

Discovery and Diversity of Terpenoids in Maize (*Zea mays*)

By

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

In

Plant Biology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2026

I'd like to dedicate this thesis to those who have supported my journey. To my family, whose love has given me the ability to pursue my passions and chase my dreams: without you, none of this would have been possible. To my partner Brian, who has pushed me to be my best from the very beginning and has remained my sunshine throughout everything: thank you for your encouragement and love. Finally, I would like to acknowledge my advisors, whose confidence and guidance shaped me into the scientist I am today, as well as my labmates, who reminded me that science is fun.

Abstract

Terpenoids are a diverse class of specialized metabolites that contribute to plant growth, defense, and environmental adaptation. **Chapter 1** of this thesis provides a broad overview of the different types of terpenoid compounds and the roles they play in response to different biotic stimuli, ranging from beneficial interactions to defense. This chapter highlights the multifunctionality of these metabolites, many of which serve multiple roles within the plant depending on tissue type and environmental context. Diterpenoids, a class of terpenoids, have well-established roles in pest and pathogen defense across diverse plant species, including both gymnosperms and angiosperms. Specifically in maize, several pathogen-induced diterpenoid pathways, including the dolabralexin and kauralexin pathways, have been characterized. **Chapter 2** expands upon this body of work and describes a novel diterpenoid pathway, termed the rosalexin pathway, which is mediated by the class I diterpene synthase (diTPS) ZmTPS42/KSL1 and is inducible alongside other defense pathways in maize. **Chapter 3** further extends this research by using an *Agrobacterium*-mediated *Nicotiana benthamiana* co-expression platform for the discovery of novel metabolites from the combination of maize terpene synthases and cytochrome P450 monooxygenase enzymes (P450). Together, these findings expand the known maize terpenoid metabolic network and identify candidate enzymes and pathways that may contribute to specialized metabolism.

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***Chapter 1: A Literature Review of the Role of Terpenes in Mediating Biotic
Plant-Environment Interactions***

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Author statement: This work is part of a larger review article to be published in Natural Product Reports (NPR). All the authors contributed to the main body of work, but the sections included in this thesis were written by SS with help from PZ.

Introduction

Terpenes compose one of the oldest and largest classes of natural organic compounds and comprise tens of thousands of metabolites distributed across all kingdoms of life.^{1,2} While these compounds first emerged around 2 billion years ago in bacteria and archaea, the vast expansion of terpene metabolism began when plants colonized land around 500 million years ago.³ The evolutionary divergence of terpenoid metabolism has resulted in a staggering array of structurally distinct natural products that are often species specific and rely on stimulus-dependent regulation. Terpenes are made up of five carbon isoprenyl building blocks that form hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), and tri-terpenoids (C30) to larger carotenoids (C40) and derived apocarotenoids, natural rubber poly-isoprenes, and a range of non-canonical terpenoid derivatives such as homoterpenoids or iridoid monoterpenoids.⁴⁻⁷ This chemical repertoire has enabled plants to deploy terpenoids as versatile mediators of dynamic ecological interactions.

Terpenoid-mediated pathogen defenses

Terpenoid-mediated pathogen defenses rank among the most widely studied and include a diverse range of complex terpenoid mixtures that serve as effective chemical defense layers against both generalist and specialist fungal and bacterial pathogens as well as viral diseases.^{8,9} A prominent example is conifer oleoresin defenses, comprised of diterpene resin acids (DRAs), mono- and few sesqui-terpenoids, that accumulate in specialized resin ducts upon tissue damage by stem-boring insects.^{10,11} In this ecological context, DRAs serve as an effective chemical and physical defensive barrier against insect pests and associated fungal pathogens such as the Blue Stained Fungus (*Grosmannia clavigera*).¹⁰ However, considerable co-evolution has equipped *G. clavigera* with several detoxification capabilities, which include enzymatic terpenoid degradation and specialized ABC transporters enabling the export of toxic terpenoids.^{12,13}

While DRAs are a largely conserved defense system in many gymnosperms, angiosperms have evolved a much larger diversity of antimicrobial and antiviral terpenoids. Particularly, members of the Poaceae such as rice (*Oryza sativa*), maize (*Zea mays*), and barley (*Hordeum vulgare*) produce species-specific terpenoid phytoalexin blends with demonstrated or predicted functions in pathogen defense.^{9,14} For example, in maize, *Fusarium* species trigger the production of several different terpenoids including sesquiterpenoids zealexins, α/β -costic acids, α -santalenoic acid, and cubebol as well as diterpenoids kauralexins and root-specific dolabralexins.^{15–20} Notably, metabolite-specific differences have been identified in the effectiveness against individual pathogens. Additionally, many of these compounds have other roles in plant-environment interactions. Zealexins and kauralexins have important roles in pest defense, and dolabralexins have a hypothesized role in oxidative and drought stress highlighting the multifunctional roles of these compounds.^{21–23}

More recently, the complexity of terpenoid-mediated plant-pathogen interactions has been highlighted by the discovery of a new class of labdane-derived diterpenoids in barley termed hordedanes which deter *Fusarium graminearum* growth.¹⁴ Interestingly, the pathogen *Bipolaris sorokiniana* can oxidize and conjugate this compound to sesquiterpenoids, changing the antimicrobial activity to instead facilitate root colonization.¹⁴ The above examples only offer a glimpse into the diversity of terpenoids that plants deploy to ward off microbial pathogens. They do, however, exemplify the long-standing evolutionary arms race in which plants continually expand and elaborate their terpenoid arsenal to deter pathogens, while pathogens, in turn, co-evolve increasingly sophisticated detoxification and metabolic countermeasures to evade, inactivate, or even exploit these bioactive compounds. Currently, proposed mechanisms of action for terpenoids inhibiting pathogen growth include increased lipid peroxidation and interfering with ergosterol biosynthesis or binding in pathogens, thereby altering cell membrane fluidity and preventing growth^{24–28}, but the actual mechanisms remain unresolved.

Terpenoid-mediated herbivore defenses

Similar to their antimicrobial bioactivities, many terpenoids serve as chemical protection against herbivory in both above- and below-ground tissues. For example, the aforementioned DRAs alongside α -pinene, limonene, 3-carene, and other terpenoids, do not only exhibit pathogen-defensive functions, but also serve as a structural and chemical defense against insect pests, notably including mountain pine beetles (MPB; *Dendroctonus ponderosae*) and spruce weevils (*Pissodes strobi*) that cause severe damage to natural and commercial conifer stands.^{10,11} Upon invasion of the tree by the insect, terpenoid-containing oleoresin stored in resin ducts is released from the wound site, resulting in the expulsion of the insect. Further polymerization of DRAs ultimately traps the insect. Parallel to this physical barrier, oleoresin terpenoids are toxic to the insect invaders and its larvae, for example, disrupting conserved insect juvenile hormone signaling and thereby impeding insect reproduction.²⁹ Like the associated pathogenic fungi, MBPs evolved detoxification mechanisms such as the CYP-catalyzed conversion of α -pinene into aggregation pheromones such as trans-verbenol that is released to attract other beetles to already infested trees.^{11,30} Moreover, fungal pathogens can aid in host colonization. In pine (*Pinus spp.*), *G. clavigera* contributes to monoterpene detoxification, enhancing MPB colonization, while in spruce (*Picea spp.*), these compounds can inhibit fungal growth and stimulate spore production, promoting dispersal by spruce beetles (*Dendroctonus rufipennis*).^{12,31} While conifers largely rely on oleoresin defenses, a vast array of volatile and higher molecular weight mono-, sesqui-, di- and tri-terpenoids with herbivore-deterrent activities evolved in angiosperms.^{8,9} For example, inducible non-volatile kauralexins and zealexins in maize display antifeedant activity and accumulate in response to the European corn borer (*Ostrinia nubilalis*).^{21,22} Likewise, in members of the Cucurbitaceae, cucurbitacin triterpenoids have demonstrated deterrent effects on insect feeding and oviposition as well as toxic effects on herbivore growth.³²

