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The case for sporadic cyanogenic glycoside evolution in plants

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Cyanogenic glycosides are α-hydroxynitrile glucosides present in approximately 3000 different plant species. Upon tissue disruption, cyanogenic glycosides are hydrolyzed to release toxic hydrogen cyanide as a means of chemical defense. Over 100 different cyanogenic glycosides have been reported, with structural diversity dependent on the precursor amino acid, and subsequent modifications. Cyanogenic glycosides represent a prime example of sporadic metabolite evolution, with the metabolic trait arising multiple times throughout the plant lineage as evidenced by recruitment of different enzyme families for biosynthesis. Here, we review the latest developments within cyanogenic glycoside biosynthesis, and argue possible factors driving sporadic evolution including shared intermediates and crossovers with other metabolic pathways crossovers, and metabolite multifunctionality beyond chemical defense.

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Abbreviations
CYP, cytochrome P450; HCN, hydrogen cyanide; FMO, flavin-containing monooxygenase; NO, nitric oxide; ROS, reactive oxygen species; UGT, UDP-glucosyltransferase.

Introduction

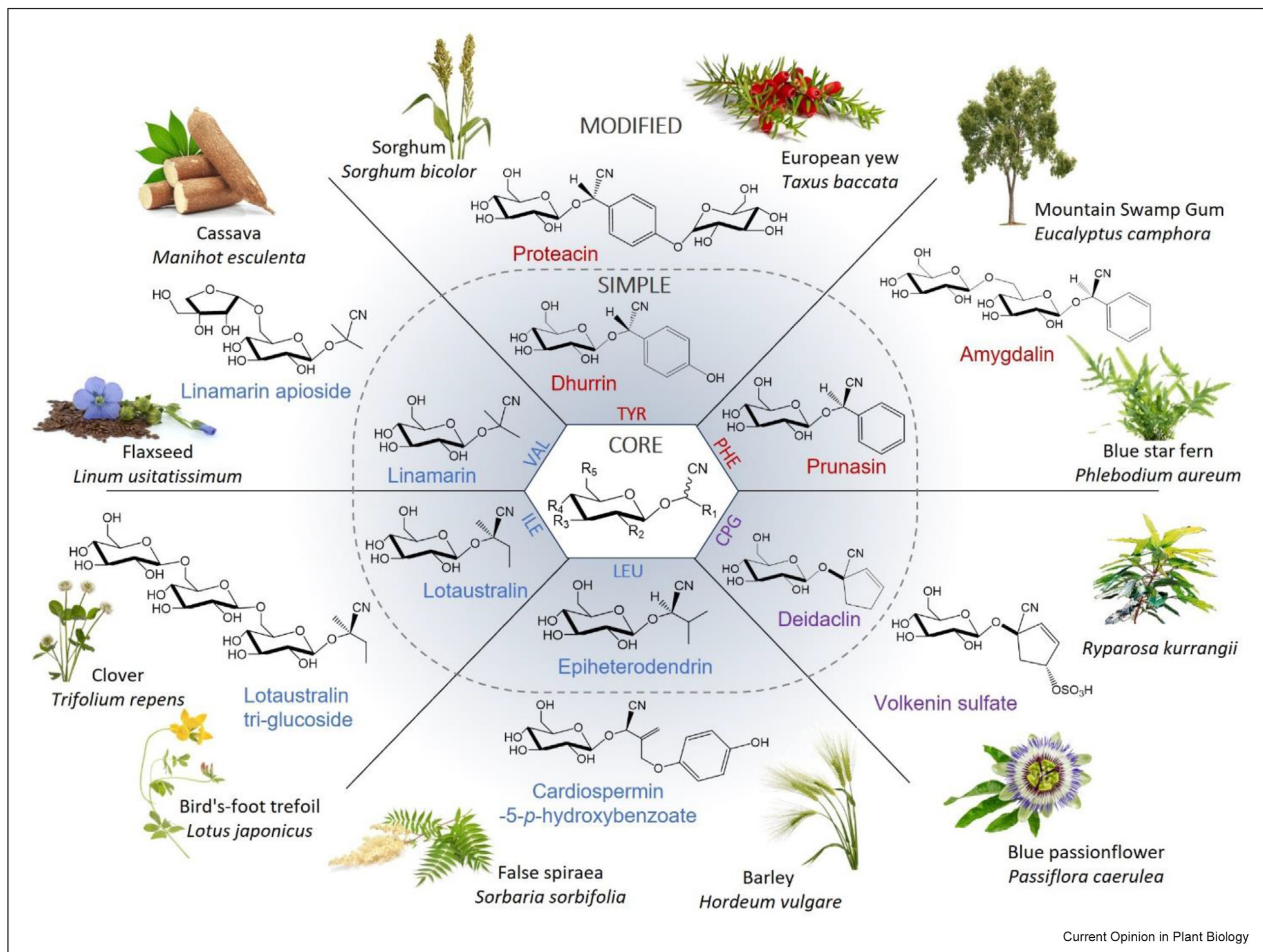
Cyanogenic glycosides are widespread bioactive natural products present in over 3000 plant species, including important food crops such as wheat, barley, sorghum, cassava, almond and apple [1]. In their structurally simplest form, cyanogenic glycosides are amino acid-derived α-hydroxynitriles (cyanohydrins), typically conjugated to a glucose moiety (Figure 1). Structural modifications include additional glycosylation, hydroxylation, sulfination and phenolic conjugation, with over 100 structurally diverse cyanogenic glucosides reported across the plant kingdom [2]. Upon tissue disruption, catabolic enzymes cleave the stabilizing glucose moiety and the resultant cyanohydrin breaks down to release toxic hydrogen cyanide (HCN) (Figure 2). This process, known as cyanogenesis, attributes cyanogenic glycosides as chemical defense molecules, providing protection against pests and pathogens. Cyanogenic glycosides have also been associated with other metabolic roles including nitrogen storage and transport, regulation of germination and dormancy release, and oxidative stress protection [1].

