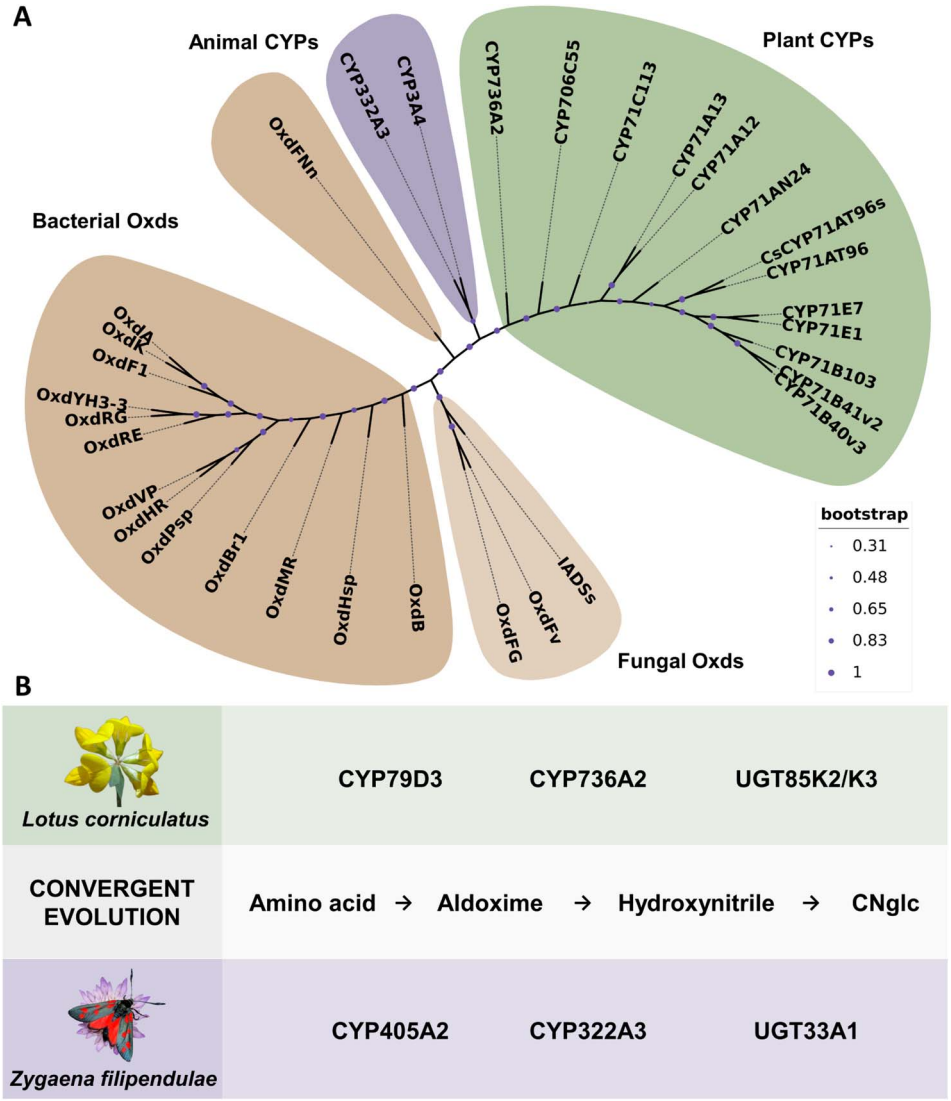

Fig. 11 Evolutionary relationships of nitrile-forming enzymes in different kingdoms of life. (A) Neighbour-joining phylogenetic tree presenting the relatedness among oxime-nitrile enzymes in nature. (B) Convergent biosynthesis of CNglcs between plant *Lotus japonicus* and moth *Zygaena filipendulae*.

6 Biological and ecological functions of nitrile biosynthesis

Nitrile biosynthesis serves a wide range of functions in different forms of life[220] (Fig. 2). In plants, CNglcs generally act as immediate feeding deterrents against herbivores, predators, and pathogens. This function attributes to CNglcs' bitter taste and the released toxic HCN (by β-glucosidases) that shows a distinct defensive odor.[221] The cyanides (predominantly HCN), acutely or chronically, cause intoxication, varying degrees of illness, or even death of predators by inhibiting the activity of metalloenzymes, predominantly cytochrome c oxidase in the mitochondrial electron transport chain.[222] Although the deterrents themselves are quite powerful, the defensive effectiveness of CNglcs also depends on the amount of CNglcs consumed, how quickly they are ingested, and the kinds of attacking organisms. Attackers usually steer clear of plants that contain CNglcs, even though they can tolerate low concentrations of CNglcs, whereas certain specialized arthropods have evolved abilities to sequester (ingestion, accumulation, and storage of CNglcs from plants) and use CNglcs as oviposition cues and/or phagostimulants, allowing these animals to consume cyanogenic plants without harm.[223]

