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Letters

# The Progesterone 5β-Reductase/Iridoid Synthase Family: A Catalytic Reservoir for Specialized Metabolism across Land Plants

Trinh-Don Nguyen and Sarah E. O'Connor*

Cite This: https://dx.doi.org/10.1021/acschembio.0c00220

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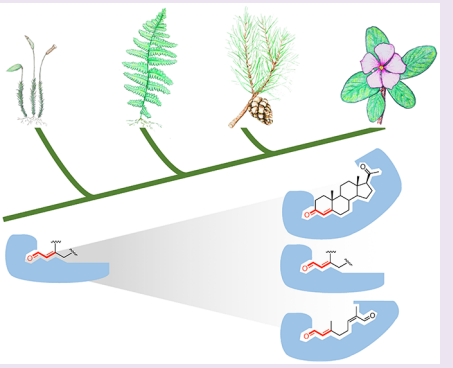
**ABSTRACT:** Iridoids are plant-derived terpenoids with a rich array of bioactivities. The key step in iridoid skeleton formation is the reduction of 8-oxogeranial by certain members of the progesterone 5β-reductase/iridoid synthase (PRISE) family of short-chain alcohol dehydrogenases. Other members of the PRISE family have previously been implicated in the biosynthesis of the triterpenoid class of cardenolides, which requires the reduction of progesterone. Here, we explore the occurrence and activity of PRISE across major lineages of plants. We observed trace activities toward either 8-oxogeranial or progesterone in all PRISEs, including those from nonseed plants and green algae. Phylogenetic analysis, coupled with enzymatic assays, show that these activities appear to have become specialized in specific angiosperm lineages. This broad analysis of the PRISE family provides insight into how these enzymes evolved in plants and also suggests that iridoid synthase activity is an ancestral trait in all land plants, which might have contributed to the rise of iridoid metabolites.





## INTRODUCTION

Iridoids constitute a noncanonical group of monoterpenoids with a distinctive cyclopentanopyran skeleton. Although members of this group were first isolated from insects (hence the name "iridoid", from the rainbow ant genus *Iridomyrmex*), most known iridoids are synthesized by plants.[1,2] Hundreds of naturally occurring iridoid structures act as defensive chemicals in 57 known families within the angiosperms.[3,4] Iridoids also serve as precursors for a wide range of high-value monoterpenoid indole alkaloids, including the anticancer drugs vinblastine in *Catharanthus roseus* and campothecin in *Camptotheca acuminata*.[5] Iridoid biosynthesis begins with the conversion of geranyl pyrophosphate, the general precursor of monoterpenoids, to geraniol via a typical monoterpene synthase, geraniol synthase. Geraniol is then hydroxylated and oxidized to form 8-oxogeranial, which, in turn, is subjected to reduction, catalyzed by a short-chain alcohol dehydrogenase called iridoid synthase (abbreviated hereafter as ISY), to form the reactive 8-oxocitronellyl enol. In the absence of additional enzymes that guide the stereoselective cyclization of this intermediate, it is converted nonenzymatically in aqueous solution to a combination of iridodial and nepetalactol stereoisomers (see Figure 1, as well as Figure S2 in the Supporting Information).[6−9] The specific short-chain dehydrogenase family to which ISY belongs is known for its progesterone 5β-reductase activity, specifically, the stereoselective reduction of progesterone to 5β-pregnan-3,20-dione that occurs in the biosynthesis of cardenolide variety of triterpenoids.[10,11] This enzyme family, named PRISE (for progesterone 5β-reductase/iridoid synthase activity), appears to play a critical ecophysiological role, since both iridoids and cardenolides are major groups of signaling molecules and semiochemicals.[12] Intriguingly, however, members of the PRISE family have been reported in iridoid- and cardenolide-free species such as *Arabidopsis*.[13−17] Earlier reports showed that both PRISEs from *Catharanthus roseus*, an iridoid-producing species, and PRISEs from the Brassicaceae, including those that do not produce iridoids and/or cardenolides, had different reductase activities toward 8-oxogeranial and progesterone.[16,18,19] Promiscuous enzymatic activities are likely starting points for specialization under different conditions, as demonstrated by several examples in enzyme evolution.[20−24] To determine whether such a process within the PRISE family played a role in iridoid metabolism evolution, we identified PRISE homologues from an encompassing range of plant lineages. We found that PRISEs appear to be ubiquitous in plants, and the substrate specificities of these enzymes for either 8-oxogeranial and progesterone, as measured within a phylogenetic framework, may provide clues regarding how iridoid pathways evolved.