To deal with autotoxicity from bioactive terpenoids, plants can utilize modifications such as glycosylation and cellular compartmentalization to sequester these compounds.³³ Interestingly, in *Nicotiana attenuata*, 17-hydroxygeranyllinalool diterpene glycosides are modified by two CYP736As to prevent autotoxicity, but are hydroxylated when ingested by pests and inhibit sphingolipid synthesis highlighting a clever mechanism of using metabolic modifications to avoid autotoxicity while gaining herbivory defense.^{33,34}

Beyond such local defenses at the site of attack, plants emit complex VOC blends that mediate indirect defenses^{35,36}, including monoterpenoids such as β -ocimene and linalool, sesquiterpenoids such as α -farnesene and β -caryophyllene, and the homoterpenes 4,8-dimethyl-1,3,7-nonatriene (DMNT) and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) to name a few.^{37–39} Many of these terpenoids have multiple functions in pathogen defense, herbivore defense, as well as pollinator attraction, therefore requiring strict regulation of their composition and dosage. For example, at lower concentrations, linalool and (*E*)- β -caryophyllene in maize attract adult fall armyworm (FAW), but upon infection and increased volatile emission, deter oviposition of female FAW.⁴⁰ Next to direct herbivore deterrence, plants deploy terpenoids in complex tritrophic interactions as indirect defenses, whereby terpenoids are emitted upon herbivore attack to recruit predators or parasitoids.⁴¹ Notably, in maize, (*E*)- β -caryophyllene attracts predators of the stem borer *Chilo partellus* as well as entomopathogenic nematodes that infect attacking larvae below ground.⁴² Plants also utilize volatile terpenes to prime defense pathways within the plant and in neighboring plants. Commonly, defense priming happens through jasmonic acid signaling such as in Norway spruce where priming triggers wound-induced oleoresin response, resulting in faster terpene production following damage.⁴³ Interestingly, in sweet potato (*Ipomoea batatas*), herbivory induced volatile homoterpene DMNT in sweet potato sufficiently primes neighboring plants independent of jasmonic acid signaling offering an example of highly specific, single volatile mediated plant-plant communication.⁴⁴

The role of terpenoids in pollinator attraction

Beyond defensive roles, terpenoids play important roles in cooperative interorganismal interactions. In addition to carotenoid pigments, terpenoid volatile organic compound (VOC) blends serve as major components of pollinator attraction.⁴⁵ A survey of 305 plant species across 66 families detected terpenoids in 88.2% of species, with monoterpenoids such as limonene, trans- β -ocimene, and linalool among the most common compounds, with the latter two being the most frequently predominant.⁴⁶ Unsurprisingly, terpenoid VOC blends are species-specific in both abundance and composition although they do share frequently occurring compounds. For example, while linalool is considered a generalist attractant, plants with higher linalool levels have been shown to be more attractive to Lepidoptera.⁴⁶ This effect has been exemplified by common fig (*Ficus carica*) studies, demonstrating that fig wasp (*Blastophaga psenes*) attraction decreases with altered concentrations of linalool and derivatives thereof.⁴⁷ Extensive co-evolution has occurred in shaping dynamic plant-pollinator interactions. For example, distinct terpenoid floral scent chemotypes have been identified in orchid sub-species of the genus *Gongora*, which impact specific interactions with VOC-collecting bee pollinators and, in turn, have driven VOC divergence in *Gongora* orchids.⁴⁸ Recent work by Darragh et al. further supports that pollinators cause selection pressure influencing floral volatiles as exemplified by a decrease in floral scent during a transition from bee to hummingbird pollination.⁴⁹ A complex interplay exists between the use of terpenoid VOCs in pollinator associations and chemical defense. For instance, D-limonene and S-(+)-linalool function as pollinator attractants, whereas R-limonene deters aphids and R-(-)-linalool acts as a herbivore deterrent, highlighting the impact of stereochemical variations in plant-insect communication.^{50,51} Similarly, nectar also contains species-specific terpenoids among other specialized metabolites with predicted roles in deterring undesired floral visitors.⁵² Recent studies have found that some nectar terpenoids, such as thymol and carvacrol, can reduce the

growth of parasites in bees, suggesting that self-medication by pollinators may be a new form of pollinator attraction.^{53,54} These examples highlight the evolutionary and ecological balance between pollinator attraction and chemical defense that have influenced the chemical diversification of terpenoids, especially in flowering plants.⁵⁵

The role of terpenoids in root microbiome interactions

In addition to the well-established functions of terpenoids in above-ground plant-environment interactions, the past decade of research has underscored the role of terpenoids also in root-environment interactions. Since vascular plants and microbes began coexisting millions of years ago, plants have been selectively enriching or excluding certain microbiota to shape their rhizosphere communities.^{56,57} Increasing evidence has begun to emerge to suggest that terpenoid compounds are thought to play specialized roles in mediating plant-microbe interactions beyond influencing carbon and nutrient cycles of microbes.⁵⁸ A well-known example is the role of strigolactones (SLs) in promoting symbiotic interactions with arbuscular mycorrhizal fungi (AMF) and other beneficial soil microbes to enhance nutrient uptake.^{59,60} SLs further play important roles in plant-plant communication, particularly in interactions with the parasitic plant *Striga*.⁶¹ While microbiome-mediated changes in the root metabolome - including SLs - have been shown to suppress *Striga* parasitism in sorghum, numerous studies have illustrated the ability of *Striga* to repurpose SLs for host recognition and stimulating seed germination.^{59,62,63} For example, Li et al. demonstrated that maize mutants with altered SL composition exhibit altered *Striga* germination and enhanced resistance.⁶⁴

More recently, Huang and colleagues showed that root-exuded triterpenoids, including thalianin, arabinin, and related triterpene fatty acid esters, impact the microbiome assembly in *Arabidopsis*.⁶⁵ Likewise, triterpenoid saponins and other triterpenoids such as cucurbitacins in members of the *Cucurbitaceae* have been discovered to enrich bacterial taxa such as

Enterobacter and *Bacillus* with possible roles in increasing pathogen resistance.⁶⁶ Outside of the triterpenoid class, *Arabidopsis* mutants deficient in selected sesterterpenoids⁶⁷ as well as maize mutants lacking species-specific diterpenoids feature altered microbiome composition.⁶⁸ Additionally, terpenoids can modify the root microbiome by restricting diversity and selecting for certain organisms, thus creating a tailored microbial community. For example, non-volatile sesqui and di-terpenoids in maize, switchgrass, barley, and rice restrict growth of certain soil-dwelling bacterial and fungal species.^{14,68–70} Considering the increasing number of species-specific root terpenoids that have been identified especially in monocot plants, including maize dolabralexins,¹⁸ barley hordedanes,¹⁴ and panicoloids in switchgrass,⁷¹ it appears plausible that at least some of these compounds may contribute to root-microbiome interactions. Notably, Kudjardjie et al. recently demonstrated that rice diterpenoids impact the root microbiome assembly by recruiting nematode-suppressive microbes, thus, providing indirect resistance to the root nematode (*Meloidogyne graminicola*).⁶⁹

Strikingly, terpenoid-mediated plant-microbe interactions are not limited to the rhizosphere. Above-ground, volatile terpenoids involved in long-range plant-plant communication and pollinator attraction are hypothesized to influence the root microbiome through priming. For example, in tomato (*Solanum lycopersicum*), receiver plants can perceive β -caryophyllene, triggering changes in root exudates which, in turn, alter the root microbiome.⁷² Similarly, in maize, exposure to linalool induces neighboring plants to exude root benzoxazinoids, causing an altered root microbiome and increased salicylic acid levels to restrict plant growth while potentially priming plants for pre-emptive defense.⁷³ These collective findings speak to wider-ranging terpenoid roles in root-environment interactions that are yet to be unearthed.

