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Erysimum cheiranthoides, an ecological research system with potential as a genetic and genomic model for studying cardiac glycoside biosynthesis

Tobias Züst · Mahdieh Mirzaei · Georg Jander

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Abstract At least twelve plant families contain species that synthesize cardiac glycosides as defense against herbivory. These inhibitors of animal Na^+, K^+-ATPases also have medical uses in treating congestive heart failure and other diseases. However, despite extensive ecological research and centuries of use in both traditional and modern medicine, the complete cardiac glycoside biosynthesis pathway has yet to be elucidated in any plant species. To a large extent, this research deficit results from the fact that cardiac glycosides are produced exclusively by non-model plant species such as *Digitalis* that have not been amenable to the development of mutagenesis, cloning, and genetic mapping approaches. Recent advances in genome sequencing, transcript profiling, plant transformation, transient expression assays, and plant metabolite analysis have provided new opportunities for the investigation and elucidation of cardiac glycoside biosynthesis pathways. The genetic tools that have been developed for Brassicaceae, in particular *Arabidopsis thaliana*, may be directly applicable to *Erysimum*, a Brassicaceae genus that characteristically produces cardiac glycosides as defensive metabolites. We propose that *Erysimum cheiranthoides* (wormseed wallflower), a rapid-cycling, self-pollinating species with a relatively small, diploid genome, would be a suitable model system to advance research on the biosynthesis of cardiac glycosides in plants.

Keywords *Erysimum cheiranthoides* · Wallflower · Cardiac glycoside · Cardenolide · Model system

T. Züst
Institute of Plant Sciences, University of Bern, Altenbergrain 21, 3013 Bern, Switzerland

M. Mirzaei · G. Jander (✉)
Boyce Thompson Institute, 533 Tower Road, Ithaca, NY 14853, USA
e-mail: gj32@cornell.edu

Introduction

Cardiac glycosides are a diverse group of natural products that act as allosteric inhibitors of Na^+, K^+-ATPase, an essential membrane ion transporter that is found in almost all animal cells. Broadly, cardiac glycosides can be categorized as cardenolides and bufadienolides, which have a common steroid core (5β,14β-androstane-3β14-diol) and differ according to the presence of a five- or six-membered lactone ring. This steroid-lactone core structure is highly conserved among cardiac glycosides and mediates the specific binding of cardiac glycosides to Na^+, K^+-ATPase (Dzimiri et al. 1987). While some cardenolides and bufadienolides naturally occur as aglycones (genins), most are linked to one or several sugar moieties in a linear chain, resulting in a glycoside 'tail' that significantly increases the binding affinity and inhibitory effect of these compounds (Dzimiri

et al. 1987). Several hundred cardenolide and bufadienolide structures, which differ by substitutions to functional groups on the steroid core, stereoisomeric conformation, or the incorporation of different types of sugars in the glycoside tail, have been described (Kreis and Müller-Uri 2010; Melero et al. 2000; Singh and Rastogi 1970). Although most cardiac glycosides have been identified and isolated from plants, they are also found in certain insects and toads (hence the name bufadienolide). Cardiac glycosides have no known function in the core metabolism of the plants that produce them, and thus likely act primarily in defense against insects and other herbivores.

The ability to produce cardiac glycosides is scattered across the plant phylogenetic tree, with at least a dozen plant families (Apocynaceae, Asparagaceae, Brassicaceae, Celastraceae, Crassulaceae, Euphorbiaceae, Fabaceae, Malvaceae, Moraceae, Plantaginaceae, Ranunculaceae, and Zingiberaceae) containing cardiac glycoside-producing species (Agrawal et al. 2012; Melero et al. 2000; Steyn and van Heerden 1998). In the Apocynaceae, cardiac glycosides are the predominant secondary metabolites in most species and likely represent an ancestral trait of this family. In the other plant families, there are only sporadic occurrences of cardiac glycoside in subclades or single known species, which likely represent cases of relatively recent, repeated pathway evolution. Such convergent evolution of plant toxins with highly similar inhibitory effects on animal Na^+, K^+-ATPases suggests both a strong selective pressure for plant defense against herbivory and a common metabolic origin that facilitates repeated evolution of similar or identical molecular structures.

The discovery of the metabolic pathways involved in cardiac glycoside synthesis has been limited by the fact that these compounds are not found in traditional genetic model species. The elucidation of the biosynthetic pathways of other important plant metabolites such as glucosinolates, benzoxazinoids, and flavonoids was greatly facilitated by the genomic and molecular resources available for model plants such as *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*, *Solanum lycopersicum*, and *Medicago truncatula.* Even though the biosynthesis of cardiac glycosides has been studied extensively in *Digitalis* spp. (foxglove; Kreis 2017; Luckner and Wichtl 2000), the relatively large plant size, complex pollination requirement, and long, often biennial life cycle of this genus has significantly hindered progress in unravelling the full cardiac glycoside metabolic pathway. In contrast, the cardenolide-producing Brassicaceae genus *Erysimum* is closely related to *A. thaliana* (Huang et al. 2016), and has been proposed as a more suitable model for investigating the molecular biology of cardiac glycoside biosynthesis (Munkert et al. 2011). Within this genus, *Erysimum cheiranthoides* (wormseed wallflower; Fig. 1), a rapid-cycling diploid species with a relatively small genome size, would provide an excellent model system for the use of genetic and genomic approaches to investigate the biosynthesis, ecological function, and evolutionary origins of cardiac glycoside biosynthesis. Here, we provide an overview of currently known steps in cardiac glycoside synthesis in general, and in *Erysimum* in particular. Additionally, we review chemical, ecological, evolutionary, and ethnobotanical literature on *Erysimum* to highlight its relevance as a model system in diverse research areas, and conclude by outlining a set of research methods that would be required for developing *E. cheiranthoides* as a new plant genetic and genomic model system.

