Managing enzyme promiscuity in plant specialized metabolism: A lesson from flavonoid biosynthesis
Mission of a “body double” protein clarified

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Abstract
Specificities of enzymes involved in plant specialized metabolism, including flavonoid biosynthesis, are generally promiscuous. This enzyme promiscuity has served as an evolutionary basis for new enzyme functions and metabolic pathways in land plants adapting to environmental challenges. This phenomenon may lead, however, to inefficiency in specialized metabolism and adversely affect metabolite-mediated plant survival. How plants manage enzyme promiscuity for efficient specialized metabolism is, thus, an open question. Recent studies of flavonoid biosynthesis addressing this issue have revealed a conserved strategy, namely, a homolog of chalcone isomerase with no catalytic activity binds to chalcone synthase, a key flavonoid pathway enzyme, to narrow (or rectify) the enzyme’s highly promiscuous product specificity. Reducing promiscuity via specific protein–protein interactions among metabolic enzymes and proteins may be a solution adopted by land plants to achieve efficient operation of specialized metabolism, while the intrinsic promiscuity of enzymes has likely been retained incidentally.

KEYWORDS chalcone isomerase, chalcone isomerase-like protein, chalcone synthase, enhancer of flavonoid production, flavonoids, plant specialized metabolism, promiscuity

INTRODUCTION
“Specificity” is a fundamental property of enzymes. Viewed from different perspectives, various enzyme specificities exist, including substrate, product, reaction, and stereo specificities. Many introductory biochemistry textbooks only describe enzymes that show strict specificities. Examples include Jack bean urease (EC 3.5.1.5)[1] and Streptomyces L-glutamate oxidase (EC 1.4.3.11),[2] which basically only act on urea and L-glutamate, respectively. Many other enzymes are not so specific; however, being able to more or less act on other substrates besides primary substrates, produce other products, or catalyze other types of reactions. This ability of an enzyme to catalyze reaction(s) other than those related to its original function is referred to as “promiscuity” or “ambiguity” (see Information Box 1).[3,4]

Plants produce a diverse array of metabolites that are apparently non-essential to growth, such as flavonoids (see below), isoprenoids, and alkaloids. The structures of these metabolites, which may vary according to plant lineage, may total more than 1,000,000.[11] Previously referred to as “secondary metabolites,” these compounds have been distinguished from “primary metabolites” that are unambiguously

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Information Box 1—what is “promiscuity” of enzymes and why is it important?

The term “promiscuity” has been used in various contexts by different scientists. Some scientists (e.g., those involved in enzymatic synthesis of organic compounds or metabolic engineering to produce structurally different metabolites) simply use this term to mean the ability of enzyme active sites to catalyze different chemical transformations involving different functional groups and/or catalytic mechanisms, irrespective of their biological relevance. Such enzyme promiscuity is important to the research community because the repertoire of compounds to be synthesized may be modified by engineering the specificity of promiscuous enzymes via a protein engineering approach. Other scientists, such as evolutionary biochemists, restrict the use of this term to reactions whose biological or physiological relevance is absent or unknown. If an enzyme can catalyze reactions of no known biological or physiological relevance, it is regarded as a promiscuous enzyme; in contrast, an enzyme displaying multiple biologically relevant activities is considered to be multi-functional, multi-specific, or of broad specificity, but not promiscuous. Enzyme promiscuity has been shown to serve as a basis of enzyme functional evolution (see text for details) and has become a focus of attention in an evolutionary context. In this article, we use the term “promiscuous” in this latter context.

Just as with enzyme specificities, various promiscuities exist—for instance, substrate promiscuity, product promiscuity, and catalytic promiscuity. Substrate promiscuity refers to the ability of an enzyme to use multiple substrates in the same type of reaction. Among enzymes involved in plant specialized metabolism, one such example is flavonoid 3′-hydroxylase (CYP75B [EC 1.14.14.82]; see Supplementary Figure S1). Product promiscuity, which is less studied, refers to the ability of an enzyme to produce different products from a single substrate. Enzymes showing product promiscuity include CHS (this article), aureusidin synthase of snapdragon (EC1.21.3.6) and sesaminol synthase (CYP91C) of sesame seeds. Finally, catalytic promiscuity is the ability of an enzyme to carry out different reactions using different substrates, as illustrated by 2-hydroxyisoflavanone dehydratase of soybean (EC 4.2.1.105), which catalyzes the dehydration of 2-hydroxyisoflavanone to produce isoflavone and shows esterase activity when carboxyl esters (such as p-nitrophenylobutyrate) are used as substrates.