Chapter 2: Discovery of the rosalexin pathway expands the modular network of maize diterpenoid chemical defenses

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Author statement: This work is part of a larger paper. For the work presented in this thesis, PZ and EAS conceived the original research and oversaw the study. AEC, GW, YD, EP, AH, EAS, and PZ conducted gene discovery, genomics, phylogenetic, and enzyme biochemical analyses. SS performed the enzyme biochemical studies shown in this thesis. SS performed the metabolite and transcript profiling studies with help from GW. DH, SB, GW, and AEC conducted in vitro antifungal assays. GW conducted NMR experiments and analyses.

Introduction

Plants employ complex blends of specialized metabolites to mediate interactions with their environment and thrive in various ecological niches.^{1,74} Among these metabolites, the large group of 20-carbon diterpenoids serves multifaceted functions with direct impact on plant vigor and fitness.⁷⁵ Widely conserved diterpenoids such as gibberellin (GA) phytohormones have critical functions in plant development. By contrast, specialized diterpenoids are narrowly distributed across plant lineages, often display stimulus- and tissue-specific accumulation, and function in mediating the outcome of defensive and cooperative plant-environment interactions.^{4,75} Driven by environmental selection pressures, this expansion of diterpenoid metabolism has been enabled by the repeated duplication and functional divergence of ancestral pathway genes of general metabolism.^{74,76,77} In particular, GA-biosynthetic terpene synthases and cytochrome P450 monooxygenases have provided a genetic reservoir for the duplication and functional evolution of specialized enzyme functions.^{6,76} The resulting diTPS and P450 gene families comprised of functionally distinct members form modular pathway networks, where different combinations of class II and class I diTPSs and P450s generate various structural scaffolds and functional decorations that yield diterpenoids with specific physiological functions.^{6,75,78} In maize, this modular metabolic network generates unique groups of protective diterpenoids, including kauralexins and dolabrallexins.^{18,19,79,80} Due to their evolutionary relationship, GAs, kauralexins and dolabrallexins share a common ent-copalyl diphosphate (*ent*-CPP) precursor (**Figure 1**).^{19,76}

Dysregulation of GA and defensive diterpenoid pathways in maize is mitigated by the presence of two duplicated class II diTPSs, ZmTPS37/CPS1/ZmAN1 and ZmTPS38/CPS2/ZmAN2, that convert the common geranylgeranyl diphosphate (GGPP) substrate into *ent*-CPP, yet display distinct spatiotemporal regulation and stress-inducibility. While ZmTPS37/CPS1/ZmAN1 functions in GA metabolism⁸⁰, ZmTPS38/CPS2/ZmAN2 is stress-inducible and involved in

specialized diterpenoid metabolism.^{81,82} Downstream, two class I diTPSs, namely ZmTPS43/KSL2 and ZmTPS45/KSL4, control key pathway nodes by converting *ent*-CPP into the kauralexin precursor *ent*-isokaurene or the dolabralalexin precursor dolabradiene, respectively.^{18,19,23} Catalytically promiscuous P450s of the CYP701A and CYP71Z subfamilies that are capable of functionally modifying both precursors then bring about the biosynthesis of different, bioactive kauralexins and dolabralalexins.^{18,19} Presence of two additional class II diTPSs, ZmTPS39/CPS3 and ZmTPS40/CPS4 that form (+)-CPP and 8,13-CPP, respectively, further shows that the diversity of maize specialized diterpenoids extends beyond kauralexins and dolabralalexins.⁸⁰

Here, we report that the maize class I diTPS, ZmTPS42/KSL1, catalyzes a previously unrecognized reaction that transforms *ent*-CPP into a 5-rosanol scaffold. Modular activity of ZmTPS38/CPS2/ZmAN2, ZmTPS42/KSL1, and the promiscuous P450 enzyme, ZmCYP71Z18, form a third *ent*-CPP-derived pathway branch in maize en route to specialized diterpenoids, termed rosalexins (**Figure 1**). In vitro antimicrobial activity of epoxyrosanol along with pathogen-induced ZmTPS42/KSL1 transcript and rosalexin accumulation in aerial tissues indicated a defense-related function. However, no clear pathogen-susceptible phenotype was observed in ZmTPS42/KSL1-deficient maize plants, suggesting a more specialized stress-responsive function within the network of bioactive kauralexin, dolabralalexin, and rosalexin diterpenoids in maize.

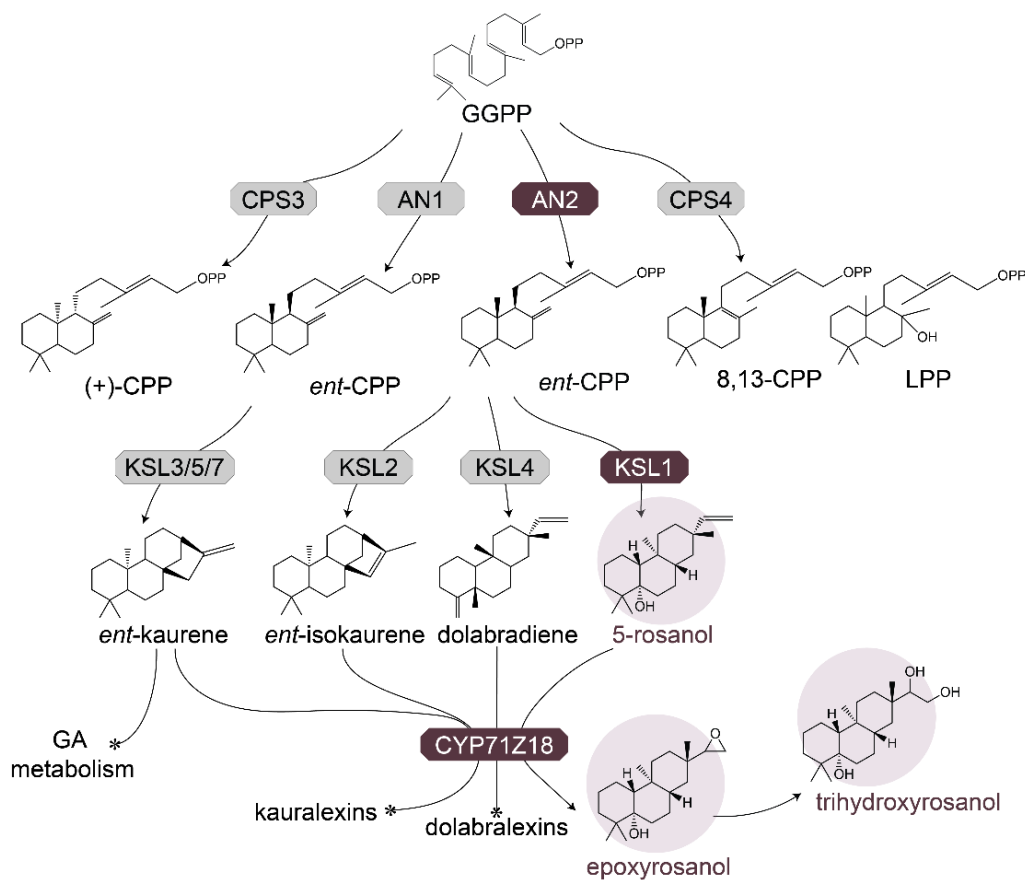


Figure 1. | Simplified overview of diterpenoid pathways in maize. Maize contains a modular diterpenoid-metabolic network to form specialized compound groups with distinct bioactivities, including kauralexins, dolabrallexins, and rosalexins (this study). Abbreviations: GGPP, geranylgeranyl diphosphate; CPP, copalyl diphosphate; KSL, kaurene synthase-like; CYP, cytochrome P450.