Cyanogenic glycosides represent a definitive example of sporadic metabolic evolution, with this compound class present in many phylogenetically diverse plant lineages [3]. Ferns (monilophytes) constitute the earliest diverging plant lineage to produce cyanogenic glycosides, with repeated evolution observed in later diverging gymnosperms, monocots and dicots (reviewed by Ref. [4]). Beyond the plant kingdom, cyanogenic glycosides also occur in different invertebrate species, with the trait apparently arising independently in insects and millipedes [5]. Recent discoveries within pathway elucidation support sporadic evolution of cyanogenic glycosides in plants, with multiple recruitment events of nonorthologous gene members to the biosynthetic pathway observed. This review presents different drivers for repeated evolution of cyanogenic glycosides throughout the plant kingdom, showcasing recent advances within biosynthetic pathway crossovers. Metabolite multifunctionality beyond chemical defense is likely driving repeated evolution of this metabolite class, with cyanogenic glycoside biosynthesis and recycling apparently occurring at critical points in plant development.

Sporadic evolution of cyanogenic glucoside biosynthesis

Sporadic evolution of cyanogenic glycosides is evidenced by the convergent evolution of the biosynthetic pathway in phylogenetically unrelated plant species. Cyanogenic glycosides are derived from aromatic (Phe, Tyr) and aliphatic (Ile, Leu, Val) amino