Biosynthesis of cardiac glycosides

Production of cardenolides and bufadienolides likely evolved from pathways for the biosynthesis of phytosterols (24 alkyl sterols) and endogenous plant steroid hormones, e.g. brassinosteroids. Early isotope labeling studies suggested that cardiac glycosides are synthesized from cholesterol with progesterone as an intermediate (Kreis et al. 1998; Theurer et al. 1994). However, despite decades of research with *Digitalis*, only a relatively small number of enzymes in the cardenolide biosynthesis pathway have been identified. Progesterone 5β-reductase, which catalyzes the conversion of progesterone to 5β-pregnane-3,20-dione (Fig. 2), was first cloned from *Digitalis purpurea* (Gärtner et al. 1994) and is expressed in all tested *Digitalis* species (Kreis 2017). Two other early steps in cardenolide biosynthesis, dehydrogenation of pregnenolone to isoprogesterone and reduction of 5β-pregnane-3,20-dione (Fig. 2), are both catalyzed by 3β-hydroxysteroid dehydrogenases. After their initial identification in *Digitalis lanata* (Finsterbusch et al. 1999; Herl et al. 2006), 3β-

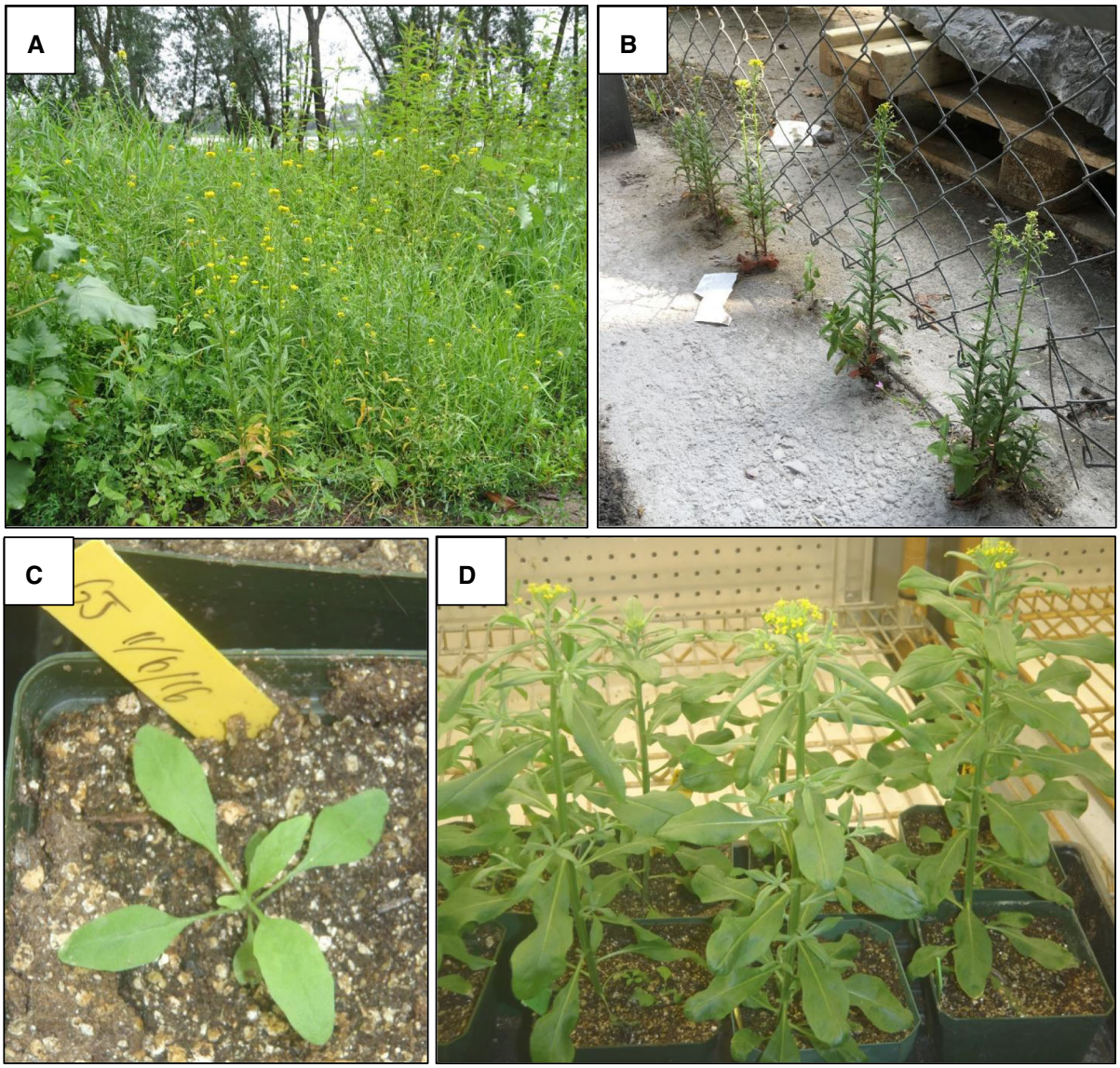

Fig. 1 *Erysimum cheiranthoides.* **a** Natural growth in the Lenzen-Elbtalaue (Elbe River floodplain) in Germany. **b** Growth in a disturbed habitat near the Aare River in Bern, Switzerland. **c**, **d** Plants after 2 and 6 weeks growth, respectively, in a growth chamber with fluorescent lights