Results

ZmTPS42/KSL1 forms a rosane-type diterpene scaffold

One reason ZmTPS42/KSL1 had yet to be characterized until now is that in the B73 line, the most well-studied line of maize, ZmTPS42/KSL1 lacks the γ - and β -domains as well as the DDxxD motif critical for class I diTPS catalytic activity and, thus, likely represents a pseudogene. Similar to B73, several lines including Oh43 and B75 featured major sequence variations and gaps, predictably rendering the encoded proteins non-functional. By contrast, Oh7B, CML103, Ki3, and CML277 among other lines contain full-length ZmTPS42/KSL1 genes with predicted protein sequence identities of 74-100%.

To test the biochemical function of ZmTPS42/KSL1 in the context of the maize diterpenoid network, we selected the full-length ZmTPS42/KSL1 from Oh7B to assay pairwise enzyme activities with known maize class II diTPSs: the *ent*-CPP synthase ZmTPS38/CPS2/AN2 (catalytically identical to ZmTPS37/CPS1/AN1)⁸¹, the (+)-CPP synthase ZmTPS39/CPS3, and the 8,13-CPP synthase ZmTPS40/CPS4⁸⁰. Agrobacterium-mediated transient co-expression assays in *Nicotiana benthamiana* were conducted by pairing ZmTPS42/KSL1 individually with each class II diTPS. Co-expression of ZmTPS42/KSL1 with ZmTPS38/CPS2/AN2 resulted in the formation of a major product (compound **1**) and a minor byproduct (compound **2**) (**Figure 2a**). Compound **1** featured m/z 275 and m/z 290 mass ions indicative of a labdane-type diterpene alcohol and product **2** featured m/z 257 and m/z 272 mass ions characteristic of a labdane-type olefin (**Figure 2b**). In addition, GC-MS analysis of the extracted enzyme products of the pairwise activity of ZmTPS42/KSL1 with the (+)-CPP synthase ZmTPS39/CPS3 showed several labdane diterpenoids (compounds **3-8**), albeit at very low abundance. Among these, **3**, **6**, **7**, and **8** were distinct ZmTPS42/KSL1 products and **3** could be identified as sandaracopimaradiene based on comparison to an authentic standard (**Figure 3**). Combined activity of ZmTPS42/KSL1 with the 8,13-CPP synthase ZmTPS40/CPS4 yielded a range of

unidentified labdane scaffolds (compounds **9-12**) at low abundance (**Figure 3**). However, these products represented byproducts of ZmTPS40/CPS4 rather than unique compounds resulting from ZmTPS42/KSL1 activity. To elucidate the structure of the ZmTPS42/KSL1 product **1**, we enzymatically produced and purified >1 mg of product **1** for NMR analysis and identified the metabolite as 5-hydroxy-rosa-15-ene (hereafter named 5-rosanol) (data not shown). Low product abundance prevented NMR analysis of the ZmTPS42/KSL1 byproduct **2**. However, based on characteristic m/z 257 and m/z 272 mass ions and the biosynthetic mechanism forming 5-rosanol, we hypothesize that **2** represents a rosadiene olefin scaffold.

Given that the biosynthesis of related kauralexin and dolabralalexin diterpenoids requires the functional modification of the respective diTPS-produced scaffolds by specialized P450 enzymes of the CYP701A and CYP71Z subfamilies^{15,18,19}, we next performed co-expression studies combining ZmTPS38/CPS2/AN2 and ZmTPS42/KSL1 (Oh7B) with known diterpenoid-metabolic maize P450s. Co-expression with ZmCYP71Z18 yielded a new major (compound **13**) and minor (compound **14**) product, featuring indicative mass ions of m/z 306 and m/z 288, respectively, that are characteristic of oxygenated labdane diterpenoids (**Figure 4**). Purification and NMR analysis of **13** and **14** verified these structures as 5-hydroxy-15-epoxyrosa-5(6)-ene (hereafter named epoxyrosanol) and epoxy-rosa-5(6)-ene (named epoxyrosaene), respectively. While epoxyrosanol is derived from 5-rosanol, the formation of small quantities of epoxyrosaene supported our hypothesis that the minor ZmTPS42/KSL1 product **2** is rosaene and serves as a precursor for epoxyrosaene. In keeping with the nomenclature of other maize diterpenoids, we collectively designated 5-rosanol (**1**), rosaene (**2**), epoxyrosanol (**13**), and epoxyrosaene (**14**) as rosalexins.

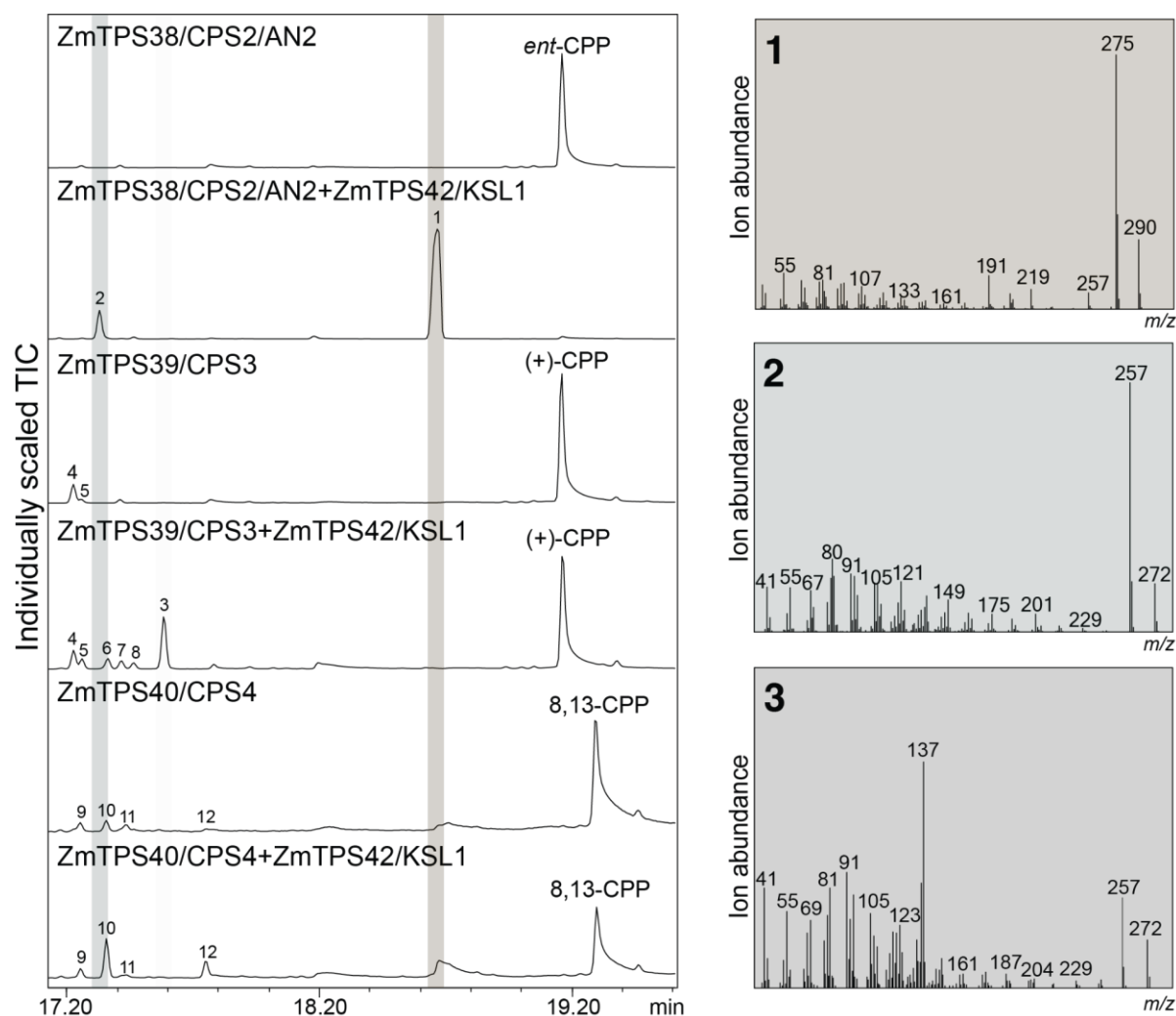


Figure 2. | Functional characterization of *ZmTPS42/KSL1*. **a**, Total ion chromatograms (TIC) of reaction products resulting from *Agrobacterium*-mediated *Nicotiana benthamiana* co-expression assays of *ZmTPS42/KSL1* with *ZmTPS38/CPS2/AN2*, *ZmTPS39/CPS3*, or *ZmTPS40/CPS4*. **b**, Mass spectra of compound 1 and 2 resulting from the pairwise reaction of *ZmTPS38/CPS2/AN2* and *ZmTPS42/KSL1*.