essential to plant growth (i.e., carbohydrates, proteins, lipids, and nucleic acids). A more recent perspective, however, is that the roles of these metabolites in plants should not be considered “secondary”; instead, they have a wide variety of lineage-specific physiological roles, such as reproduction and responses to biotic and abiotic stresses. Plant secondary metabolites are now called “plant specialized metabolites”, with an emphasis on the fact that both their roles and distributions are specialized for specific plant lineages. This huge diversity of plant specialized metabolites is produced via a variety of reactions catalyzed by enzymes, which, at least in vitro, generally show lower efficiency and greater promiscuity of catalysis than primary metabolic enzymes. (For example, $k_{cat}$ values of enzymes involved in specialized metabolism are estimated to be approximately 30-fold lower on average than those involved in primary metabolism.) Moreover, growing evidence suggests that these enzymes commonly interact with each other to form a metabolon, which is defined as “a transient and dynamic supramolecular complex of cooperating, often consecutive enzymes of a metabolic pathway, which is often associated with cellular structural elements and non-enzymatic proteins.” In addition, structural and phylogenetic analyses of specialized metabolic enzymes suggest that such promiscuity serves as an important basis for the evolution of new enzyme functions and new metabolic pathways. These observations are well exemplified by enzymes and proteins involved in the biosynthetic pathway of flavonoids. In this article, we discuss the evolution of the flavonoid pathway in conjunction with the evolutionary origin and promiscuity of enzymes. A recent finding concerning a strategy used by plants to manage highly promiscuous specificity of a flavonoid enzyme is also discussed in an evolutionary context and in terms of metabolon formation.

FLAVONOIDS ARE PRODUCED BY LAND PLANTS

Flavonoids are a class of plant specialized metabolites with a basic $C_6-C_3-C_6$ carbon framework that can be categorized into 10 major subclasses mainly based on structural differences in the $C_3$ portion of their structures (Figure 1). With a few exceptions (detailed below), flavonoids are produced by all land plants (embryophytes; i.e., bryophytes [liverworts and mosses, but possibly not hornworts], pteridophytes, gymnosperms, and angiosperms), and distributions of flavonoid subclasses vary according to the land-plant subgroups. In nature, these flavonoid aglycons further undergo hydroxylation, glycosylation, acylglycosylation, methylation, and/or prenylation. These processes enhance the structural diversity of flavonoids, for which at least 7000 structures have been described to date.

The first committed step of flavonoid biosynthesis is catalyzed by chalcone synthase (CHS; EC 2.3.1.74), a member of the type-III polyketide synthase family of enzymes. CHS catalyzes three consecutive condensations of $C_3$ units of malonyl-CoA to p-coumaroyl-CoA to produce 2,4,4′,6-tetrahydroxychalcone (THC). p-Coumaroyl-CoA, the starter substrate of the CHS-catalyzed reaction, is derived from the amino acid L-phenylalanine via the general phenylpropanoid pathway and also serves as a precursor for other important plant
specialized metabolites, such as lignin, lignans, and coumarins.[26] CHS is, therefore, considered to be a key enzyme located at the entry of the flavonoid pathway that directs the general phenylpropanoid pathway to flavonoid synthesis (Figure 1). In vitro, the product THC is spontaneously isomerized to produce a racemic mixture of naringenin, a flavanone.[27] In planta, however, THC undergoes stereo-specific isomerization catalyzed by chalcone isomerase (CHI; EC 5.5.1.6) to produce 2S-naringenin,[28] a compound further metabolized to produce various flavonoid aglycons via branched pathways (Figure 1) that depend on the plant species.[19] Some enzymatic oxidations in these pathways are catalyzed by cytochrome P450 proteins, which are endoplasmic reticulum-localized heme-dependent monoxygenases.[17] In several plant species, CHS, CHI, and other flavonoid enzymes located further down the pathway associate with each other using cytochrome P450 proteins as a nucleus to form multi-enzyme complexes called flavonoid metabolons.[29]