hydroxysteroid dehydrogenases have been identified and cloned from several other *Digitalis* species. Given the relatively broad substrate specificities of the identified progesterone 5β-reductase and 3β-hydroxysteroid dehydrogenase enzymes, it is quite possible that their in vivo substrates are not those that are postulated in Fig. 2, but rather other endogenous plant steroids. Malonyl coenzyme A:21-hydroxypregnane 21-*O*-malonyltransferase, which is required for the synthesis of the cardenolide lactone ring (Fig. 2), has been characterized enzymatically in *D. purpurea* (Kuate et al. 2008). Other enzymes of cardiac glycoside biosynthesis, including those catalyzing the addition of sugar side chains and those modifying the aglycone functional groups, remain to be identified.

The investigation of cardenolide biosynthetic enzymes in *Erysimum* is a direct extension of the prior work that has been done with *Digitalis.* Predicted progesterone 5β-reductase genes have been

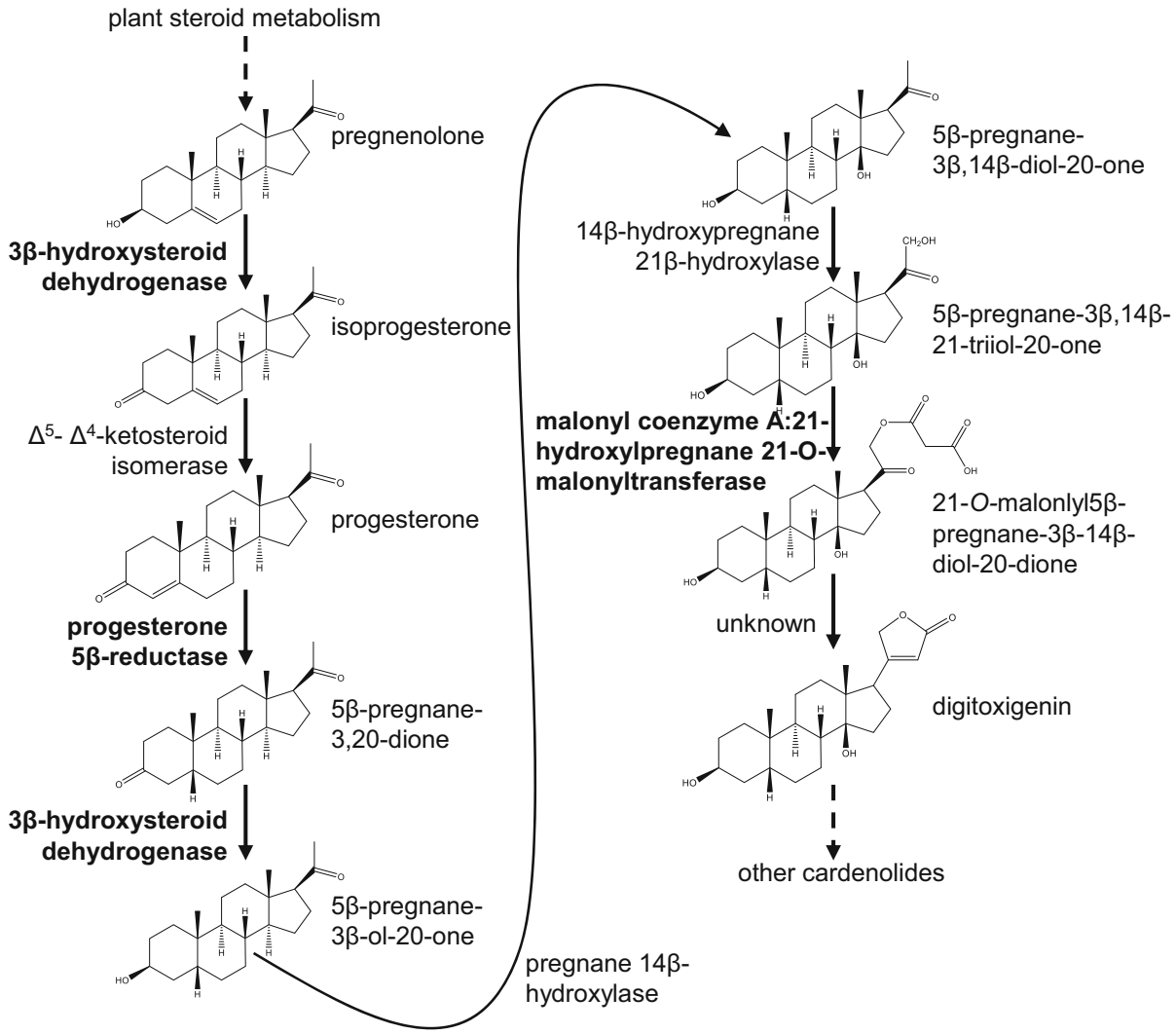

Fig. 2 Biosynthesis of cardenolides. Predicted steps in the biosynthesis of the cardenolide aglycone, digitoxigenin. Enzymes for which there is evidence of enzymatic function in Digitalis are marked in bold. Adapted from Kreis and Müller-Uri (2010)