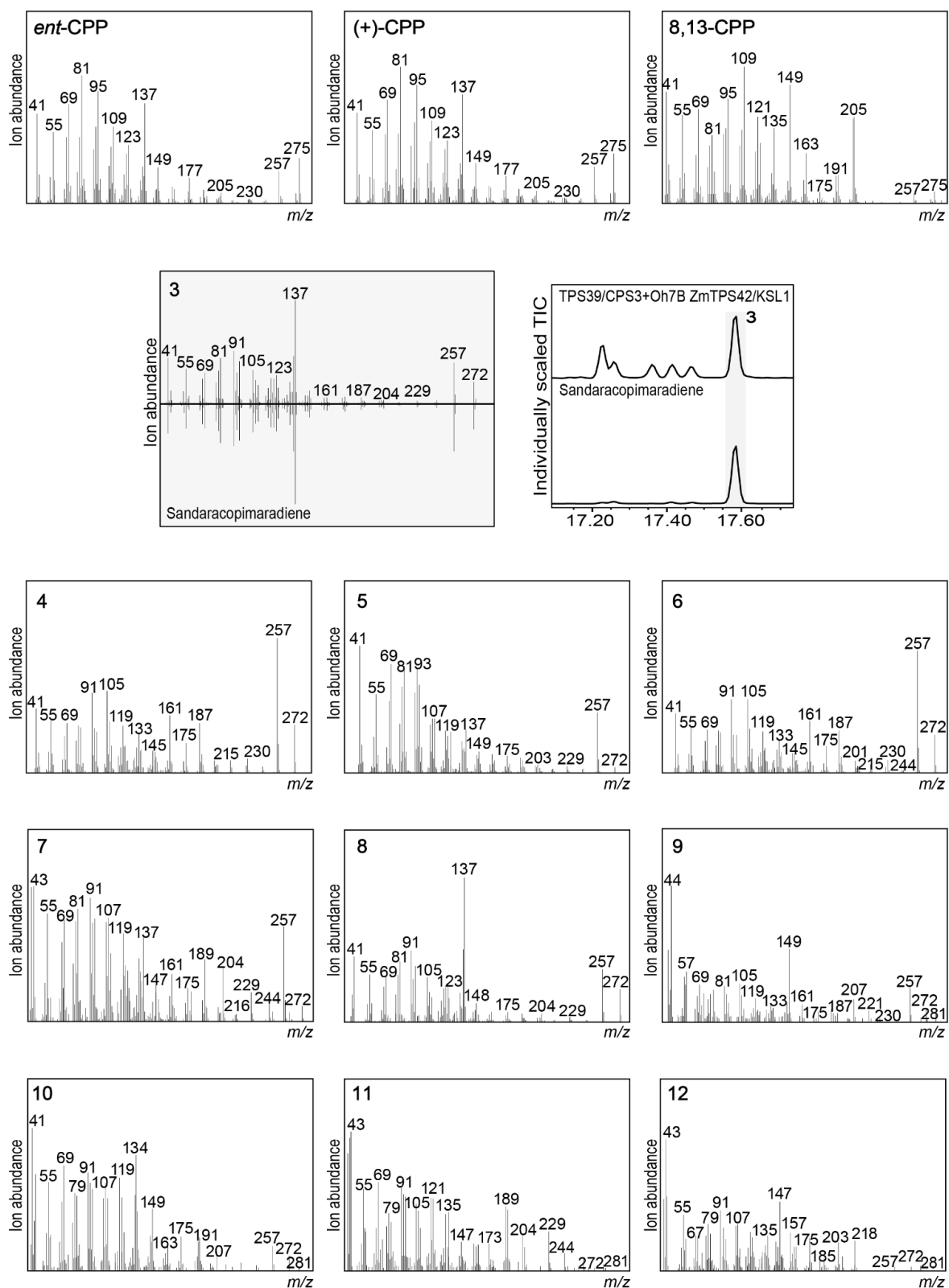


Figure 3. | Mass spectra of products from the pairwise reaction of ZmTPS42/KSL1 with different maize class II diterpene synthases. Mass spectra of *Agrobacterium*-mediated

Nicotiana benthamiana co-expression assays of ZmTPS38/CPS2/AN2, ZmTPS39/CPS3, and ZmTPS40/CPS4 alone as well as in combination with ZmTPS42/KSL1. **Ent-CPP** is the major product of ZmTPS38/CPS2/AN2. **(+)-CPP** is the major product of ZmTPS39/CPS3 and compounds 4 and 5 are minor products. Compounds **3-8** were found in the ZmTPS39/CPS3 + ZmTPS42/KSL1 combination and compounds **3, 6-8** are unique to ZmTPS39/CPS3 + ZmTPS42/KSL1 combination. Compound 3 was identified as sandaracopimaradiene using a standard. **8,13-CPP** is the major product of ZmTPS40/CPS4 and compounds **9-12** are minor products.