**HOW HAVE FLAVONOID FUNCTIONS EVOLVED IN LAND PLANTS?**

Plants are believed to have originally produced flavonoids for adaption to harsh terrestrial conditions when they extended their habitats from aquatic to terrestrial environments approximately 450 million years ago. Phylogenetic analysis has suggested that all land plants—from bryophytes to angiosperms—share a common aquatic plant ancestor, most likely an alga closely related to charophytes.[30] Throughout their evolution, plants have been involved in close interactions with microbes, and land plants are believed to have arisen as a result of an ancient symbiosis between an alga and an aquatic fungus.[31] The first land plant to emerge is considered to have been a liverwort-like species.[32] By 420–400 million years ago, the level of atmospheric oxygen was presumably similar to present levels.[33,34] Unlike algae, the survival of very early land plants was threatened by various
Information Box 2—Physiological roles of flavonoids in extant land plants and the effects of these compounds on various organisms

**Stress responses**\(^{39}\): Flavonoids accumulate in plants in response to abiotic stresses, such as strong UV light, low temperatures, and high osmotic pressures. Flavonoids serve as sunscreen agents and are excellent reactive-oxygen-species scavengers in plants.

**Plant reproduction**\(^{39}\): In some plant species, flavonoids are essential for male fertility. For example, a CHS mutant of rice produces flavonoid-depleted pollen and is male sterile.\(^{40}\) In addition, flavonoids occur in flower petals and, through their involvement in flower coloration, play an important role in attracting pollinators and seed dispersers. The orange, pink, red, purple, and blue colors of flowers are in most cases due to anthocyanins.\(^{41}\) The bright yellow color of some flowers arises from chalcones and aurones.\(^{41}\) Other flavonoids are almost colorless to human eyes but serve as co-pigments in flower coloration.

**Plant–insect interactions**: Oviposition\(^{42}\)—Oviposition of swallowtail butterflies is stimulated by a mixture of at least 10 different substances present in leaf exudates, including flavonoids. More specifically, swallowtail larvae only eat leaves of specific types of rutaceous plants. To identify such plants prior to oviposition, female butterflies touch and scrape leaf surfaces with their forefeet, which contain chemical sensors for specific substances in leaf exudates.

**Feed stimulants**\(^{43}\)—The mulberry silkworm only eats mulberry leaves. A range of substances in mulberry leaves is closely related to this very specific feeding behavior. These substances have precise roles in insect-feeding responses—as olfactory attractants, swallowing factors, and biting factors—the latter of which includes flavonoids.

**Plant–microbe interactions**\(^{39}\): Roots of leguminous plants, such as soybean, exude flavonoids into the soil environment that serve as chemo-attractants for nitrogen-fixing symbiotic bacteria inhabiting soil. The symbiosis with these bacteria allows leguminous plants to grow even on nitrogen-poor soil where other plants cannot. In addition, flavonoids, such as soybean isoflavones, play an important role in defense mechanisms against pathogenic microorganisms.

**Health benefits to humans**\(^{44}\): Some dietary flavonoids have been shown to have various health benefits to humans, including lowering risks of hormone-dependent cancers, cardiovascular diseases, and osteoporosis; reducing body fat; and preventing dental caries.

terrestrial environmental stresses, such as a high concentration of gaseous oxygen, harmful UV-B light, temperature fluctuations, and drought.\(^{35}\) Plants may also have had to interact with microbes, including symbiotic as well as pathogenic ones (plant–pathogen co-evolution).\(^{31,36}\) The intracellular concentration of flavonoids in early land plants is presumed to have been very low—in the nanomolar concentration range.\(^{35,37}\) In this context, microalgae from divergent evolutionary lineages (such as Cyanobacteria, Rhodophyta, Chlorophyta, Haptophyta, and Ochrophyta) are known to accumulate trace amounts of flavonoids;\(^{38}\) this may be an example ofpreadaptation, in which some algae may already have had the potential to synthesize flavonoids prior to colonization of land.