amplified from ten *Erysimum* species, and His-tagged versions of two *Erysimum crepidifolium* proteins were purified after gene expression in *Escherichia coli* (Munkert et al. 2011, 2015b). In vitro enzyme assays demonstrated conversion of progesterone to 5β-pregnane-3,20-dione by the two *E. crepidifolium* enzymes, as well as reductase activity with additional substrates (Munkert et al. 2011, 2015a, b). Functional progesterone 5β-reductases are present in all tested Brassicaceae, and there is no significant difference in the utilization of progesterone as a substrate by enzymes from species that do and do not produce cardiac glycosides (Munkert et al. 2015a). Three genes encoding 3β-hydroxysteroid dehydrogenases have been cloned from *E. crepidifolium* (Munkert et al. 2014), and in vitro enzyme assays demonstrated dehydrogenation of pregnenolone and the 3-reduction of 5α/β-pregnane-3,20-dione. As in the case of progesterone 5β-reductase, genes encoding functional 3β-hydroxysteroid dehydrogenases are also present in species that do not produce cardenolides (Rahier et al. 2006), indicating a broader role in plant steroid metabolism. However, despite this evidence of in vitro enzymatic activity, *Erysimum* mutant lines or other reverse genetics approaches will be required confirm the in vivo function of the identified

progesterone 5β-reductases and 3β-hydroxysteroid dehydrogenases in cardenolide biosynthesis.

The diversity of cardiac glycosides found in *Erysimum*

More than 50 different cardiac glycosides have been isolated from various *Erysimum* species. Cardiac glycosides are highly concentrated in seeds of *Erysimum*, with total concentrations often reaching 10–50 mg g^{-1} of dry weight, while concentrations in leaves more commonly range from 0.2 to 5 mg g^{-1} dry weight (Makarevich et al. 1994). In *Erysimum*, all known cardiac glycosides belong to the cardenolides, i.e. consisting of a steroid core linked to a five-membered lactone ring (Rodman et al. 1982; Lei et al. 1996, 1998, 2000, 2002; Makarevich and Kolesnikov 1965; Makarevich et al. 1994; Sachdev-Gupta et al. 1990, 1993). Cardenolides are further distinguished as 5α- or 5β-based on the conformation between the A and B rings of the steroid core (Fig. 3a), with compounds in the 5β-conformation being generally more abundant in *Erysimum* (Makarevich et al. 1994; Nielsen 1978b). Cardenolides of *Erysimum* accumulate either as aglycones or as glycosides, the latter having one, two, or three monosaccharides linked in a linear chain to the steroid core. To date, 15 different aglycones have been identified in *Erysimum* (Makarevich et al. 1994), with glycosides of the four aglycones strophanthidin, digitoxigenin, cannogenol, and bipindogenin being the most common (Fig. 3a). At least 10 different monosaccharides are incorporated into glycosides, two of which are exclusively found within cardenolides (Fig. 3b; Makarevich et al. 1994).

Cardenolides produced by *E. cheiranthoides* include at least seven mono- and di-glycosides of strophanthidin, cannogenol, and digitoxigenin (Fig. 4; Makarevich and Kolesnikov 1965; Sachdev-Gupta et al. 1990, 1993), with the three compounds erysimoside, erychroside (both strophanthidin), and erycordin (cannogenol) generally being the most abundant. Total cardenolide concentrations in *E. cheiranthoides* can reach up to 3 mg g^{-1}, and there is substantial qualitative and quantitative variation among plants from different populations (Latowski et al. 1979; Züst 2018, personal observations).

The evolutionary history of the genus *Erysimum*: a role for cardenolides?

Plant–herbivore co-evolution is frequently likened to a chemical arms race: A plant that produces a chemical defense to protect its resources from herbivores is frequently attacked by specialists that have evolved tolerance strategies to cope with this defense. In turn, this increases the selective pressure for the evolution of additional defenses in the plant (Ehrlich and Raven 1964; Fraenkel 1959). Many of the several thousand metabolites that are found in any given plant species (Bino et al. 2004) may have evolved as defenses against herbivores and pathogens. Although the acquisition of novel chemical defenses in evolutionary recent times is likely widespread in the plant kingdom, this phenomenon has been reported most commonly in the well-studied Brassicaceae (Feeny 1977). For instance, the production of saponins in *Barbarea vulgaris* (Shinoda et al. 2002), alkaloids in *Cochlearia officinalis* (Brock et al. 2006), and alliarinoside in *Alliaria petiolata* (Frisch and Møller 2012) represent recently evolved chemical defenses that allow these species to resist attack from specialized herbivores that have evolved tolerance of glucosinolates, the most characteristic chemical defenses of the Brassicaceae (Dimock et al. 1991; Haribal and Renwick 2001; Nielsen et al. 2010; Shinoda et al. 2002).