## RESULTS AND DISCUSSION

To probe the evolution of PRISE, we used *C. roseus* ISY (PRISE5) as a query to search for PRISE homologues in the

Received: March 26, 2020
Accepted: June 5, 2020
Published: June 5, 2020

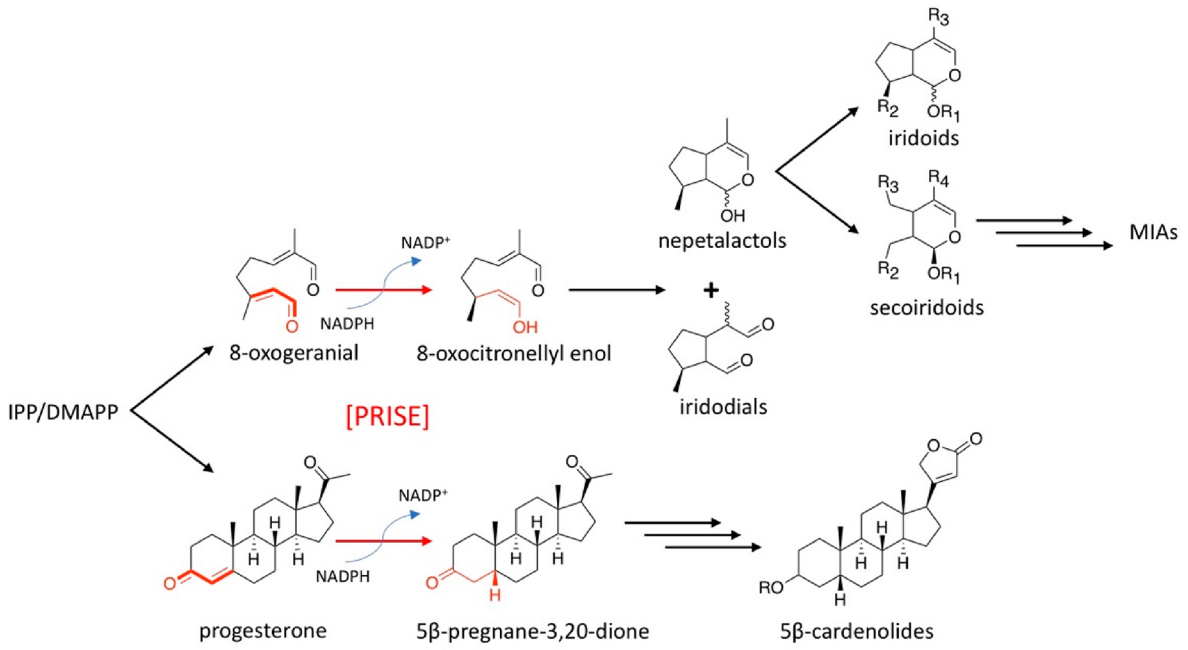



**Figure 1.** Progesterone 5$\beta$-reductase/iridoid synthase (PRISE) in the biosynthetic pathways of iridoids and monoterpenoid indole alkaloids (MIAs), and 5$\beta$-cardenolides. Bold red lines indicate the characteristic 4-en-3-one moeity of PRISE substrates. [IPP = isopentenyl pyprophosphate, DMAPP = dimethylallyl pyrophosphate.]

publicly available nucleotide databases, including NCBI, the 1KP Project, and the bryophyte genome databases.[25−27] BLAST searches revealed that PRISE homologues occur in all reported plant lineages. Outside angiosperms, we found full-length PRISE sequences in several families of gymnosperms (46%−56% sequence identity to *C. roseus* ISY), lycophytes (41%−56% sequence identity to *C. roseus* ISY), nonvascular plants (moss and liverwort) (46%−53% sequence identity to *C. roseus* ISY, and green algae (31%−47% sequence identity to *C. roseus* ISY).

We selected 113 sequences for phylogenetic reconstruction (see Table S1 in the Supporting Information). Although our sequence selection was biased toward plants that have genome or cDNA sequence data available, the available sequences cover major plant lineages including representative members of the core eudicots (orders Lamiales, Brassicales, Apiales, Caryophyllales, Cornales, Sapindales, Cucurbitales, Gentianales, Malvales, Asterales, Fabales, Malpighiales, and Vitales), monocots (orders Asparagales, Zingiberales, and Poales), basal angiosperm (order Amborellales), gymnosperms (order Pinales), lycophytes (orders Lycopodiales, Isoetales, and Selaginalles), and bryophytes (orders Marchantiales, Notothyladales, Metzgeriales, and Funariales). Importantly, the selected PRISE homologues include those from species that produce iridoids but not cardenolides (e.g., *C. roseus*), cardenolides but not iridoids (e.g., *Digitalis lanata*), neither iridoids nor cardenolides and with relatively limited observed secondary metabolites in general (e.g., the nonvascular bryophytes). Anywhere from 1−10 PRISE homologues were found in each of the species examined.