The evolution and function of flavonoid biosynthesis still needs to be fully elucidated. The signaling and regulatory functions of flavonoids in response to environmental stress are proposed to represent their primary roles in early land plants, as these functions can likely be accomplished at low cellular concentrations.\(^{35,37}\) Consistent with this idea, some flavonoids in extant land plants are found in cell nuclei and act as signaling molecules involved in genetic regulation.\(^{35}\) Moreover, recent studies have also suggested an early role for flavonoids in defense against oomycete invasion.\(^{136}\) More specifically, characterization of the genetic response of an early divergent liverwort (Marchantia polymorpha) to infection with an oomycete pathogen (Phytophthora palmivora) revealed the robust transcription activation of conserved gene families, including those related to general phenylpropanoid and flavonoid pathways. This pattern paralleled the response observed in extant angiosperm plants (e.g., Nicotiana benthamiana). The role of R2R3-Myb transcription factors in this activation was also found to be conserved among these plants. This phenylpropanoid- and flavonoid-mediated defense mechanism against oomycete infection is likely conserved throughout land plants, and oomycete invasion may have been one of the early selective pressures shaping the evolution of general phenylpropanoid and flavonoid pathways.\(^{36}\) Subsequently, land plants have evolved to exhibit enormous species diversity in which flavonoid structures and physiological functions are also diversified in a lineage-specific manner. Known physiological functions of flavonoids in extant plants are summarized in Information Box 2. These flavonoid functions may have gradually evolved at later stages after cellular concentrations and the spectrum of flavonoids increased and reached levels observed in modern land plants.

**FLAVONOID ENZYME SPECIFICITIES ARE PROMISCUOUS**

Similar to other enzymes involved in plant specialized metabolism, flavonoid enzymes generally show promiscuity.\(^{14,16–18}\) Promiscuous enzymatic “substrate” specificity (substrate promiscuity; see Information Box 1) is physiologically irrelevant if the substrate for promiscuous activity is absent or not encountered by the enzyme in a cell. The availability of two or more different substrates for an enzyme in a cell, however, may allow the formation of a “metabolic grid,” which can be illustrated by flavonoid biosynthesis in some plants\(^{17}\) (see Supplementary Figure S1 for an example). In the metabolic grid, one enzyme catalyzes...
WAKI ET AL.

FIGURE 2 Reaction pathway of chalcone synthase catalysis. R, CoA– or Enz-Cys–

parallel reactions on structurally related substrates, some of which may be accessed by other enzymes, thereby contributing to the diversification of flavonoid chemical structures.

Unlike substrate promiscuity, promiscuity of “product” specificity (product promiscuity) of an enzyme may negatively impact the efficiency of overall metabolism if the product(s) arising from such promiscuity are inert as metabolic intermediates. For example, CHS is a highly promiscuous enzyme in terms of product specificity, at least in vitro, despite having a vital role as a key enzyme at the entry of the flavonoid pathway.[25,45] In the CHS-catalyzed reaction, more specifically, the production of linear diketide, triketide (1), and tetraketide (2) intermediates is followed by intramolecular Claisen cyclization of 2, which gives rise to THC (Figure 2). Intermediates 1 and 2 may diffuse away from the active site; however, followed by intramolecular lactonization to produce bis-noryangonin (BNY) and p-coumaroyltriacetic acid lactone (CTAL). Moreover, intramolecular aldol-type cyclization of 2 may take place, which results in the production of resveratrol (i.e., a stilbene with health-conferring functions identified in red wine)[46] (Figure 2). Using p-coumaroyl- and malonyl-CoA molecules as starter and extender substrates, respectively, CHS, thus, potentially produces BNY, CTAL, and resveratrol as by-products in addition to THC, and, at least in vitro, relative percentages of these by-products in total CHS products may be 50%–90% (mol/mol), depending on plant enzyme sources[45] and reaction conditions. BNY, CTAL, and resveratrol are
all inert as flavonoid precursors. The idea that these by-products could exactly operate as functional surrogates for flavonoids is highly unlikely, although resveratrol is known to serve in grapevines as a phytoalexin produced in response to fungal infections.\[47\] This promiscuous product specificity of CHS, if no means exists to overcome it, should reduce the efficiency of flavonoid biosynthesis and adversely affect the flavonoid-mediated responses of land plants to detrimental environmental conditions.