Figure 1

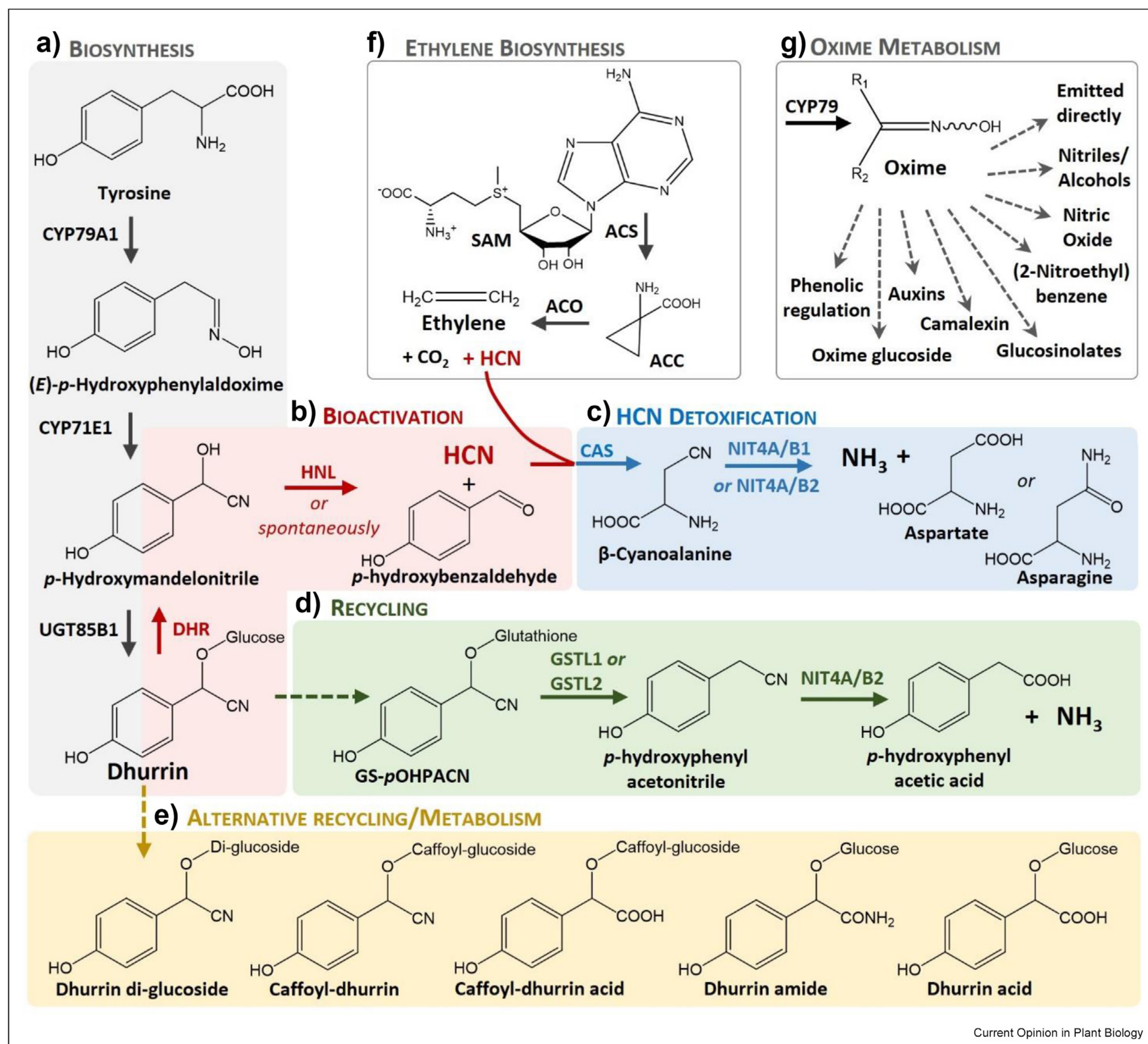


Diversity of cyanogenic glycosides in plants. Representative structures of simple and modified cyanogenic glycosides derived from the aliphatic amino acids valine, isoleucine and leucine (blue), aromatic amino acids tyrosine and phenylalanine (red), and the nonprotein amino acid cyclopentenyl-glycine (CPG; purple). Examples of phylogenetically diverse cyanogenic plant species are shown.

acids and the nonproteinogenic amino acid, cyclopentenyl glycine (Figure 1). To date, the amino acid-derived cyanogenic glucoside pathways have been elucidated from different plant species. The pathway is relatively short, consisting of 3–4 pathway steps, which may facilitate repeated evolution. In Sorghum (*Sorghum bicolor*), the biosynthetic enzymes are organized in a protein complex (metabolon) on the ER membrane, facilitating efficient channeling of pathway intermediates [6]. Several cyanogenic species have the biosynthetic gene members arranged within a gene cluster [7,8], but not all (e.g. almond; *Prunus dulcis* [9]). Where present, biosynthetic gene clusters have aided identification of pathway members and associated transporters [10–13].

The first pathway step involves two successive *N*-hydroxylations of the primary amino acid, followed by a dehydration and decarboxylation step to produce an aldoxime [14]. This enzymatic step is conserved in seed plants, catalyzed by a multifunctional cytochrome P450 enzyme (CYP) from the CYP79 family [7,15,16](Figure 3). In ferns, the same reaction was recently identified to be catalyzed by class B flavin-containing monooxygenase (FMO), fern oxime synthase 1 (FOS1) [17]. While convergent evolution of the first step has occurred between ferns and seed plants, FOS1 shares a remarkable 98% gene sequence identity between two cyanogenic fern species *Phlebodium aureum* and *Pteridium aquilinum*, despite 140 million years of evolutionary separation. The aldoxime intermediate is then dehydrated and hydroxylated by multifunctional CYPs (CYP71, CYP736 or CYP83) to form a cyanohydrin [7,12]. In *Eucalyptus* and *P. aureum*, this reaction is split into separate enzymatic reactions. The dehydration and hydroxylation is performed by a