The accumulation of cardenolides by species in the genus *Erysimum* is one of the longest- and best-studied examples of the evolutionarily recent gain of a novel chemical defense (Jaretzky and Wilcke 1932; Makarevich et al. 1994; Nagata et al. 1957; Singh and Rastogi 1970). Cardenolide biosynthesis is likely to be present in all *Erysimum* species, while one of the most closely-related sister genera, *Malcolmia*, lacks these chemical defenses (Moazzeni et al. 2014; Nagata et al. 1957). There are also reports of cardiac glycoside occurrence in two more distantly related Brassicaceae genera, *Syrenia* and *Draba* (Makarevich et al. 1994; Munkert et al. 2015a), but these have been studied less extensively.

A long-standing postulate in the ecological literature is that development of key adaptive traits, such as a novel chemical defense that enable escape from herbivory, allow expansion into new habitats and rapid speciation (Weber and Agrawal 2014). Consistent with this hypothesis, molecular analysis of the

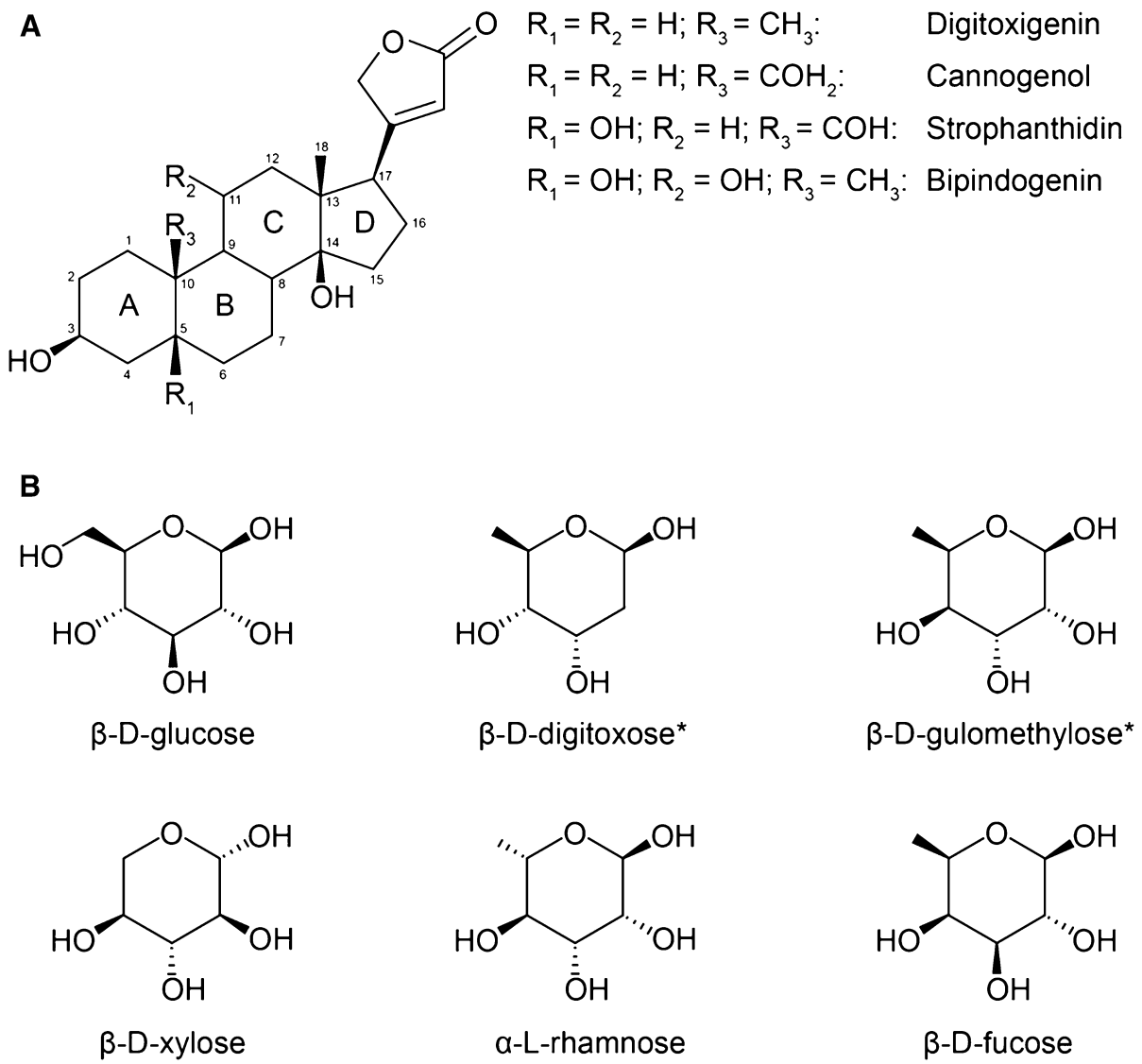

Fig. 3 Cardiac glycosides in *Erysimum*. **a** Structures of the four most common cardenolide aglycones of *Erysimum*. The skeleton structure of a cardenolide is composed of the steroid core (four fused rings, *a–d*), the lactone group at position 17, and a glycoside binding site at C3. **b** Structures of the most common monosaccharides that are incorporated into glycosylated cardenolides. Monosaccharides exclusive to cardenolides are indicated by asterisks