The phylogenetic analysis of this protein family indicates early duplications of PRISEs in land plants (Figure 2). All flowering plants examined here have at least one PRISE homologue in both clades IV and V (middle and top clades in Figure 2), while PRISEs from nonvascular plants (mosses) are found in the basal clade and clade I (bottom portion of Figure 2, which consists exclusively of homologues from nonvascular plants). Clade V (top) includes homologues from gymnosperms, and all functionally characterized ISYs involved in iridoid biosynthesis (e.g., *Catharanthus*), as well as PRISEs known to be involved in cardenolide biosynthesis (e.g., *Digitalis*).

While a few PRISEs in clades IV and V from angiosperms have been assayed with the naturally occurring 4-en-3-ones, such as 8-oxogeranial and progesterone,[15,16,19,28] we set out to biochemically characterize PRISE homologues from representative members of the angiosperms (clades IV and V), lycophytes (nonseed, vascular plants, clades II−IV), and nonvascular plants (basal clade). PRISEs from the model plant *A. thaliana* (clades IV and V), basal angiosperm *Amborella trichopoda* (clades IV and V), lycophyte *Selaginella moellendorffii* (clades II−IV), and the moss *Physcomitrella patens* (basal)—all species reported to lack iridoids and cardenolides—were compared to the reported PRISE enzymes in *Catharanthus* (iridoid producer) and *Digitalis* (cardenolide producer). In addition to members in land plants, a full-length PRISE homologue from green algae, *Coccomyxa subellipsoidea*, was also found and included in the analyses.

Given that PRISEs are found ubiquitously across the plant kingdom, it is likely that these enzymes have a more central role in plant metabolism beyond iridoid and cardenolide metabolism. Since sterols are one of the few plant central metabolites that contain the characteristic 4-en-3-one moiety characteristic of these enzyme substrates and are also found in all plant lineages, it is possible that sterols beyond progesterone may serve as substrates for these ubiquitous enzymes. Therefore, in addition to the two known, physiological substrates of the previously characterized PRISEs, 8-oxogeranial and progesterone, we tested each selected enzyme with two additional commercially available steroid substrates: testosterone, which is a steroid naturally occurring in some gymnosperm; and cholest-4-en-3-one, a close analogue of the