Another role of CNglcs in the life-cycle of plants and insects is to participate in nitrogen metabolisms, retrieving nitrogen from CNglc degradation for other metabolic processes, without the release of HCN. The stability of CNglcs renders the host organisms ideal readily mobilizable repositories for carbohydrates and reduced nitrogen, which could act as nitrogen buffers to counteract imbalances in primary metabolism under variable environmental conditions.[224,225] In rubber tree *Hevea brasiliensis*, upon germination, plenty of linamarin in the seed is glycosylated into diglucoside linustatin and transported over a long distance

to the seedling, where it undergoes a series of conversions into asparagine or aspartate for transamination reactions required by seedling development.[226] MAN, in peach *Prunus persica* L., can be metabolized into the plant hormone salicylic acid, which is involved in diverse biological processes.[227] The butterfly *Heliconius sara* is able to convert cyclopentenoid CNglc epivolkenin into the non-cyanogenic compound sarauriculatin *via* a unique cyano-to-thiol transformation.[228]

In addition, CNglcs play a role in inter- and/or intraspecies communications in insects. Under low concentrations of volatile benzaldehyde which is released from MAN, millipedes tend to aggregate, whereas high concentrations make them disperse.[222] These compounds can also be utilized as nuptial gifts to ensure better protection of the mate and offspring.[229] As observed in *Zygaena* moths and *Heliconius* butterflies, the powerful biosynthesis ability of CNglcs promotes mating, as it denotes the vigor of the male and his ability to father healthy offspring. CNglcs are transferred from the male to the female as a nuptial gift while mating and deposited in the spermatophore as a paternal investment in offspring.[230,231] As mentioned earlier, the PAN biosynthesized in migratory locusts serves as an olfactory aposematic signal and an indicator of HCN toxicity to avoid cannibalism. The production of PAN is possibly restricted to crowded conditions, as no high amounts of PAN release can be observed in solitary locust individuals. The resistant function of PAN in reducing the substantial risk of being eaten by conspecifics plays an important role in locust population ecology.[202]

Cyanides produced by cyanogenic pseudomonads exhibit a suppressive effect on plant growth.[232] For example, in plant inoculation studies with cyanogenic *Pseudomonas fluorescens,* reductions in bean growth were observed to be associated with the increasing content of cyanides in the rhizosphere.[233] Moreover, cyanogenic *Pseudomonas aeruginosa* and *Pseudomonas protegens* exert inhibitory effects towards the growth of *A. thaliana*.[234]

7 Applications of nitrile-forming enzymes in industry

Nitrile compounds are of critical relevance to human life, engaging in a broad range of industry segments, such as pharmaceuticals, solvents, fragrances, polymers, and fine/bulk chemicals. Besides, nitriles can be used as precursors to synthesize other valuable chemicals, including amines, amides, carboxylic acids, *etc*. For example, nitrile-based syntheses of aromatic, heterocyclic, and aliphatic primary, secondary and tertiary amines have been achieved in recent studies.[235] The production of nitrile is commonly chemically achieved by ammoxidation using ammonia and alkanes through high-temperature gas-phase reactions and hydrocyanation through substitution/addition reactions with hydrogen cyanide or its derivatives.[236] These methods, nonetheless, present considerable toxicity and hazards, which makes it urgent to find green alternative ways for nitrile synthesis in industry.

The nitrile-forming enzymes from nature serve as excellent cyanide-free enantioselective biocatalysts for the efficient synthesis of chiral nitriles under mild aqueous conditions.[43] The exciting utilizations and prospects of HNLs for industrial stereoselective cyanohydrins synthesis have been discussed in section 2.2. Other than that, Oxds from microorganisms have also shown great potential in industrial nitrile synthesis. Oxds stand out from traditional chemical strategies in three major ways: (1) geometric isomers of aldoxime (*E*/*Z* mixture) are dehydrated into the corresponding nitriles in enantiomerically enriched forms by the same enzyme in an enantioselective fashion; (2) Oxds can tolerate a broad substrate range of aldoximes and impressively high substrate loadings; (3) aldoxime substrates are easy to obtain from spontaneous condensation of readily available fatty aldehydes with hydroxylamine; and (4) the whole catalytic process is under mild conditions in water (non-aqueous systems are also acceptable) at room temperature with water as the only side-product.[237] When using OxdA for the dehydration of *E*-rac-*p*-Br-2-aryl-2-methylacetaldoximes in a recombinant *E. coli* whole-cell system, the (*S*)-configured nitrile was obtained with a 35% conversion ratio and outstanding enantiomeric excess of 98%.[238] In the linear aliphatic *n*-octanenitrile synthesis with whole cells containing OxdB as biocatalyst, a conversion of 93% after 24 h was achieved from a high substrate loading up to 1.4 kg L^{-1} of *n*-octanaloxime.[239] In a modified whole-cell system expressing OxdB, citronellyl-oxime was fully converted into the fragrance ingredient citronellyl nitrile in 90 h under solvent-free conditions. Besides, the whole-cell biosynthesis of PAN from L-Phe *via* *E*/*Z*-PAOx has been achieved in *E. coli* by combining CYP79A2 in the plant glucosinolate biosynthetic pathway and bacterial OxdB, raising the possibility of producing nitrile-related compounds without toxic chemicals.[240] To improve the reaction efficiencies, many efforts have been made, such as construction of more desirable enzyme variants, development of immobilized enzymes or whole-cell catalysts *via* flow chemistry and superabsorbers, and the incorporation of Pickering emulsion systems.[241–243] The growing number of exciting examples have been demonstrating the possibility of using these nitrile-forming enzymes in more industrial applications.