Erysimum genus provides evidence for both recent development of cardenolide biosynthesis and rapid speciation in this genus. Phylogenetic studies based on sequencing a ribosomal internal transcribed spacer region from 128 *Erysimum* species indicate that the genus began rapidly diversifying in Eurasia between 0.5 and 2 million years ago, and in North America between 0.7 and 1.65 million years ago (Moazzeni et al. 2014). In this relatively short time period, the *Erysimum* genus has expanded into at least 150 known species (and perhaps many more, including both diploid and polyploid species), has colonized a range of habitats across the northern hemisphere, and has developed diverse morphology, growth habits (herbaceous annual or perennial, and woody perennial), pollination strategies, and chemical defenses (Al-Shehbaz 1988, 2010; Gomez et al. 2015; Makarevich et al. 1994; Polatschek

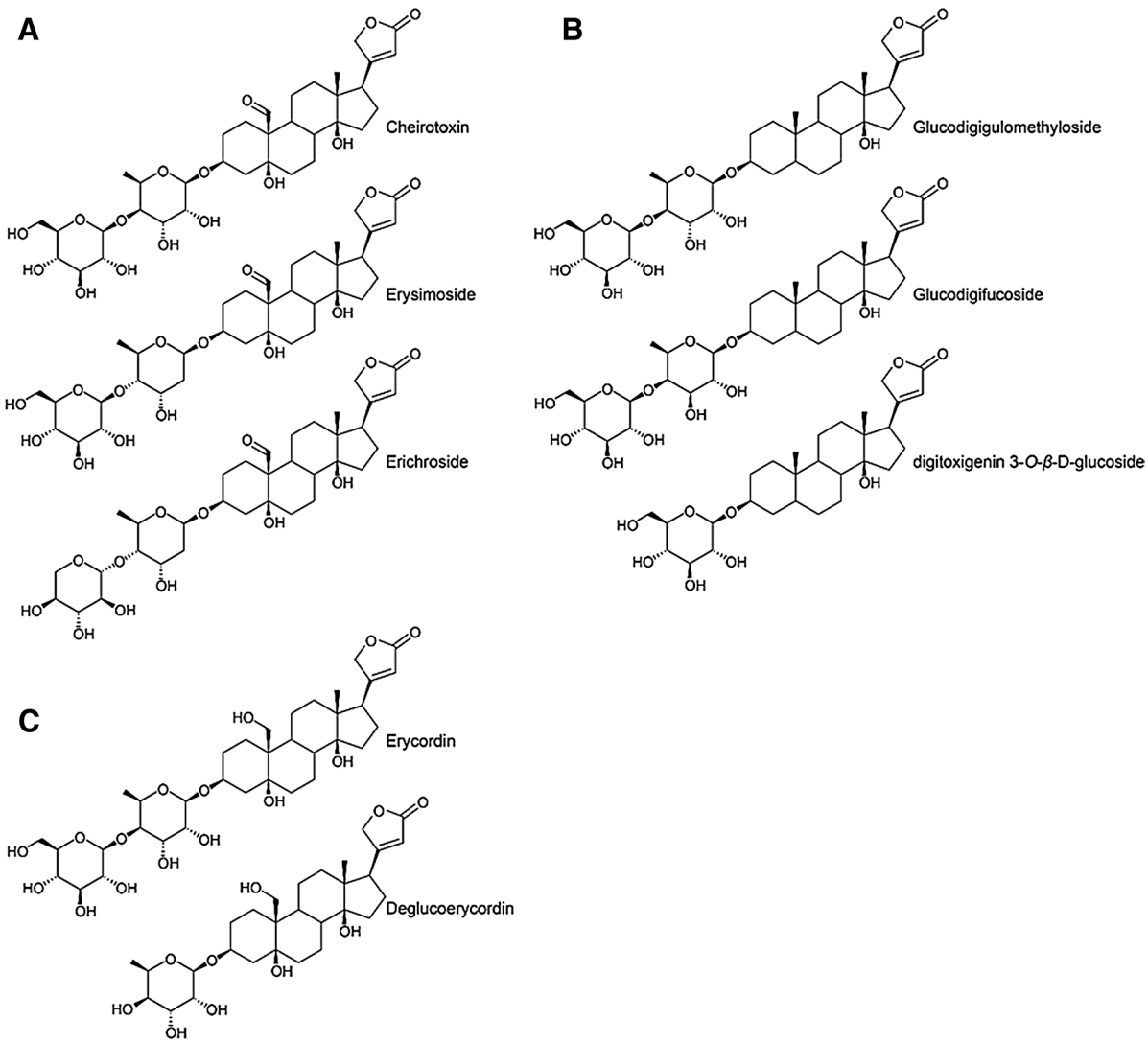

Fig. 4 Cardenolides identified from *Erysimum cheiranthoides*. Compounds are grouped by their respective aglycone: **a** three di-glycosides of strophanthidin. **b** Two di-glycoside and one mono-glycoside of digitoxigenin, and **c** a mono- and di-glycoside each of cannogenol

2010, 2011, 2012; Polatschek and Snogerup 2002; Zhou et al. 2001). In addition to its potential as a new molecular model system, the genus *Erysimum* thus also provides a unique opportunity to study the role of defense evolution in a rapidly diversifying clade.