https://dx.doi.org/10.1021/acschembio.0c00220
ACS Chem. Biol. XXXX, XXX, XXX−XXX

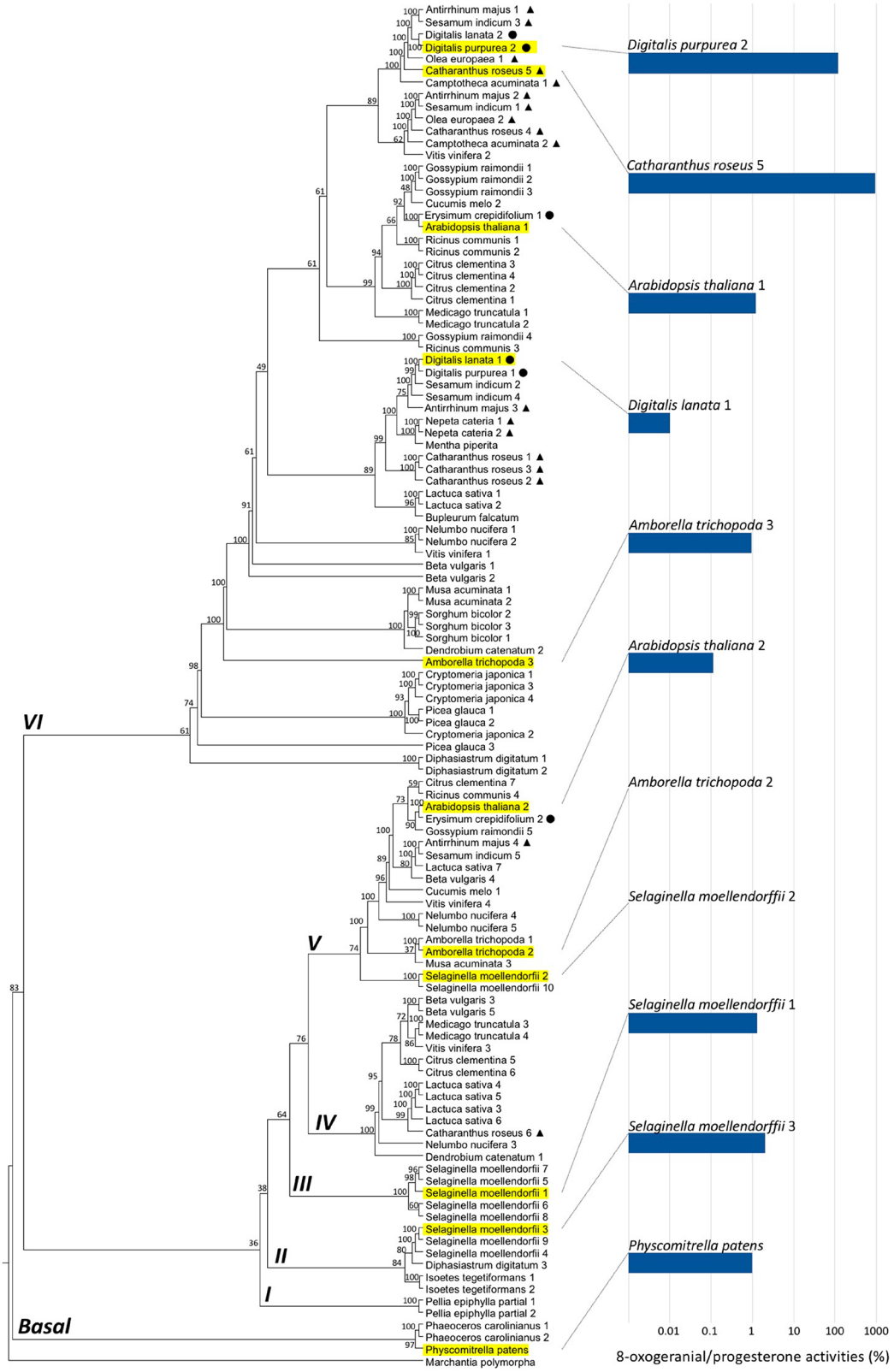



**Figure 2.** Phylogeny and 8-oxogeranial reduction activities of representative ISY/PRISE throughout land plants. Phylognetic tree inference was performed with W-IQ-Tree (http://iqtree.cibiv.univie.ac.at/) using maximum-likelihood method and JTT+I+G4 substitution model. Branch support values were calculated using ultrafast bootstraping with 1000 replicates. A tree was visualized using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Bars represent catalytic efficiencies ($s^{-1}$ $M^{-1}$) of 8-oxogeranial reduction activity, compared to those of progesterone reduction (as percentage) for selected ISY/PRISEs. [Circles (●) and triangles (▲) indicate the occurrence of cardenolides and iriroids, respectively, in the species to which the sequences belong.]



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ACS Chem. Biol. XXXX, XXX, XXX−XXX