8 Conclusions and outlook

Nitriles occur naturally in various compound forms across plants, animals, and microorganisms, and the ability to biosynthesize nitriles, especially the ancient biomolecules CNglcs, can be traced back to at least 300 million years ago.[64] Bioactive nitrile compounds, such as CNglcs and cyanohydrins, are involved in a variety of biological processes. They may initially serve as feeding deterrents against herbivores, predators, and pathogens and have been evolving additional functions under the influences/pressures of external environments. These molecules play an important role in carbon and nitrogen metabolisms, wounding response, and intraspecies communications and act as a key interface for plant–microorganism–animal interactions.

Given the high toxicity of cyanides, biosynthesis of nitriles with the necessity of cyanides *in vivo* seems inexpedient. Therefore, alternative pathways for nitrile formation in nature must have developed and evolved. The aldoxime–nitrile pathway is the most widespread and crucial route of nitrile biosynthesis in different kingdoms of life. This pathway

comprises the dehydration reaction from aldoximes to their corresponding nitriles, which is mainly achieved by two types of enzymes: the oxime-metabolizing CYPs broadly existing in plants, animals, and bacteria and the members of aldoxime dehydratase Oxds that have only been found in microorganisms so far, including bacteria and fungi. CYP-mediated biosynthetic pathways of CNglcs and the functional proteins involved have been well characterized in several plant species, whereas key information about species distribution, sub-cellular storage sites, other biological functions, and evolutionary trajectories, still needs to be pinned down. In cyanogenic arthropods, the understanding of CNglc biosynthesis is still quite limited. The significant disparity in sequence identity between the genes of functional CYPs in plants and arthropods clearly reveals the convergent evolution of nitrile-forming enzymes in nature. To better understand the convergent evolution of CNglc metabolisms, more detailed phylogenetic investigations and evolutionary analyses are demanded in the future.

Glucosinolate breakdown systems, HNL-catalyzed processes, and other unusual routes, such as the QueC/ToyM-catalyzed carboxylic acid–nitrile pathway and the iron-dependent enzyme AetD-mediated pathway, are also important components of nitrile biosynthesis in nature and have been discovered continuously. However, detailed understandings of their catalytic mechanisms and self-resistance *in vivo* remain unclear. Nonetheless, it can be indicated from the imbalance between the diverse natural nitrile compounds discovered and the limited biosynthesis characterized that hidden treasures are waiting to be mined and new breakthroughs are likely being nurtured in this very important but underexplored field.

Other than expanding the basic understanding of nature, the discovery and characterization of aldoxime dehydratases have shown great potential as key biocatalysts in organic synthesis. Nitriles constitute fundamental and economically important intermediates in the production of pharmaceuticals, agrochemicals, pigments, fragrances, and other bulk/fine chemicals. Until now, the synthesis of cyano groups in industry mostly depends on organic synthetic chemistry, which is sometimes not environmentally friendly enough, especially when using cyanide as a reagent. Thus, the use of these biocatalysts to synthesize nitriles is economically attractive and coincides with the trend of green chemistry. Aldoxime dehydratases have enabled cyanide-free chiral nitrile synthesis under ambient temperature and pressure and with excellent turnover efficiency. The whole-cell catalysts of Oxds demonstrate impressive performance towards aliphatic aldoximes in a specific chain length range of C6 to C10 and exhibit considerable stability during processing. Besides, continuous discoveries of nitrile-forming enzymes have been opening up the possibility to efficient chemoenzymatic nitrile synthesis. For example, the stereoselective chemoenzymatic cascade synthesis of nitriles with chain lengths of C6, C8, and C10 from biorenewable fatty acids was recently achieved with overall yields of up to 70%.[244] However, large-scale synthesis of aromatic nitriles was unattainable by wild-type Oxds. To solve this problem, the computer-aided design of OxdRE mutants has empowered Oxds to dehydrate a variety of differently substituted benzaldoximes, presenting a perspective for industrial preparation of aromatic nitriles in the future.[245] Protein engineering, culture condition optimization, and chemo-enzymatic cascade of characterized aldoxime dehydratases are in fast growing development, and more exciting progresses can be expected.[246,247]

9 Author contributions

Mingyu Liu: investigation, visualization, and writing – original draft; Shengying Li: conceptualization, funding acquisition, and writing – review & editing.

10 Conflicts of interest

There are no conflicts to declare.

11 Acknowledgements

This work was supported by National Natural Science Foundation of China (32025001 and 22237004) and the Shandong Provincial Natural Science Foundation (ZR2019ZD20). Fig. 2 was created with **BioRender.com**. The photos in Fig. 6 were purchased from **http://ppbc.iplant.cn** except that the photo of *Eucalyptus cladocalyx* was acquired from **https://selectree.calpoly.edu** with permission.

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