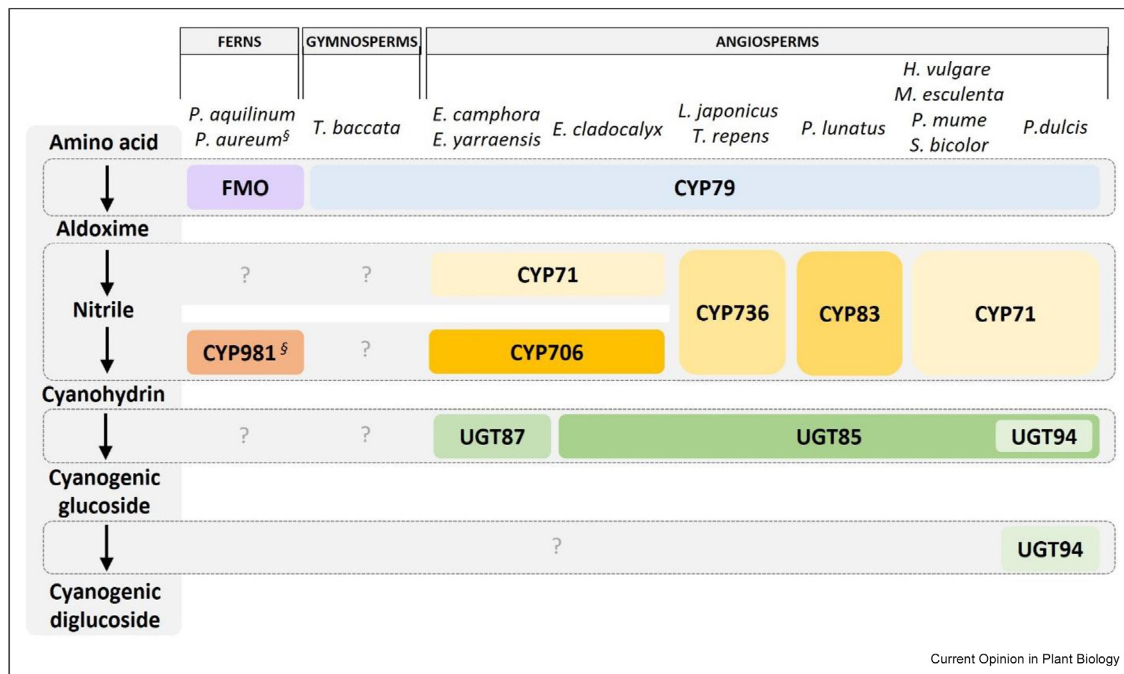
Figure 2



Overview of diverse functional pathways related to cyanogenic glucosides represented by dhurrin metabolism in *Sorghum bicolor*. a) Metabolic pathways related to the tyrosine-derived cyanogenic glucoside dhurrin in *S. bicolor* has been most exhaustively characterized. Biosynthesis (grey panel) involves two multifunctional cytochromes P450 (CYPs) and a UDP-glucosyltransferase (UGT). **b)** Bioactivation of dhurrin (red panel) releases toxic HCN via the action of a dhurrinase (DHR) and an α-hydroxynitrile lyase (HNL). **c)** HCN is detoxified (blue panel) via the action of a β-cyanoalanine synthase (CAS) and nitrilase (NIT) heteromers to produce aspartate, asparagine and ammonia. **d)** Recycling of dhurrin (green panel) without the release of HCN follows replacement of the glucose with glutathione, which is subsequently cleaved by lambda class glutathione transferases (GSTL) to release *p*-hydroxyphenyl acetonitrile. This nitrile is hydrolysed by NIT4 heteromer to produce *p*-hydroxyphenyl acetic acid and free ammonia. **e)** Alternate recycling and metabolism of dhurrin (yellow panel) is evidenced by the presence of modified dhurrin metabolites, but related enzymatic steps currently remain uncharacterized. Other cyanogenic species (e.g. *Prunus*) also produce related “anitriles” [54]. **f)** Seed plants possess the capacity to detoxify HCN due to it being a biosynthetic product in the production of the hormone ethylene. **g)** Oximes are the first intermediate for cyanogenic glucoside biosynthesis, and are also produced by plants for a wide range of metabolic functions.