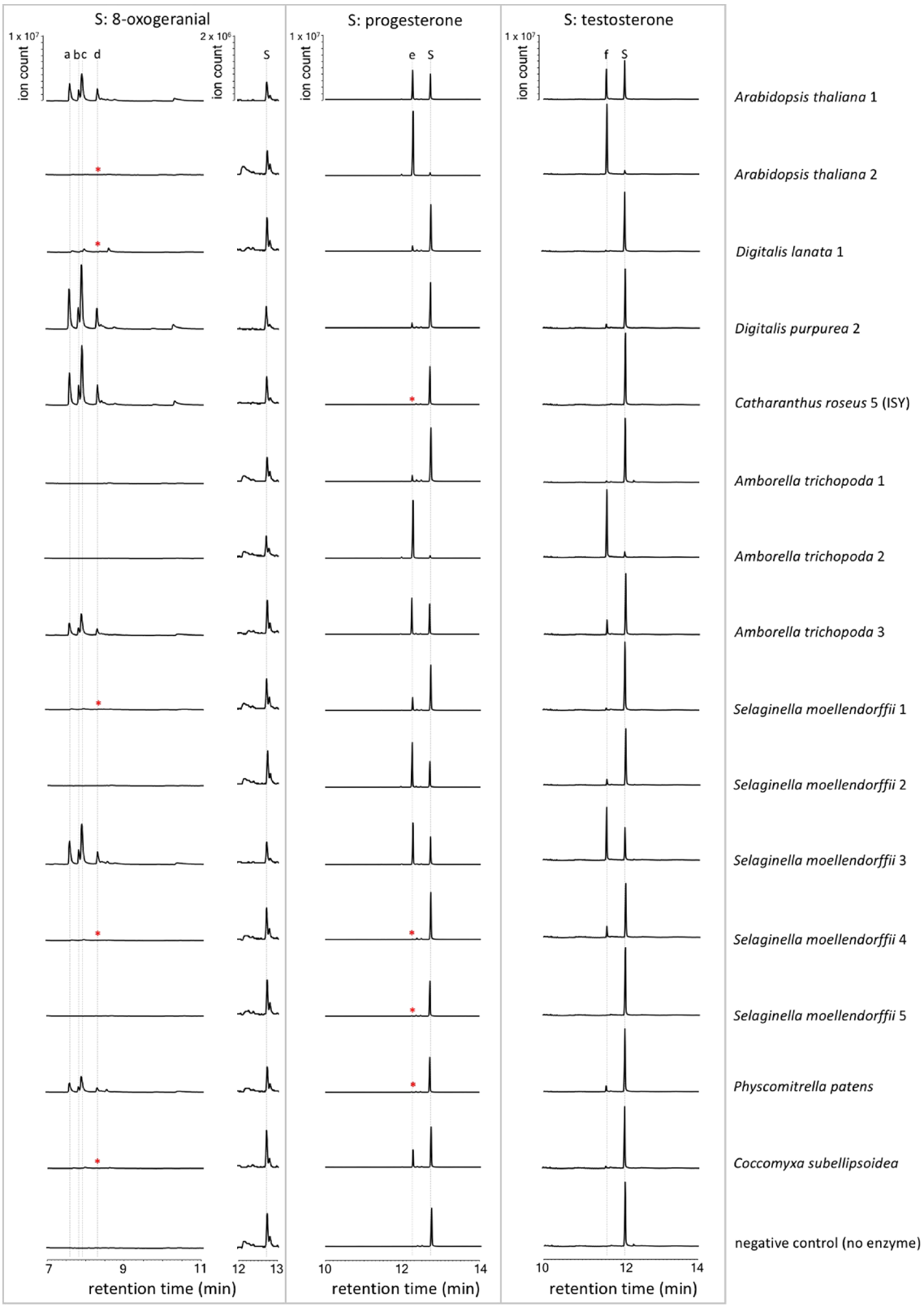


**Figure 3.** GC-MS analysis of activities of selected PRISEs. Total ion scan chromatograms are shown for each enzyme assayed with 8-oxogeranial (left), progesterone (middle), and testosterone (right). No activity on cholest-4-en-3-one was detected. [Legend: S, substrate; a−c, iridodials; d, nepetalactol; e, 5$\beta$-androstan-17$\beta$-ol-3-one (5$\beta$-dihydroprogesterone); and f, putative 5$\beta$-androstan-17$\beta$-ol-3-one (5$\beta$-dihydrotestosterone). Asterisk (*) denotes a detectable signal (see Figures S2 and S3).]

precursor 24-methylcholest-4-en-3-one in brassinosteroid biosynthesis (see Figure S1 in the Supporting Information).[29,30] Results showed that PRISEs displayed various activities against 8-oxogeranial, progesterone, and testosterone with nepetalactol/iridodial, 5$\beta$-pregnane-3,20-dione (or 5$\beta$-dihydroprogesterone), and possibly 5$\beta$-androstan-17$\beta$-ol-3-one


https://dx.doi.org/10.1021/acschembio.0c00220
ACS Chem. Biol. XXXX, XXX, XXX−XXX

Table 1. Comparison of Activities on 8-Oxogeranial and Progesterone in Selected PRISE Homologues (Mean ± SD, $n$ = 4)

| | 8-Oxogeranial | | | Progesterone | | |
|---|---|---|---|---|---|---|
| | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) | $K_M$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) |
| *Coccomyxa subellipsoidea* | 7.0 ± 1.6 | 0.025 ± 0.001 | 3578.5 | 23.6 ± 5.8 | 1.611 ± 0.148 | 68 360.9 |
| *Physcomitrella patens* | 31.3 ± 8.6 | 0.066 ± 0.005 | 2103.2 | 9.5 ± 3.1 | 2.101 ± 0.204 | 220 392.3 |
| *Selaginella moellendorffii* 1 | 4.6 ± 1.7 | 0.006 ± < 0.001 | 1273.0 | 11.7 ± 4.6 | 1.148 ± 0.143 | 98 119.7 |
| *Selaginella moellendorffii* 2 | n.d. | | | 11.5 ± 3.0 | 1.832 ± 0.157 | 159 443.0 |
| *Selaginella moellendorffii* 3 | 409.9 ± 74.2 | 2.029 ± 0.096 | 4950.0 | 8.5 ± 3.5 | 2.124 ± 0.254 | 249 032.7 |
| *Amborella trichopoda* 2 | n.d. | | | 3.1 ± 0.8 | 1.412 ± 0.089 | 458 441.6 |
| *Amborella trichopoda* 3 | 227.8 ± 29.7 | 0.321 ± 0.018 | 1410.0 | 4.5 ± 1.7 | 0.678 ± 0.067 | 149 958.8 |
| *Arabidopsis thaliana* 1 | 41.8 ± 2.75 | 0.155 ± 0.004 | 3706.6 | 6.0 ± 2.5 | 1.865 ± 0.216 | 312 081.7 |
| *Arabidopsis thaliana* 2 | 7.4 ± 3.0 | 0.002 ± < 0.001 | 308.1 | 5.7 ± 2.3 | 1.566 ± 0.148 | 275 026.3 |
| *Digitalis lanata* 1 | 3421.0 ± 1102.0 | 0.064 ± 0.015 | 18.6 | 6.5 ± 2.0 | 1.230 ± 0.111 | 189 376.4 |
| *Digitalis purpurea* 2 | 17.7 ± 5.9 | 6.317 ± 0.615 | 356 489.8 | 6.3 ± 1.9 | 1.881 ± 0.170 | 298 571.4 |
| *Catharanthus roseus* 5 | 8.5 ± 1.5 | 5.835 ± 0.302 | 688 739.4 | 28.8 ± 11.5 | 2.094 ± 0.332 | 72 784.2 |