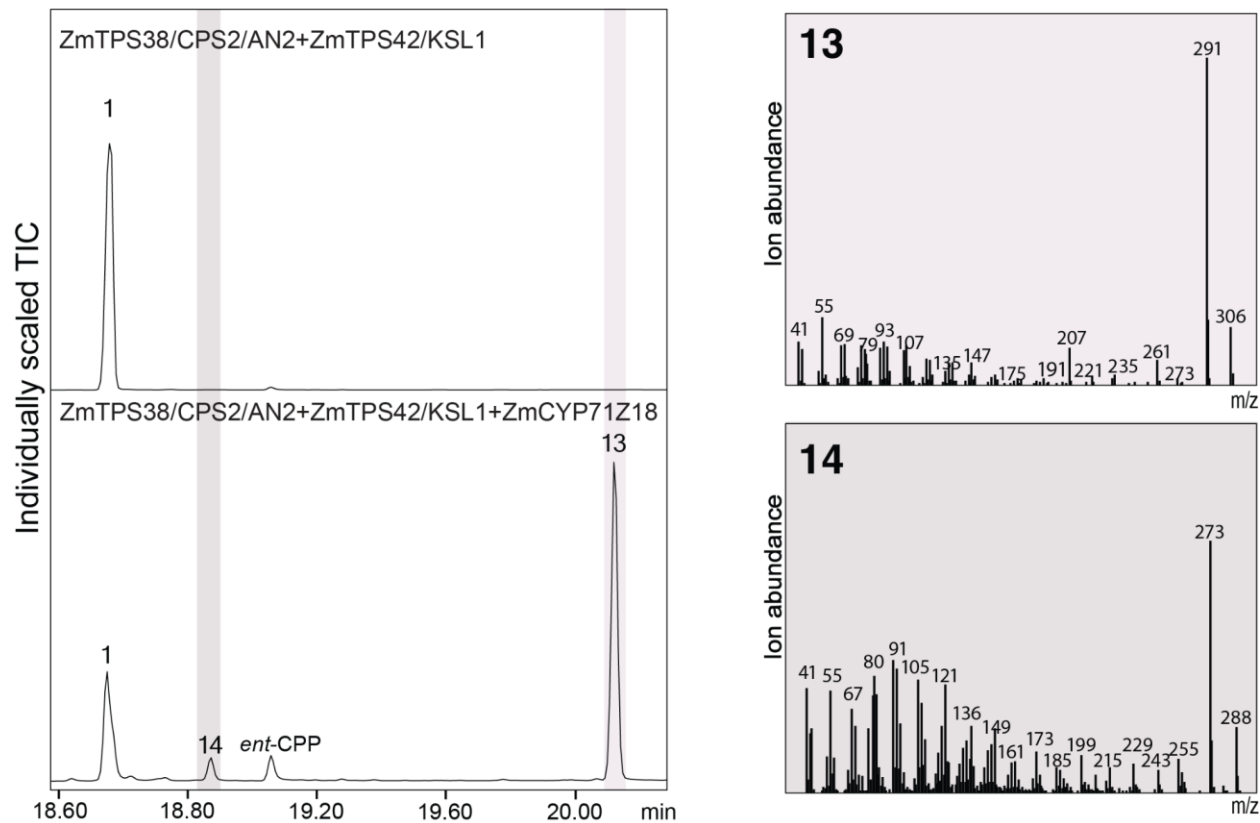


Figure 4. | Identification of epoxyrosanol. Total ion chromatograms (left) and mass spectra (right) of reaction products resulting from *Nicotiana benthamiana* co-expression assays of ZmTPS38/CPS2/AN2 and ZmTPS42/KSL1 only, or with the addition of ZmCYP71Z18.

ZmTPS42/KSL1 co-expresses with other specialized metabolism genes

Gene expression profiles available in public repositories, including the Sekhon gene expression atlas, showed highest expression of ZmTPS42/KSL1 in above-ground tissues including stem internodes, first and post-pollination leaves^{83,84} as well as embryo tissue⁸⁵. In addition, integrating mutual rank (MR) and Pearson's correlation (PCC) analyses of in-house transcriptome data of maize stems infected with the major maize pathogen *Fusarium verticillioides* showed patterns of co-expression of ZmTPS42/KSL1 and known genes of maize specialized diterpenoid metabolism (**Figure 5**). Specifically, ZmTPS42/KSL1 gene expression was strongly correlated with ZmTPS43/KSL2, ZmTPS45/KSL4, ZmCYP71Z18, and CYP701A43/KO2 involved in kauralexin and dolabralexin metabolism. Notably, among the four maize class II diTPSs, ZmTPS42/KSL1 showed the strongest co-regulation with the *ent*-CPP synthase ZmTPS38/CPS2/AN2. By contrast, GA-metabolic genes, including ZmTPS37/CPS1/AN1, ZmTPS44/KS(L3)/D5, and ZmTPS46/KSL5 showed distinct expression patterns from ZmTPS42/KSL1 (**Figure 5**). These findings suggest a role in specialized metabolism in comparison to gibberellin metabolism.

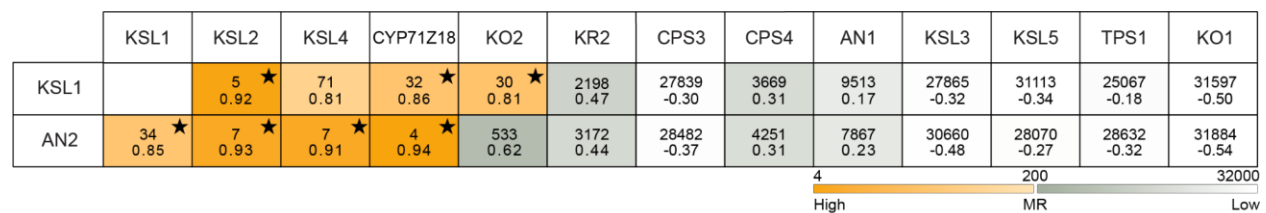


Figure 5. | Co-expression of ZmTPS42/KSL1 with specialized metabolism and GA

metabolism. Heat map illustrating the pairwise gene correlation for ZmTPS38/CPS2/AN2 and ZmTPS42/KSL1 genes with class II diTPS and GA metabolism genes. Strong co-expression was defined by Pearson's correlation coefficient (PCC) >0.7 and mutual rank (MR) of <200 and

are shaded in orange (using $-\log_{10}(\text{MR})$) whereas weak correlations are shaded in grey. PCC and MR values are printed on the bottom and top, respectively. Stars denote gene pairs with $\text{PCC} > 0.8$ and $\text{MR} < 50$. Abbreviations: CPS, copalyl diphosphate synthase; TPS, terpene synthase; KSL, kaurene synthase-like; CYP, cytochrome P450; KO, kaurene oxidase; KR, kauralexin reductase

Rosalexins are stress-inducible alongside other specialized maize diterpenoid pathways

To investigate associations of rosalexins with pathogen responses *in planta*, seven available maize genotypes featuring ZmTPS42/KSL1 genes demonstrated or predicted to be functional (Oh7B, Ki3, CML103, CML277) or containing non-functional ZmTPS42/KSL1 genes (Oh43, B73, and B75) were selected for plant pathogen stress studies. Here, longitudinal stem incisions between nodes 3 and 4 of 62-day old plants as well as the roots of the plants were infected with either *F. verticillioides* or water as a control. However, mapped fungal read counts suggest that while the infection of the stem versus control was successful, control roots have a similar percentage of mapped reads as infected roots suggesting possible cross-contamination (data not shown).

Consistent with the absence of a functional ZmTPS42/KSL1, LC-MS/MS analysis via quantifying relative ion intensity and abundance showed that Oh43, B73, and B75 tissues did not contain rosalexin metabolites at any detectable level irrespective of pathogen or mock treatment (**Figure 6**). Control Oh7B plants also did not contain detectable rosalexin levels. However, accumulation of 5-rosanol and epoxyrosanol was observed in pathogen-infected stems of Oh7B, Ki3, and CML103 plants. Unexpectedly, 5-rosanol and epoxyrosanol were not detected in CML277 despite possessing a functional ZmTPS42/KSL1 sequence. 5-rosanol and epoxyrosanol were not detected in roots consistent with lower ZmTPS42/KSL1 gene expression in roots. Standard-verified 5-rosanol was detected by a predominant m/z 273 mass fragment

representing a neutral loss of water $[M-H_2O+H]^+$. On average, 5-rosanol was detected at $49.57 \pm 16.1 \mu\text{g g}^{-1}$ (FW) for Oh7B, $21.00 \pm 9.91 \mu\text{g g}^{-1}$ (FW) for Ki3, and $90.62 \pm 10.48 \mu\text{g g}^{-1}$ (FW) for CML103 in infected stem tissue (**Figure 6**). One Ki3 control replicate accumulated 5-rosanol at $8.42 \mu\text{g g}^{-1}$ (FW) in stems, but 5-rosanol was not detected in the other replicates. Ion spectra analysis of epoxyrosanol revealed a m/z 307 fragment $[M+H]^+$ consistent with the NMR molecular formula of the purified compound, as well as a predominant m/z 330 ion representing a sodium adduct $[M+Na]^+$. Based on these ions, epoxyrosanol was detected exclusively in infected Oh7B, Ki3, and CML103 stems at very low concentrations of $0.002 \pm 0.001 \mu\text{g g}^{-1}$ (FW), $0.007 \pm 0.002 \mu\text{g g}^{-1}$ (FW), and $0.006 \pm 0.002 \mu\text{g g}^{-1}$ (FW) respectively (**Figure 6**). In infected Oh7B, Ki3, CML103, and CML277 stem tissue, another abundant metabolite was detected, which also featured a m/z of 307 mass fragment and a nearly identical MS/MS fragmentation pattern as compared to epoxyrosanol, yet showed an earlier retention time as compared to epoxyrosanol. We hypothesized that the detected metabolite could result from the enzymatic or spontaneous opening of the epoxide ring of epoxyrosanol. We tested this hypothesis using a chemical conversion approach. Subjecting enzymatically produced, purified epoxyrosanol to slightly acidic conditions indeed yielded the same compound, and further NMR analysis indeed identified it as trihydroxyrosanol (THR), adding another compound to the group of rosalexins that features the loss of the epoxide and gain of two hydroxyl groups at C-15 and C-16. THR was present in both control and infected roots, albeit at trace amounts at similar concentrations, and looking at the mapped fungal read counts suggests that this is most likely due to cross-contamination of the roots during the experiment (data not shown). Conversely, THR accumulates at much higher abundance in infected stem tissue at $2.52 \pm 0.70 \mu\text{g g}^{-1}$ (FW) in Oh7B, $1.62 \pm 0.45 \mu\text{g g}^{-1}$ (FW) in Ki3, $8.05 \pm 1.91 \mu\text{g g}^{-1}$ (FW) in CML103, and $0.43 \pm 0.18 \mu\text{g g}^{-1}$ (FW) in CML277. THR was detected in control plants for CML103 and CML277 at $0.53 \pm 0.15 \mu\text{g g}^{-1}$ (FW) and $0.74 \pm 0.71 \mu\text{g g}^{-1}$ (FW) respectively (**Figure 6**). Despite root infection of all

samples, the lack of rosalexins in control samples supports a localized response at the site of infection as compared to being part of a systemic defense response.