CYP71 and CYP706 in *Eucalyptus* species, respectively [18,19**]]. Only the nitrile hydroxylation is known for *P. aureum*, performed by a fern-specific CYP981 [20]. In some cyanogenic species, metabolism of the aldoxime forms the branching point for the formation of cyanogenic (α-) and noncyanogenic (β- and γ-) hydroxynitriles. Recent pathway characterization in barley (*Hordeum vulgare*) demonstrated that while all three genome clustered CYP71s can catalyze the conversion of the aldoxime to its corresponding cyanogenic

Figure 3



Sporadic evolution of cyanogenic glycoside biosynthesis in ferns, gymnosperms and angiosperms. Recruitment of different enzyme flavin-containing monooxygenase (FMO) cytochrome P450 (CYP) and UDP-glucosyltransferase (UGT) family members is observed. Unknown steps are indicated. [§]Indicates the step only identified in *P. aureum*.

cyanohydrin *in vivo* [11], only one CYP71 is responsible for cyanogenic glycoside biosynthesis *in planta* [13*]. All three CYP71s, however, form a tight interaction in the production of the four noncyanogenic β- and γ-hydroxynitriles.

The stabilizing glucosylation step for cyanogenic glycoside biosynthesis is typically performed by a UDP-glucosyltransferase (UGT) from the UGT85 family. Recently, dynamic convergent evolution within a single subgenus was reported for this final step. Cyanogenic *Eucalyptus cladocalyx* biosynthesizes prunasin via a UGT85, while the same glucosylation is performed by a member of the UGT87 family in *E. camphora* and *E. yarraensis* [19**]. Biosynthetic modifications on a cyanogenic mono-glucoside has only been described in almond [21]. Three UGT94s were found to be positioned in a tandem repeat, and two members can glucosylate the mono-glucoside prunasin to form the cyanogenic diglucoside, amygdalin. Interestingly, the third UGT94 catalyzes the same catalytic steps as a previously characterized almond UGT85 [22], glucosylating mandelonitrile to form the cyanogenic monoglucoside prunasin. Given plants likely possess multiple UGTs with promiscuous capabilities to stabilize the labile cyanohydrin [23,24], recruitment of new UGTs for cyanogenic monoglucoside production would be a relatively straightforward evolutionary progression, thereby facilitating repeated evolution.

Overall, cyanogenic glycoside biosynthesis and the recruitment of diverse FMO, CYP and UGT families in different plant lineages showcases repeated and dynamic evolution of this pathway. The fern FOS1 represents the first time shared catalytic activity was observed between FMOs and CYPs in plant metabolism, but shared functionality has since been reported for benzoxazinoid biosynthesis in dicots and monocots, respectively [25*]. Beyond plants, cross-kingdom evolution of cyanogenic glycoside biosynthesis also occurs. For example, the cyanogenic specialist moth *Zygena filipendulae* both ingests cyanogenic glucosides from its cyanogenic plant source *Lotus corniculatus*, or -self-biosynthesizes the metabolites via a three-step parallel pathway involving two CYPs and a UGT (CYP405A2, CYP332A3 and UGT33A1) [26]. The independent recruitment of these biosynthetic genes in *Z. filipendulae* demonstrates cross-kingdom convergent evolution of cyanogenic glycosides rather than horizontal gene transfer, as first hypothesized. The uncharacterized biosynthetic pathways of structurally diverse and cyanogenic glycoside-rich *Passiflora* species, as well as interactions with specialized cyanogenic *Heliconius* butterflies would serve as an excellent model to investigate

the cyclopentenyl glycine-derived cyanogenic glycoside cross-kingdom co-evolution [27].

Pathway crossovers may facilitate sporadic evolution of cyanogenic glycosides

Sporadic evolution of cyanogenic glycosides in plants is likely facilitated by conserved preexisting biochemical intermediates and metabolic process (i.e. storage and transport) for biosynthesis and catabolism (Figure 2a–c). With amino acids as precursors, accessible substrates are easily available for repeated cyanogenic glycosides evolution. Furthermore, the first intermediate – an aldoxime – is a key intermediate for many different physiological processes operating within both general and specialized metabolism, providing a basis for evolutionary expansion (Figure 2g) [28,29]. Indeed, members of the CYP79 family, and presumably the presence of aldoximes, are highly conserved in seed plants [17].