(or 5$\beta$-dihydrotestosterone) as products, respectively (Figure 3 and Figures S2−S4 in the Supporting Information). No turnover of cholest-4-en-3-one could be observed in any assay, suggesting that an oxygenated group on the opposite end of the substrate (on the D-ring for steroid-like structures; see Figure S1) plays an essential role in substrate recognition. Steady-state kinetic parameters were characterized for 8-oxogeranial (the acyclic monoterpenoid iridoid precursor), and progesterone (a steroid substrate) in 12 homologues. These represent PRISEs from angiosperms, lycophytes, bryophytes, and green algae, and they include representatives of the same species in more than one clade where applicable.

PRISEs in all clades displayed significant activities toward progesterone (see Figure 3, as well as Figure S3 in the Supporting Information). Interestingly, although optimized reduction/cyclization activities toward 8-oxogeranial are only found among clade V members as shown here and elsewhere,[6,7] trace levels of 8-oxogeranial reductase activity were observed in PRISEs of the basal clade (moss, $k_{cat}$ = 0.066 $s^{-1}$), and the vascular plants in clades II and III (*Selaginella*, $k_{cat}$ as high as 2 $s^{-1}$) and of the basal angiosperms in clade V (*Amborella*, $k_{cat}$ = 0.321 $s^{-1}$) (see Figure 3, as well as Figure S2). These activities seem to be maintained and further optimized in clade V ($k_{cat}$ > 5 $s^{-1}$) while significantly reduced or completely lost ($k_{cat}/K_M$ < 1000 $s^{-1}$ $M^{-1}$) in members of clade IV. This significant reduction and loss of ancestral 8-oxogeranial reduction activity is accompanied by the specialized activity on progesterone as seen in *A. thaliana* PRISE2 and *A. trichopoda* PRISE2 in clade V (see Table 1 and Figure 2).

From the universal occurrence of PRISE members in all plants, it is reasonable to speculate that PRISE members are involved in a reaction in primary metabolism that is central to plants, and this might be why they appeared early in plant evolution. Substrate tolerance for both progesterone and 8-oxogeranial has been reported in an PRISE homologue from various plant species, including *A. thaliana* and a gymnosperm,[17,19] but our studies for the first time demonstrate that this ancestral promiscuity was retained throughout land plants from moss to angiosperms. We also cloned, for the first time, a PRISE homologue from green alga *C. subellipsoidea*, which showed broad substrate specificity toward steroidlike 4-en-3-ones (e.g., progesterone, testosterone) and acyclic allylic aldehydes (8-oxogeranial), although these activities were minimal.

To the best of our knowledge, iridoids and/or cardenolides are not reported in nonvascular plants and *Selaginella*. BLAST searches using enzymes in the iridoid pathway from *C. roseus*, as queries only produced significant hits for PRISE (~45% amino acid identity) and no significant hits for geraniol synthase and geraniol-8-hydroxylase (<40% amino acid identity). This analysis suggests that, although low levels of iridoid synthase activity can be found in these plants, the precursor enzymes of the iridoid pathways do not occur in the basal plant lineages. Therefore, the ISY activity may not be the determining factor in the emergence of iridoids. Instead, other factors such as availability of 8-oxogeranial substrate may have played a greater role. In this regard, the emergence of ISYs/iridoids could be considered a type of "exaptation", where the ancestral enzymes are able to catalyze 8-oxogeranial reduction but have no substrate. Once the substrate is available, these enzymes are primed for co-option into a new pathway.[22]

The capacity to reduce a variety of substrates could have been a detoxification mechanism for highly reactive $\alpha$−$\beta$-unsaturated carbonyl compounds, as previously proposed by Kreis and coworkers.[19] As land plants evolved, PRISE members appear to have been recruited for different pathways, and we see that certain groups seem to be optimized for steroidlike 4-en-3-ones while others accept acyclic allylic aldehydes almost exclusively. Examples for the latter group include geranial in the biosynthesis of the semiochemical (*S*)-$\beta$-citronellol in orchids, in addition to 8-oxogeranial.[31]