Since rosalexins are significantly more abundant in stems as compared to roots, we focused transcriptomic analysis on stem tissue. ZmTPS42/KSL1 was upregulated in response to *F. verticillioides* in lines featuring functional genes as well as B75 which possesses a non-functional gene and does not produce rosalexins (**Figure 6, Figure 7**). Genes in the zealexin, kauralexin, and dolabralalexin were also upregulated in response to fungal infection for all lines in stem tissues (**Figure 7**).

Notably, when comparing absolute gene expression, ZmTPS42/KSL1 transcript abundance was higher in stem tissue in comparison to root tissue, supporting this pathway being predominantly localized to the aerial tissue (**Figure 8**). In stem tissue, ZmTPS42/KSL1 was lower as compared to ZmTPS43/KSL2 but higher when compared to ZmTPS45/KSL4, which are the core genes controlling the committed kauralexin and dolabralalexin pathway nodes, respectively. Shared pathway genes, including ZmTPS38/CPS2/AN2 and ZmCYP71Z18, showed relatively higher gene expression than ZmTPS42/KSL1. Out of the maize class II diTPS, ZmTPS42/KSL1 showed an expression pattern most similar to ZmTPS38/CPS2/AN2 further supporting a role in specialized metabolism from *ent*-CPP (**Figure 8**). In root tissue, control versus infected treatments did not show differences in gene expression, due to the possible root contamination. However, ZmTPS17, ZmCYP71Z16, ZmTPS14, and ZmTPS40/CPS4 show stronger gene expression in roots than stems, suggesting a role in below-ground terpenoid production (**Figure 8**).

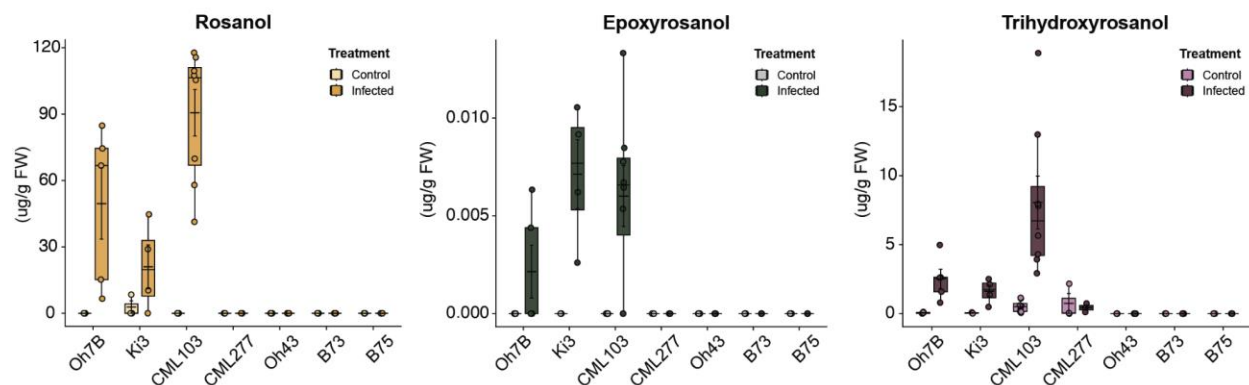


Figure 6. | LC-MS/MS analysis of rosalexins across maize lines. LC-MS/MS average metabolite abundance in stems for 5-rosanol, epoxyrosanol, and trihydroxyrosanol in Oh7B, Ki3, CML103, CML277, Oh43, B73, and B75 plants in mock-treated (control) and plants infected with *F. verticillioides* (infected).

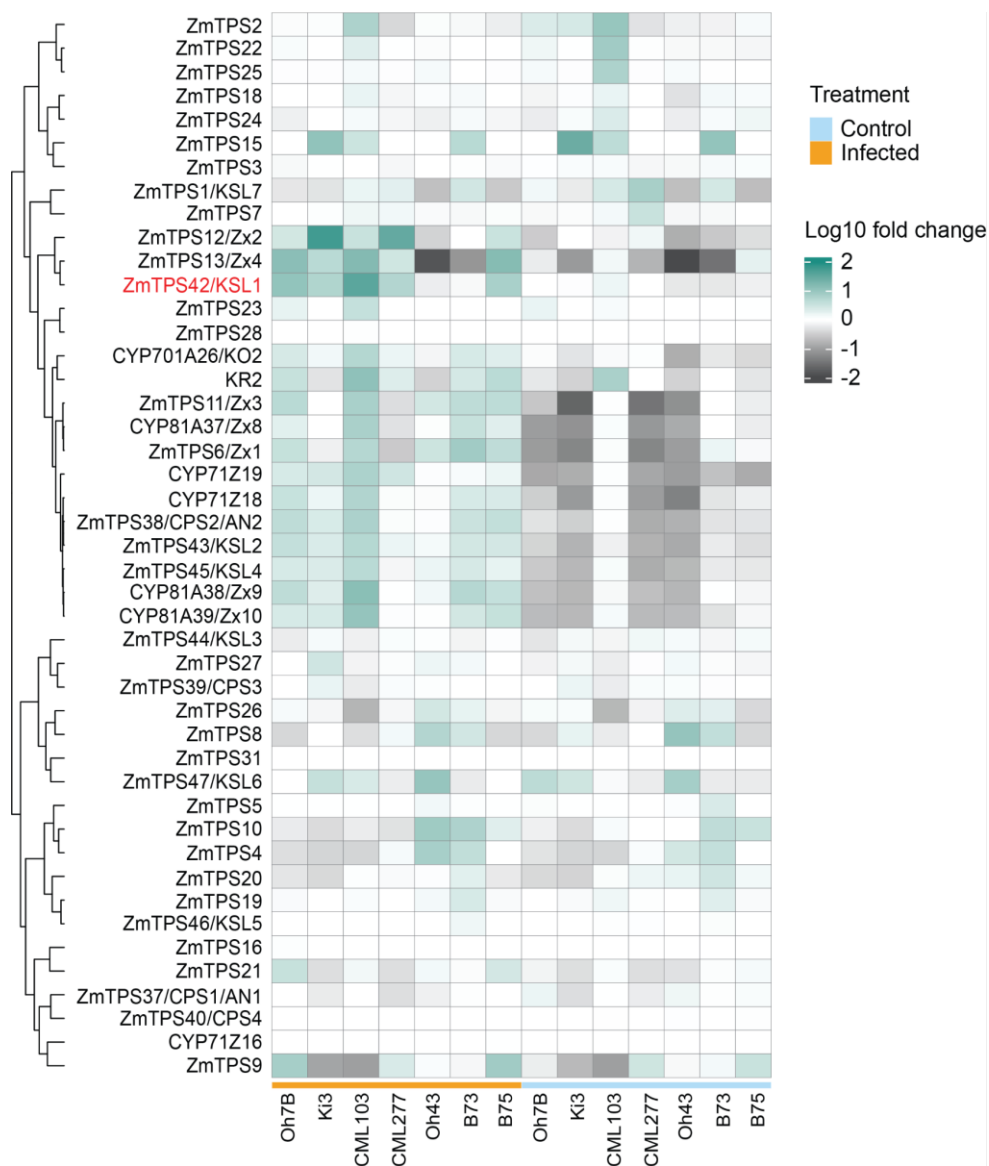


Figure 7. | Differential gene expression in control and infected stem tissue. Heat map depicting log10 transformed fold change of known maize terpene synthases, P450 genes, and kauralexin reductase 2 (KR2) in response to water control or *F. verticillioides* infection.