Beyond cyanogenic glycosides, aldoximes are involved in other facets of chemical defense. In Tococa (*Miconia microphysca*), aldoximes were recently reported to be biosynthesized and stored as aldoxime glucosides for several days following herbivory, proposed to function as a rapid defense against future herbivory [30*]. The biosynthetic pathway for the aldoxime glucoside in *M. microphysca* shares high similarly with cyanogenic glucosides, catalyzed by a CYP79 and UGT85. In *M. microphysca*, and other plant species, aldoximes and related CYP71-catalyzed volatile nitriles are emitted as a direct and indirect defensive response to insect herbivory, but are also emitted to attract specific pollinators [29]. Aldoximes have long been linked as intermediates for volatile nitro compounds, and recently CYP79s and a CYP94 was reported to catalyze the production of (2-nitroethyl)benzene as a floral scent in loquat (*Eriobotrya japonica*) [31]. In the order Brassicales, aldoximes serve as critical intermediates for glucosinolate production, and the phytoalexin camalexin [29,32].

As important players in general metabolism, CYP79-catalyzed aldoximes are precursors for the auxins indole-3-acetic acid (IAA) and phenyl acetic acid (PAA) [33,34], a new oxidative pathway for nitric oxide (NO) biosynthesis [35**] and operate as negative regulators of phenylpropanoid biosynthesis [36]. Auxins are essential phytohormones, orchestrating virtually all processes in plant growth and development. The aldoxime-derived IAA and PAA pathways operate in parallel to the primary route for auxin biosynthesis involving TAA/TAR and YUCCA proteins. Given that CYP79-derived auxin biosynthesis was recently found to be conserved in both mono- and di-cotyledonous species [34], this alternative pathway for IAA and PAA is likely more widespread than originally believed. NO plays an essential signaling role in plants, involved in a multitude of growth, development and defense-related processes. NO can be derived via different routes and a recent study identified CYP-derived aldoximes as intermediates in a new oxidative pathway for NO production likely catalyzed by peroxidase enzymes [35]. Physiological links of aldoxime-derived NO was evidenced by altered root organogenesis *in vivo*, and may be the cryptic link for how aldoxime-mediated negative regulation of phenylpropanoid biosynthesis [36].