**ENZYME PROMISCUITY SERVES AS A BASIS OF THE EVOLUTION OF SPECIALIZED METABOLISM**

Despite its potential negative impacts on the survival of growing plants, enzyme promiscuity has been regarded as a key to the evolvability of new enzyme functions and the establishment of novel specialized metabolic pathways.\[4,16–18\] In most cases, plant specialized metabolites are ultimately derived from the intermediates of primary metabolism, and phylogenetic analyses of enzymes suggest that specialized metabolic enzymes are also evolutionarily distantly related to enzymes and proteins involved in primary metabolism.\[4,16–18\] These observations imply that plants do not build up new protein scaffolds de novo to produce new enzyme functions; instead, they take full advantage of scaffolds of pre-existing primary metabolic enzymes. This idea is well exemplified by CHS and CHI in flavonoid biosynthesis. Both of these enzymes are phylogenetically related to enzymes and proteins involved in fatty acid biosynthesis: CHS is structurally and mechanistically related to \(\beta\)-ketoacyl-acyl carrier protein synthase (EC 2.3.1.41),\[25\] while CHI is proposed to have evolved from the fatty acid binding protein (FAP) of prokaryotes and plants, which has no known catalytic activity (see below for details).\[28,48\]

On the basis of general observations of the evolutionary origin of enzymes involved in specialized metabolism, the following scenario can be proposed as an example of the emergence of a new pathway of specialized metabolism in plants.\[4,16–18,49\] Primary metabolic enzymes generally show an intrinsic promiscuity. One such enzyme, termed E1, exhibits a major catalytic activity, \(\epsilon_1\), along with a relatively weaker ancillary activity, \(\epsilon_4\) (see Figure 3). The activity ratio \(\epsilon_4/\epsilon_1\) may be changed via amino acid substitutions or by varying reaction conditions. The activity \(\epsilon_1\) might be potentially involved in the synthesis of a metabolite (Z) from a primary metabolite W (see below and Figure 3). When \(\epsilon_1\) is stochastically combined with the weak ancillary activities of other enzymes (i.e., \(\epsilon_{11}\) and \(\epsilon_{18}\) of E11 and E8, respectively) from other pathways, it potentially gives rise to a sequence of latent underground reactions to produce Z from W. The metabolites X, Y, and Z may initially be sporadic, selectively neutral, non-physiological by-products, and their cellular concentrations may be very low. During successive generations, a host plant species may experience environmental challenges (e.g., global warming or cooling, drought, spread of pathogens, and herbivore attacks). If Z has a selective advantage to the host plant under such circumstances, this Z-producing pathway will be fixed in subsequent generations and further improved through repeated duplications of genes coding for enzymes E1, E11, and E8 and their positive selections; in this way, an efficient specialized metabolism will be established in which enzyme variants E1', E11', and E8' respectively show major activities \(\epsilon_{11}\), \(\epsilon_{111}\), and \(\epsilon_{18}\). The metabolites X, Y, and Z may initially be sporadic, selectively neutral, non-physiological by-products, and their cellular concentrations may be very low. During successive generations, a host plant species may experience environmental challenges (e.g., global warming or cooling, drought, spread of pathogens, and herbivore attacks). If Z has a selective advantage to the host plant under such circumstances, this Z-producing pathway will be fixed in subsequent generations and further improved through repeated duplications of genes coding for enzymes E1, E11, and E8 and their positive selections; in this way, an efficient specialized metabolism will be established in which enzyme variants E1', E11', and E8' respectively show major activities \(\epsilon_{11}\), \(\epsilon_{111}\), and \(\epsilon_{18}\). The metabolites X, Y, and Z, thus, provide an incentive for recruitment of enzymes promoting their synthesis, and the “survival of the fittest” principle is applicable to both enzymes (E1', E11', and E8') and metabolites (X, Y, and Z) (metabolite-enzyme co-evolution\[49\]).

Plant specialized metabolism is still in the process of evolution. Metabolic enzymes constituting extant pathways exhibit promiscuity, which potentially serves as a basis for the generation of an enzyme with a new catalytic function via birth-and-death processes of paralogous genes following repeated gene duplications. This concept is well illustrated by the relationship among CHS, stilbene synthase (STS; EC 2.3.1.95) of grapevine (Vitis vinifera L.),\[50\] and \(p\)-coumaroyltriacetic acid synthase (CTAS) of hydrangea (Hydrangea macrophylla L.).\[51\] Using
FIGURE 4  Phyllogenetic trees of CHS, CHIL, and related enzymes and proteins. (A) A phylogenetic tree of CHS, STS, CTAS, and related enzymes, in which *Escherichia coli* β-ketoacyl-acyl carrier protein synthase (EcKAS III) has been used as a reference outgroup. Branches to STS and CTAS are shown in blue. Stereo structures of EcKAS III and *Arabidopsis thaliana* CHS (each highlighted in red) are shown to the right of the tree. (B) A phylogenetic tree of FAP, CHIL, and CHI. Stereo structures of FAP, CHIL, and CHI of *A. thaliana* (from top to bottom) are shown to the right of the tree.