With its promiscuity feature, the PRISE family appears to be another example that supports the general hypothesis of evolution of specific enzyme activity from ancestral promiscuity.[20,21] Our phylogenetic and biochemical analyses indicate that the promiscuity of the PRISE family is widespread and ancestral, and that the ability to reduce acyclic unsaturated carbonyl substrates such as *C. roseus* ISY's reduction of 8-oxogeranial to nepetalactol and iridodials has not been recently "invented" but, instead, is very ancient (see Figure S2). Notably, this activity has been lost or reduced to negligible levels multiple times over the course of evolution of plants, as shown in in clade IV and certain subgroups of clade V in this study. This finding is also supported by a study on *Antirrhinum majus* PRISEs, in which *A. majus* PRISE1 (clade V) displayed comparable 8-oxogeranial reduction activity to that of *C. roseus* PRISE5 (ISY), while other *A. majus* PRISEs in clades IV and V only showed trace activities.[8] Perhaps when 8-oxogeranial-like substrates were lost and/or new metabolic pathways that involved steroid-like enones emerged, certain ancient PRISEs


https://dx.doi.org/10.1021/acschembio.0c00220
ACS Chem. Biol. XXXX, XXX, XXX−XXX

were selected for alternative functions. From our data, it is also important to note that there exist PRISE homologues with nonexistent activity toward 8-oxogeranial and high activity toward steroidlike structures including progesterone in the iridoids- and cardenolides-free species *A. thaliana*, *A. trichopoda*, and *S. moellendorffii*. In contrast, progesterone 5$\beta$-reductase activity was observed at various degrees in all PRISE homologues, including *C. roseus* ISY. In PRISEs that accept such linear enones, the molecular flexibility of the substrate, as shown in at least one study on *Plantago major* PRISE,[32] might render some promiscuity toward other structures. In PRISEs that only accept steroidlike enones, the rigidity of these four-ring structures could affort higher specificity at the expense of activities toward linear enones. This specialization, as evident in PRISEs in clade V here, suggests a committed role in yet-to-be-identified pathways, as opposed to general detoxification activities. Furthermore, PRISEs could also serve as another example of a plant's "silent metabolism", in which enzymes with broad-substrate specificity is retained and readily allow plants to chemically adapt to new conditions.[33,34] To the best of our knowledge, except nonvascular plants (such as *Physcomitrella* and *Marchantia*), all plant species have more than one PRISE, allowing the optimization of specific activities in at least one homologue while retaining some promiscuity in others. Finally, the promiscuity of PRISE members is remarkable as progesterone and 8-oxogeranial reductions are catalyzed by two different types of enzymes in animals' cardenolide and iridoid biosynthesis, respectively.[2,28] This evolvable promiscuity provides not only an advantageous starting point in the establishment of novel metabolic pathways in plants, but also materials for many potential biochemical applications.

## METHODS

**Phylogenetic Analysis.** PRISE homologues across land plant lineages and green algae were identified by BLAST search using *C. roseus* ISY/PRISE5 as query. From available sequences on NCBI, 1KP Project, the *Marchantia* genome database, we selected 113 sequences, representing major orders covering angiosperms, gymnosperms, lycophytes, and bryophytes. These sequences were aligned with the multiple sequence alignment tool PRANK, and their maximum-likelihood phylogeny were reconstructed using the W-IQ-TREE server.[35,36]

**Cloning.** PRISE homologues from *Physcomitrella patens*, *Selaginella moellendorffii*, *Amborella trichopoda*, *Digitalis purpurea*, and the green alga *Coccomyxa subellipsoidea* were synthesized by ThermoFisher, in accordance with their published sequences without the start and stop codons, and with the additional sequences of AAGTTCTGTTTCAGGGCCCG and TAAAGCTTTCTAGACCAT at the 5′- and 3′-end, respectively (see Table S1 in the Supporting Information). PRISE1 (At4g24220) and PRISE2 (At5g58750) from *Arabidopsis thaliana* were cloned from cDNA using Phusion High-Fidelity DNA Polymerase (NEB) and the primer pairs of 5′-AAGTTCTGTTTCAGGGCCCGAGTTGGTGGTGGGCTGG-3′ (forward) and 5′-ATGGTCTAGAAAGCTTTAAGGTACGATCTTGAACGCC-3′ (reverse), and 5′-AAGTTCTGTTTCAGGGCCCGGGGTCTGAAAATGGCAGC-3′ (forward) and 5′-ATGGTCTAGAAAGCTTTACAAAGGAATGAGTTTTTCATCTCTCATC-3′ (reverse), respectively. *C. roseus* PRISE5 (ISY) and *D. lanata* PRISE1 (progesterone 5$\beta$-reductase) sequences codon-optimized for expression in *Escherichia coli* were obtained from a previous study.[18] All PRISEs were cloned using In-Fusion HD Cloning Kit (Clontech Laboratories) into pOPINF expression vector as described in earlier reports, allowing expression of proteins with N-terminal fusion of a hexa-histidine tag.[18,37] The constructs were confirmed by sequencing.