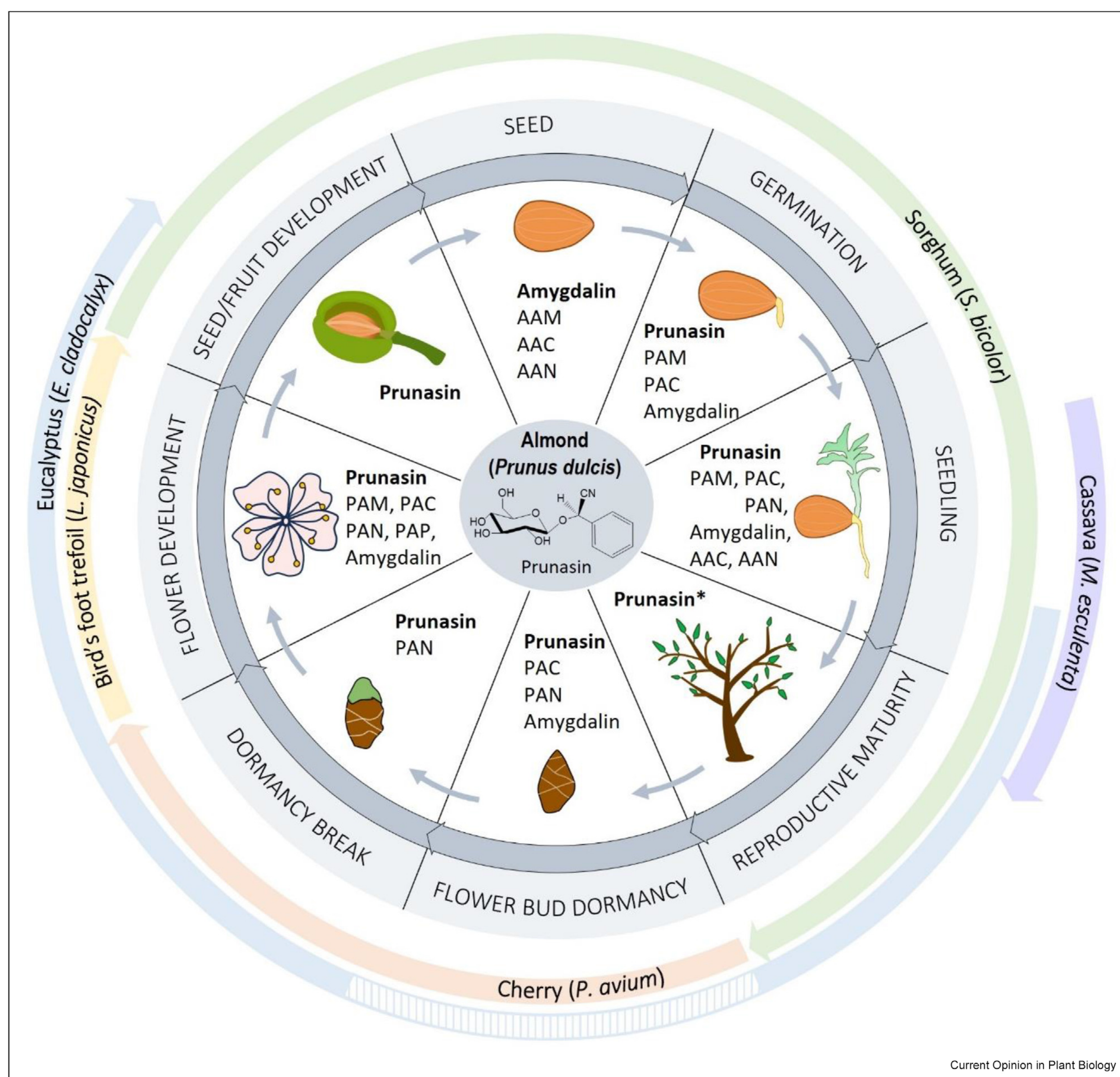
Pathway crossovers linked with cyanogenic glucoside catabolism may also facilitate their sporadic evolution. As part of cyanogenic glycoside catabolism, the cyanohydrin is metabolized into HCN and a ketone or aldehyde (Figure 2b). All seed plants presumably possess a HCN detoxification pathway (Figure 2c), as HCN is a byproduct of ethylene biosynthesis following the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) via an ACC synthase (Figure 2f) [37,38]. Ethylene is an essential phytohormone, regulating key processes within development such as germination, dormancy release, flower development and fruit ripening, and is also a major phytohormone mediating different biotic and abiotic stress responses [39–41]. Accordingly, selection likely favors repeated evolution of cyanogenic glycosides whereby a cost-effective HCN detoxification system is already in place, and the resultant HCN is converted into ammonia and aspartate/asparagine, and recycled back into the general metabolite pool (Figure 2) [42].

Cyanogenic glycoside multifunctionality as a driver for repeated evolution

Once recruitment of pathway members for cyanogenic glycoside biosynthesis in plants has occurred, selection would favor trait retention that provides beneficial qualities for growth, development and performance. Indeed, cyanogenic glycosides are linked to multiple functions beyond chemical defense, including nitrogen storage and remobilization, regulation of bud dormancy break and as an antioxidant [1]. For example, an interplay between HCN release and ROS metabolism was recently proposed in *Prunus* species, where HCN release from cyanogenic glycosides may lead to altered metabolism via catalase and superoxide dismutase inhibition [43]. Furthermore, the cyanohydrin intermediate towards HCN release in peach (*P. persica*) has also been implicated in the biosynthesis of salicylic acid [44]. Cyanogenic multifunctionality is exemplified in cassava (*Manihot esculenta*) where knockdown or knockout of associated CYP79s increased insect pest susceptibility, altered stem and root development and affected root nitrogen metabolism [45,46]. Furthermore, rapid decreases in foliar cyanogenic glycoside content following light spikes also suggests their role in cassava as possible scavengers for reactive oxygen species (ROS) [47].