MANAGING ENZYME PROMISCUITY—A CLUE FROM GENETIC ANALYSES OF FLOWER COLOR VARIATION IN JAPANESE MORNING GLORY

Despite its significance in the evolution of new enzyme functions, enzyme promiscuity is highly unlikely to favor the survival of growing plants coping with various environmental stresses. For example, in vitro CHS catalysis is very inefficient: 50%–90% (mol/mol) generation of by-products, none of which are useful as precursors for flavonoid biosynthesis. If CHS catalysis is equally inefficient in vivo,
this enzyme would be unable to efficiently exert its physiological role as an entry enzyme in the flavonoid pathway. Land plants must have a mechanism to reduce such enzyme promiscuity. A clue to address this issue has been obtained via genetic studies of flower color mutants of Japanese morning glory (Ipomoea nil).[53]

Japanese morning glory produces blue flowers that accumulate a pelargonidin-based anthocyanin (Heavenly Blue anthocyanin), and its mutants produce reddish flowers that accumulate pelargonidin derivatives (i.e., Wedding Bells anthocyanin).[54] Numerous mutants with pale-colored flowers have also been known for almost 90 years.[55] In 2014, three mutant alleles responsible for pale-colored flowers due to a significant reduction in petal anthocyanin accumulation were identified.[53] Characterization of these mutants revealed that a transposon insertion into promoter or coding regions of a gene encoding a protein with predicted primary and tertiary structures very similar to CHI (CHI-like protein, CHIL; see also Figure 4B) caused a substantial reduction in the contents of anthocyanins and other flavonoids, such as flavonols, in flower petals, producing a paler flower color. On the basis of phenotypic observations associated with its loss-of-function mutations, CHIL was termed an "enhancer of flavonoid production" (EFP).[53] Judging from the results of knock-down mutational analyses of the EFP gene using distant plant species, the EFP function of CHIL was predicted to be conserved among diverse plant species.[53]

Despite the primary and tertiary structural similarities of CHIL and CHI, CHIL displays no CHI activity because amino acid residue(s) essential for CHI catalysis are replaced by other residue(s) in CHIL.[28,48,56] The physiological function of CHIL in plants remained unknown until its EFP role was uncovered. In this context, CHIL was previously misidentified as a CHI enzyme and used in the same role to heterologously engineer a flavonoid pathway in yeast cells for microbial production of flavonoids.[57] As such, CHIL looked like a "body double" of CHI. As expected from the structural similarities of CHIL, CHI, and FAP (see above), these proteins are phylogenetically closely related to each other (Figure 4B).[28,45,48,53] Similar to CHS genes, CHIL genes have been identified in all land-plant genomes, but this is not necessarily the case for CHI genes—for example, the genomes of some mosses and hornworts, which are classes of bryophytes, have not been found to encode CHI gene sequences.[121] The fact that CHIL genes, along with the CHS gene, are ubiquitously encoded in land-plant genomes strongly suggests that the EFP function of CHIL is essential to these organisms. This conclusion has led in turn to investigation of the mechanistic aspects of the EFP role of CHIL.

A MISSION OF THE "BODY DOUBLE" PROTEIN CLARIFIED

To clarify how CHIL fulfills its EFP role, an examination of whether CHIL is a component of flavonoid metabolons was first carried out in plants confirmed to contain those metabolons (i.e., snapdragon[58] and soybean[59,60]). This examination revealed that CHIL is a metabolon component that interacts in these metabolons with CHS (in snapdragon and soybean) and cytochrome P450 proteins (flavone synthase II in snapdragon and isoflavone synthase in soybean).[45] Because CHIL and CHS ubiquitously occur in land plants (see above), the next determination was whether physical interactions between these two proteins are also ubiquitous in land plants. As a result, CHIL was found to interact with CHS in all examined land plants—from bryophytes to angiosperms.[45]