Erysimum cardenolides provide an additional defense against herbivory

Although both cardenolides and glucosinolates are glycosylated defensive metabolites found in *Erysimum*, their functions are quite different. In contrast to cardenolides, glucosinolates are non-toxic in their glycosylated form. Cleavage of the glucosinolate sugar moiety by an activating glucosidase, followed by further non-enzymatic breakdown, leads to the formation of toxic and deterrent compounds (Halkier and Gershenzon 2006). Several insect herbivores that are specialized for feeding on Brassicaceae, including *Pieris rapae* (white cabbage butterfly), *Plutella xylostella* (diamondback moth), *Brevicoryne brassicae* (cabbage aphid), *Phyllotreta striolata* (striped flea beetle), and *Athalia rosae*

(turnip sawfly), have evolved strategies to prevent or re-direct glucosinolate activation, and are therefore well-adapted for consuming glucosinolate-containing plants (Beran et al. 2014; Francis et al. 2002; Jones et al. 2001; Müller and Wittstock 2005; Pontoppidan et al. 2001; Ratzka et al. 2002; Wittstock et al. 2004).

Consistent with the "escape from herbivory" hypothesis explaining the evolution of cardenolides as a novel plant defense, several species of Brassicaceae-specialist herbivores refuse to oviposit on and/or consume *Erysimum*. For example, adult *Pieris rapae* do not deposit eggs on *E. cheiranthoides* and larvae refuse to eat *E. cheiranthoides* leaves (Feeny 1977). Similarly, *Pieris napi macdunnoughii* (*Pieris marginalis*, margined white butterfly) females do not oviposit on *Erysimum asperum* (western wallflower), nor do larvae consume this species (Chew 1975, 1977). *Anthocharis cardamines* (orange tip butterfly), another pierid that uses almost all Brassicaceae species as host plants, avoids oviposition on *E. cheiranthoides* (Wiklund and Ahrberg 1978). Two specialist beetles *Phaedon* sp. and *Phyllotreta* sp., were deterred from feeding by strophanthidin glycosides applied to a non-cardenolide host plant at concentrations similar to those found in several *Erysimum* species (Nielsen 1978a, b). However, cardenolides do not provide universal defense, and larvae of another specialist lepidopteran, *P. xylostella*, are regularly observed feeding on *E. cheiranthoides* in the field (Daan Mertens and Erik Poelman, personal communication). The specialist *Eurydema oleracea* (crucifer shield bug) and the mustard aphid *Lipaphis erysimi* readily feed on *Erysimum* species in the field (Züst 2018, personal observations). A community of specialist seed-feeding herbivores also consumes *Erysimum*, despite high levels of cardenolides in the seeds (Gómez 2005).

A series of publications by Alan Renwick and coworkers at the Boyce Thompson Institute not only constitute some of the first structural identifications of *E. cheiranthoides* cardiac glycosides, but also demonstrate that these compounds deter oviposition and feeding by *P. rapae*. Chemical separation of *E. cheiranthoides* sprayed onto cabbage showed the presence of both attractants and deterrents for *P. rapae* oviposition (Renwick and Radke 1985, 1987). The oviposition stimulants were found to be 3-methylsulfinylpropyl glucosinolate and 3-methylsufonylpropyl glucosinolate (Dimock et al. 1991; Huang et al. 1993), whereas oviposition deterrents were identified as cardenolides (Renwick et al. 1989; Sachdev-Gupta et al. 1990). Although erysimoside and erychroside had strong deterrent effects, erycordin was inactive in this oviposition assay. Tarsal sensilla of *P. rapae* responded to both glucosinolate- and cardiac glycoside-containing leaf extracts, suggesting that these toxins may be perceived by contact with the leaf surface (Städler et al. 1995). Consistent with the deterrent effects on oviposition, cardiac glycosides from *E. cheiranthoides* also served as feeding deterrents for *P. rapae* larvae (Dimock et al. 1991; Sachdev-Gupta et al. 1993). Another pierid species, *Pieris napi oleracea* (mustard white butterfly), which is less sensitive to exogenously added cardiac glucosides than *P. rapae* in oviposition assays, also oviposits more readily on *E. cheiranthoides* foliage (Huang et al. 1993; Huang and Renwick 1993).

Consistent with their role in plant defense, accumulation of cardenolides in *E. crepidifolium* (Munkert et al. 2014), *E. cheiranthoides* (Züst and Mirzaei 2018, personal observations), and likely also other *Erysimum* species is induced by exogenous addition of methyl jasmonate, a well-studied plant hormone that is required for anti-herbivore defense induction in numerous plant species (Howe and Jander 2008). Mirroring induction of cardenolides, expression of one of the three known *E. crepidifolium* 3β-hydroxysteroid dehydrogenases was induced by treatment with methyl jasmonate (Munkert et al. 2014), which is consistent with this enzyme's function in cardenolide biosynthesis.

Ethnobotanical and medical uses of *Erysimum*

Matching the repeated gain of cardiac glycosides by a wide range of plant species, native cultures in Europe (*Digitalis* spp.), China (*Erysimum* spp.), North America (*Asclepias* spp.), and North Africa (*Scilla* spp.) have independently developed the use of cardiac glycoside-containing plants for treating a variety of medical ailments (Araya et al. 2012; Luckner and Wichtl 2000; Stoll 1937; Zhu 1989). The more specific application of cardenolide-containing *Digitalis* extracts in the treatment of congestive heart disease was first described in 1785 (Withering 1785). Since this initial written report, hundreds of

publications have been devoted to the investigation of cardenolide target sites, method of action, functional diversity, and applications in human medicine. As the current and potential therapeutic uses of cardiac glycosides have been summarized in several recent reviews (e.g. Fürst et al. 2017; Gurel et al. 2017; Kreis 2017; Patel 2016; Schneider et al. 2017), we will not describe them here.