Cyanogenic glycoside recycling operates via multiple pathways, with and without HCN release (Figure 2c–e). Given that all ethylene-producing plants possess the metabolic machinery to detoxify HCN giving rise to ammonia and aspartate/asparagine formation, it is not surprising that remobilization of cyanogenic glycosides via this pathway is widespread [48–50]. As not all cyanogenic glycoside-containing plants possess hydrolyzing β-glucosidase enzymes to activate this pathway (e.g. barley, clover and bracken fern), recycling presumably occurs via another route. At least in sorghum, two additional recycling pathways are in operation. The first involves replacement of the glucose moiety with glutathione, which is subsequently cleaved and hydrolyzed to produce *p*-hydroxy phenylacetic acid and free ammonia [42,51] (Figure 2d). A combination of temporal and spatial transcriptomics and metabolomics (including mass spectrometry imaging) recently revealed this pathway to be highly dynamic and tissue specific during seed germination [52*,53]. Specifically,

Figure 4



Dynamic changes in cyanogenic glycoside content, and associated metabolites at key developmental stages. The compounds listed inside the inner grey circle indicate measured presence of prunasin-related metabolites specific to almond (*P. dulcis*) life cycle, with the dominating compound at each stage marked in bold. The coloured outer arrows show developmental stages where dynamic changes in cyanogenic glycoside content is reported in other representative species. Abbreviations: PAM, prunasin amide; PAC, prunasin acid; PAN, prunasin anitrile; PAP anitrile pentoside; AAM, amygdalin amide; AAC, amygdalin acid; AAN, amygdalin anitrile. *Indicates tissues where recycling metabolites may be present, but not specifically measured.

it was shown that dhurrin is biosynthesized and localized in the growing embryonic tissue, but also in the scutellum and aleurone layer. Predominate accumulation of dhurrin in the growing embryo is speculated to provide a measure of chemical defense for this valuable and highly vulnerable tissue. In contrast, different recycling products primarily accumulate in the scutellum and/or pericarp. The spatial divergence of metabolic products therefore supports alternative metabolic roles in the germinating grain. A second proposed recycling pathway without HCN release is far less understood, evidenced by occurrence of structurally related conjugates, amides, acids and anitriles that co-occur with cyanogenic glycosides in phylogenetically unrelated species [54] (Figure 2e). Metabolite profiling of cyanogenic glycosides, associated conjugates and metabolic recycling products reveals dynamic metabolic regulation, especially at critical stages in plant development [55](Figure 4). For example, changes in cyanogenic glycosides and associated metabolites are observed throughout all stages of the almond life cycle, in both vegetative and reproductive organs. This suggests either targeted use of cyanogenic glycosides as defense compounds at specific ontogenetic stages and tissues, targeted use of these metabolites for alternative roles, or a combination of both. Dramatic shifts in metabolite content are especially observed in almond, cherry (*P. avium*) and apricot (*P. armeniaca*) buds, from dormancy to flowering [56–58]. The striking metabolic changes in *Prunus* species at key developmental stages provides an excellent system to investigate alternative functionality, whereby cyanogenic glycosides are seemingly positioned at a complex metabolic junction between ROS mediation, hormone production, nitrogen homeostasis, and environmental stimuli. Understanding the regulation driving cyanogenic glycoside biosynthesis in *Prunus* is also aided by identification of a bHLH transcription factor that determines the sweet vs bitter trait in the almond kernel [9].

Conclusions

Advances in cyanogenic glycoside pathway characterization demonstrate the sporadic evolution of this metabolite class throughout the plant kingdom. Biosynthetic and functional pathway crossovers bridging specialized and general metabolism likely facilitate their repeated evolution, such that evolutionary selection will benefit and build upon preexisting pathways and metabolic scaffolds. Recent advances within CYP79-driven aldoxime metabolism and functionality exemplifies an important hub for general and specialized metabolism. Significant knowledge gaps currently exist within the functional characterization of pathway steps involved in modified cyanogenic glycoside biosynthesis (Figure 1), as well as genes responsible for the regulation [9] and execution of metabolic functions beyond chemical defense. It is unclear if the evolutionary history of downstream metabolic pathways are species specific, as proposed for the glutathione-linked recycling pathway in sorghum [52], or are more commonly conserved. Targeted characterization within different cyanogenic lineages will shed light on the wider roles for cyanogenic glycosides, with widespread applications within plant performance and protection.

Author contributions

EHJN: Conceptualization; Visualization; Writing - original draft; Writing - review & editing. **RSP**: Conceptualization; visualization; Writing - review and editing.

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data were used for the research described in the article.

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** of outstanding interest

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