The effects of CHIL binding on CHS activity were subsequently examined. CHIL catalyzes the production of THC with the concomitant production of byproducts such as BNY, CTAL, and resveratrol (see above). Under the assay conditions used, CTAL was the major by-product, and typical molar ratios of the CHS products [THC:CTAL (mol/mol)] in the absence of CHIL were 4:6 (for snapdragon CHS) and 1:9 (for CHS of Physcomitrella patens, a bryophyte). In the presence of a 10-molar excess of CHIL; however, the ratios were 9:1 for CHS enzymes from both plants.[45] The same was also the case for CHS-CHIL systems of all other examined land plants,[45] including ferns,[61] CHIL, thus, binds to CHS to enhance the THC-producing activity of CHS, concomitant with a diminution of its CTAL-producing activity. According to the results of kinetic studies, this effect on the product specificity of CHS-catalyzed reactions is at least partly due to the CHIL-mediated enhancement of the kcat of THC synthesis.[45,62] The CHS reaction pathway forks at intermediate 2 into THC production (step d, Figure 2) and CTAL formation (step e), in which the kcat of THC synthesis is possibly related to the rate of step d. CHIL sharpens the product specificity of CHS catalysis via the specific rate enhancement of step d, resulting in the increased channeling of 2 toward THC production at the expense of CTAL formation (step e) (Figure 2). A loss-of-function mutation of CHIL in Arabidopsis thaliana, which gives rise to a phenotype of paler seed coat color with diminished contents of seed flavonoids (e.g., proanthocyanidins), has been found to be fully complemented by overexpression of CHIL in the mutant.[45] CHIL narrows the promiscuous product specificity of CHS by suppressing its CTAL-producing activity and enhancing its THC-producing activity to allow CHS to exclusively produce THC, the key precursor of flavonoids. This role of CHIL is ubiquitously conserved among land plants.

Throughout the 450-million-year history of land plant evolution, the ability of CHS to produce THC has likely been maintained by its continued ability to interact with CHIL. This continued interaction has allowed CHS to precisely fulfill its physiological function—the synthesis of THC, the key precursor of a diverse array of flavonoids indispensable for land-plant adaptation to the terrestrial environment (Supplementary Figure S2). In this context, we note that grapevine STS and hydrangea CTAS do not interact with CHIL,[45] some CHS homologs have likely lost their ability to interact with CHIL and have differentiated into enzymes that exclusively produce other polyketides rather than THC. Because this role of CHIL has been maintained during the evolution of land plants, it must have been of vital importance to their survival in the terrestrial environment.

Finally, on the basis of the above-mentioned observations, we offer the following caveat. In many cases, in vitro enzymatic assays
are carried out using a dilute concentration of a single enzyme in an aqueous buffer system. Although such conventional enzyme assays are easy to perform and provide useful information, the results may differ depending on whether or not cellular conditions have been accurately simulated; hence, comparisons of in vitro versus in vivo properties of enzymes must be made with caution when pursuing how enzymes and metabolisms operate in vivo.

**CONCLUSIONS AND OUTLOOK**

The conserved strategy used by CHIL to reduce the intrinsic promiscuity of CHS points to the importance of specific protein–protein interactions of metabolic enzymes in the management of enzyme promiscuity in vivo. Metabolic enzymes involved in plant specialized metabolism, including flavonoid biosynthesis, have been proposed to co-localize via specific protein–protein interactions into metabolons, allowing channeling of metabolites between two contiguous enzymes in a pathway. The present findings appear to add an additional layer of control for metabolon formation in plant specialized metabolism. The promiscuous specificity of metabolic enzymes may be controlled via interaction of enzymes with other proteins, which may not only be non-catalytic ones such as CHIL but may also include related enzymes in the pathway. Thus far, the evolution of metabolic pathways has been mainly discussed in terms of the evolution of an enzyme’s specificity. The acquisition of the ability to specifically interact with metabolic enzymes and related proteins in the pathway, however, is likely another important factor allowing the pathway to evolve into a more efficient one. In other words, the evolution of metabolic pathways may have been accomplished not only via the alteration of enzymatic active-site structures governing catalytic specificities but also by changes in the surface structures of enzyme molecules allowing specific protein–protein interactions.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available in reference [45].

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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