Although it is less well-known than *Digitalis* in western medicine, *Erysimum* also has long history of implementation as a medicinal plant. Likely due to its cardiac glycoside content, *E. cheiranthoides* has been used for centuries in traditional Chinese medicine to treat a variety of ailments, including heart disease (Zhu 1989). *De Materia Medica* (Dioscorides ~70), the foremost European medical pharmacopeia for more than 1500 years, describes *Erysimum cheiri* as a medicinal herb. In *Naturalis Historia* (Pliny the Elder 77), *Erysimum* is classified as a medicinal rather than a food plant. Leading medieval pharmacopeiae of herbs and the medicines, including the *Dispensatorium des Cordus* (Cordus 1542), *Bocks Kräuterbuch* (Bock 1577), and Tabermontanus' *Neuw Kreuterbuch* (Tabermontanus 1588) describe the medical uses of *E. cheiri*. In the centuries after the Middle Ages, the medical applications of *Erysimum* were largely disused in Europe (Jaretzky and Wilcke 1932). However, more recently, *Erysimum diffusum*, as well as purified helveticoside and erysimoside, have been applied in Ukrainian medical preparations (Makarevich et al. 1994).

Properties that will make *E. cheiranthoides* a tractable genetic model system

Within the *Erysimum* genus, *E. cheiranthoides* is particularly attractive for the development of a new genetic model system for studying cardenolide biosynthesis and other ecologically relevant traits. Although *E. cheiranthoides* inflorescences grow to over 1 m in height and are therefore about three times the size of *A. thaliana*, other properties are not that different from this more established model system. As a self-pollinating annual with a seed-to-seed generation time that is as short as 10 weeks for some isolates (Jander, personal observations), *E. cheiranthoides* can be cycled rapidly in the laboratory in relatively small pots (Fig. 1c, d). Although some isolates reportedly require cold stratification for germination (Karlsson and Milberg 2002), *E. cheiranthoides* isolates that we collected in Germany and Switzerland germinated immediately after seed harvest.

The *E. cheiranthoides* genome size is only about 200 Mbp across eight chromosomes (Bainard et al. 2012; Strickler, Mirzaei and Jander 2018, personal observations), placing it at the lower end of typical plant genome sizes. Genotyping and/or sequencing of multiple *E. cheiranthoides* isolates will allow genome-wide association studies of biochemical traits, using genetic mapping methods such as those that have been applied to *A. thaliana*, *Z. mays*, *O. sativa* and other species. The diploid genome of *E. cheiranthoides* also will facilitate the identification of mutants with altered cardiac glycoside content after chemical mutagenesis, an approach that has been used successfully to identify glucosinolate-deficient mutants, as well as the corresponding mutated genes, in *A. thaliana* (Haughn et al. 1991; Kim et al. 2004; Kliebenstein et al. 2007).

In vivo confirmation of candidate gene function will be essential for investigating the genetic basis of cardenolide biosynthesis. Several approaches for gene overexpression or expression silencing that are effective in other species could be tested in *E. cheiranthoides*. The "floral dip" *Agrobacterium* transformation protocol developed for *A. thaliana* (Clough and Bent 1998) has been applied successfully to transform other Brassicaceae species, including *Camelina sativa* (camelina; Liu et al. 2012), *Thlaspi arvense* (pennycress; Sedbrook et al. 2014), and *Brassica rapa* ssp. *chinensis* (pakchoi; Qing et al. 2000). Cotyledon explants from *E. cheiranthoides* produce callus in tissue culture (Pidgeon and Jander 2018, personal observations), suggesting the possibility of regenerating transformed plants using in vitro methods. Virus vectors that allow transient gene overexpression or gene expression silencing in *A. thaliana* (Burch-Smith et al. 2006) might also be effective for functional genomics assays in *E. cheiranthoides*. *Agrobacterium tumefaciens* leaf infiltration (Johansen and Carrington 2001) or generation of hairy roots using *Agrobacterium rhizogenes* (Henzi et al. 2000; Puddephat et al. 2001) are other possible approaches for engineering transient changes in gene expression. Although not all of these established methods will work equally well with *E.*

cheiranthoides, it is very likely that enough of them will be feasible to allow the functional analysis of cardiac glycoside biosynthetic genes in vivo.

Future prospects

Establishment of a genetic model system for investigating cardenolide biosynthesis will open up many new research opportunities. It is improbable that all twelve plant families that produce cardiac glycosides evolved the same metabolic pathways for these compounds. Suitable transformation protocols would not only allow investigation of endogenous *E. cheiranthoides* pathways, but could also could be used as a platform to investigate candidate genes from other plant species. This approach would involve either complementation of mutations in *E. cheiranthoides* biosynthetic pathways or modifications of the core pathways that are present in *E. cheiranthoides* by transformation with enzymes from other plant species to produce novel cardiac glycoside profiles. These and other research approaches, in conjunction with *E. cheiranthoides* as a genetically tractable model system, will facilitate further investigation of the defensive properties of different cardiac glycosides, as well as the purification of novel compounds for medicinal research.

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