**Expression and Protein Isolation.** The pOPINF vectors harboring PRISE homologues were transformed to *E. coli* soluBL21 (DE3) cells (Genlantis). Transformed cells were inoculated overnight in LB medium supplemented with 100 $\mu$g/mL carbenicillin at 37 °C. The inoculates were then transferred to 1−2 L culture in 2xYT medium supplemented with 100 $\mu$g/mL carbenicillin in Erlenmeyer flasks with an inoculate:culture ratio of 1:100 and a culture:flask volume ratio of 1:2. When $OD_{600}$ of the cultures reached ∼0.6 after 4−6 h at 37 °C, they were continued at 18 °C for 1 h, followed by addition of IPTG to a final concentration of 100 $\mu$M for induction. The induced cultures were continued for ∼16 h. Cells were collected by centrifugation and resuspended in buffer A (50 mM Tris-HCl buffer (pH 7.0) containing 50 mM glycine, 5% (v/v) glycerol, 500 mM NaCl, and one EDTA-free protease inhibitor tablet (Roche) per 50 mM buffer) containing 20 mM imidazole. Cells were lysed using a cell disruptor (Constant Systems, Ltd.) at 25 000 psi, followed by centrifugation at 35 000*g* for 20 min at 4 °C. All subsequent steps were performed at 4 °C. The supernatant was collected and mixed with Ni-NTA slurry (Qiagen) and incubated gently on rocking platform for 1 h. The slurry was subsequently collected by centrifugation at 2000*g* and washed three times with excessive amount of buffer A (15 mL of buffer for 1 mL of slurry). Target proteins were eluted by washing Ni-NTA slurry with buffer A containing 500 mM imidazole. Buffer was exchanged using PD 10 desalting columns (GE Healthcare) to 50 mM HEPES/NaOH (pH 7.0) buffer containing 100 mM NaCl.

**Enzyme Assays and GC-MS Analysis.** To test PRISE activities, each assay of 100 $\mu$L was set up with 2 $\mu$M enzyme, 500 $\mu$M NADPH, and 200 $\mu$M substrate in 50 mM HEPES/NaOH pH 7.0) buffer containing 100 mM NaCl. The steroid substrates (progesterone, testosterone, and cholest-4-en-3-one) were purchased from Sigma−Aldrich, while 8-oxogeranial was synthesized as previously described (4). The same reaction was set up without adding enzyme as a negative control. The reaction was allowed at room temperature (RT) with gentle agitation (60 rpm). After 3 h, 200 $\mu$L of ethyl acetate was added to the reaction and mixed vigorously for 30 s. The mixture was then centrifuged at 20 000*g* for 2 min using a benchtop centrifuge, and the ethyl acetate fraction was used for gas chromatography−mass spectrometry (GC-MS) analysis. The sample was injected in splitless mode using a Gerstel MPS autosampler on an Agilent 7890 GC system coupled with a Model 5973 mass-selective detector. The inlet temperatures were 100 and 250 °C for assays with 8-oxogeranial and with other substrates, respectively. GC separation was performed on an Agilent HP-5MS column (30 m × 320 $\mu$m) with helium at 1 mL/min as the mobile phase. The GC oven program was set to 80 °C for 1 min, followed by a linear gradient of 20 °C/min to 310 °C and held for 3 min.

**Kinetic Analysis.** Kinetics of PRISEs' activities on 8-oxogeranial and progesterone were measured based on NADPH consumption. Reactions were set on a 96-well plate with each well containing 5−1000 nM enzyme, 250 $\mu$M NADPH, 1−100 $\mu$M substrate in 50 mM HEPES/NaOH (pH 7.0) buffer containing 100 mM NaCl to a total volume of 200 $\mu$L. Tetrahydrofuran at 0.1% and ethanol at 1.5% were used as cosolvent to ensure solubility of 8-oxogeranial and progesterone, respectively. Reactions were allowed at 25 °C, and NADPH consumption was monitored in a 96-well plate reader at 340 nm. Initial velocity was calculated based on NADPH standard and nonlinearly fit to the Michaelis−Menten curve using GraphPad Prism (GraphPad Software, Inc.).

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.0c00220.

Table S1 and Figures S1−S4, with additional mass spectrometry chromatograms (PDF)



https://dx.doi.org/10.1021/acschembio.0c00220
ACS Chem. Biol. XXXX, XXX, XXX−XXX

## AUTHOR INFORMATION


### Corresponding Author

**Sarah E. O'Connor** − *Department of Natural Product Biosynthesis, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany;* orcid.org/0000-0003-0356-6213; Email: oconnor@ice.mpg.de

### Author

**Trinh-Don Nguyen** − *Department of Chemistry, Irving K. Barber Faculty of Science, University of British Columbia, Kelowna, BC V1V 1V7, Canada;* orcid.org/0000-0002-2759-5791

Complete contact information is available at: https://pubs.acs.org/10.1021/acschembio.0c00220



### Funding

We gratefully acknowledge NSF Genome (Grant No.1444499).


### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We would like to thank B. Lichman, P. Soltis, and D. Soltis for helpful discussions. We also thank M. Kamileen for assistance with GC-MS analysis. The plant drawing in the Table of Contents graphic is a gift from K. Davis.

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https://dx.doi.org/10.1021/acschembio.0c00220
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https://dx.doi.org/10.1021/acschembio.0c00220
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