$\text{Log}_{10}\left(\frac{\text{sample mean [FPKM+1]}}{\text{median across all samples (FPKM)}}\right)$ was used to determine fold change for each genotype per treatment (n=3)

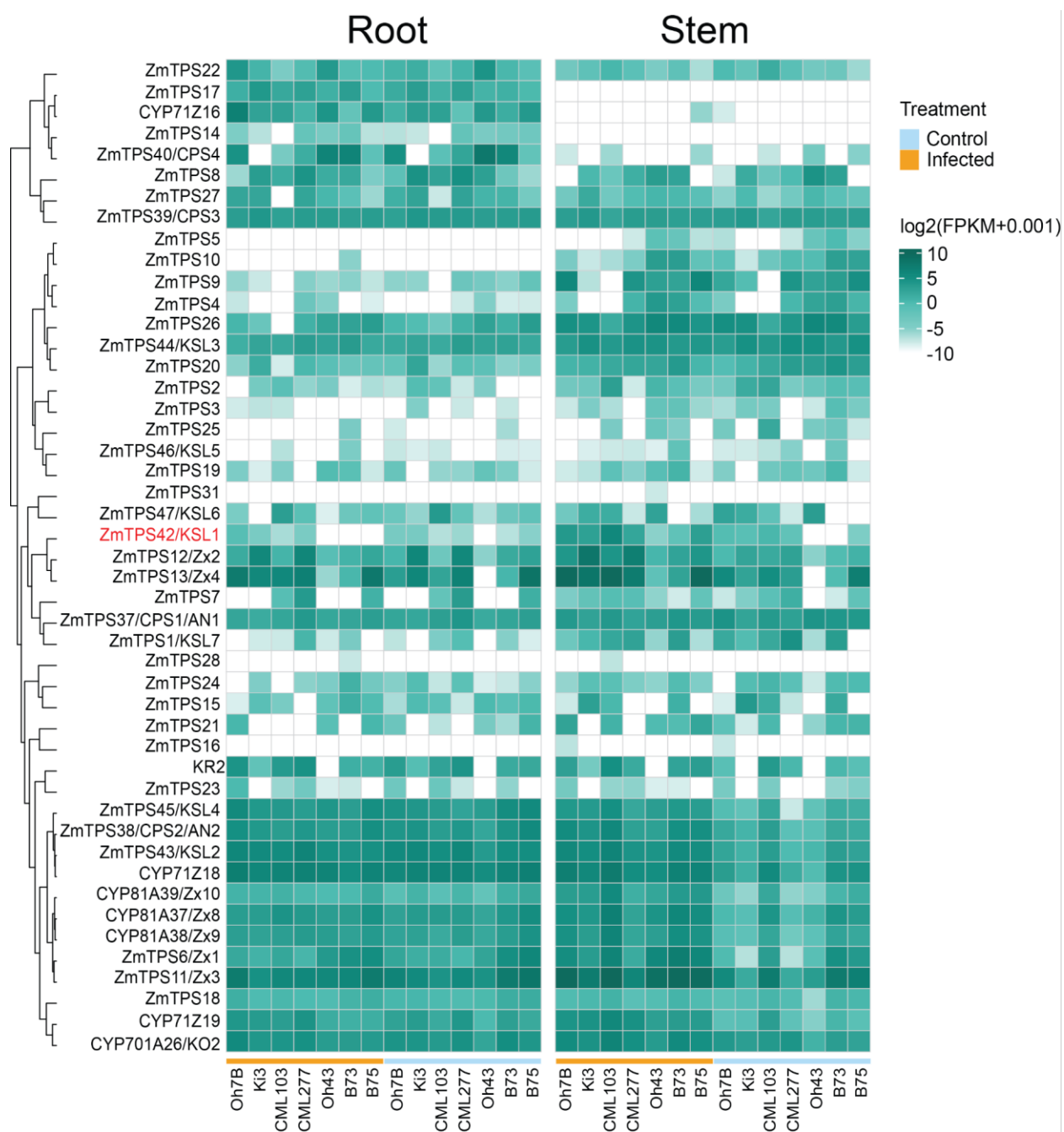


Figure 8. | Rosalexins are pathogen-induced alongside other defense terpene pathways.

Heat map depicting gene expression ($\log_2[\text{FPKM}+0.001]$) of known maize terpene synthases, P450 genes, and KR2 in response to infection with *F. verticillioides* or mock treated controls (n=3).

Bioactivity of rosallexins

Lesion size caused by *F. verticillioides* fungal infection varied across the tested maize genotypes with no distinguishable trends between lines featuring functional vs non-functional ZmTPS42/KSL1, respectively (**Figure 9**). To further test the bioactivity of the rosallexin compounds, *in vitro* fungal assays of purified rosallexin compounds against a host of common maize pathogens, including *Fusarium verticillioides*, *Aspergillus parasiticus*, and *Rhizopus microsporus* found that epoxyrosanol had the greatest growth inhibitory effect of the rosallexin compounds. 5-rosanol and THR had little to no inhibitory effect against *F. verticillioides* and *A. parasiticus* but impacted growth in *R. microsporus* for the first 32 hours. This suggests that while rosallexins are pathogen-inducible, they might play a distinct context-dependent or lesser role in the dynamic network of maize chemical defense layers.



Figure 9. | Lesion imaging of maize lines. Representative images of maize stems infected with *F. verticillioides* (infected) or water (control) using the lines Oh7B, Ki3, CML103, and CML277 (featuring a functional ZmTPS42/KSL1 gene) and Oh43, B73, and B75 (containing non-functional ZmTPS42/KSL1).

Conclusion

Plants deploy species-specific networks of specialized metabolites to mediate dynamic interorganismal interactions and ecological adaptation. In light of rising environmental pressures that threaten crop production, understanding the metabolic and regulatory circuitry that contributes to innate immunity is pivotal for improving disease control and resistance trait breeding.^{56,79,86} Species-specific blends of diterpenoids with potent antimicrobial, insecticidal, and allelopathic functions that directly impact plant protection and vigor exist in major grain crops, including rice, maize, wheat, and barley.^{9,89-90} This study highlights the capacity of integrated multi-omics approaches and combinatorial enzyme biochemical tools to uncover hidden specialized metabolic pathways even in well-studied crops such as maize.

The presence of functional and pseudogenized ZmTPS42/KSL1 genes across different maize genotypes exemplifies the high genetic diversity in maize that shapes metabolic network architecture and chemical diversity. The formation of rosalexins by the combined activity of ZmTPS38/CPS2/AN2, ZmTPS42/KSL1, and ZmCYP71Z18 adds yet another pathway branch to the portfolio of *ent*-CPP-derived diterpenoids produced in maize. Due to their distinct product specificity, ZmTPS42/KSL1, ZmTPS43/KSL2, and ZmTPS45/KSL4 serve as core pathway branch nodes en route to kauralexins, dolabrallexins, and rosalexins. Presence of ZmCYP71Z18 as a highly promiscuous P450 capable of functionally decorating each of the respective *ent*-isokaurene, dolabradiene, and 5-rosanol intermediates creates a modular pathway network for the efficient production of bioactive diterpenoid blends.^{15,19} The absence of significant co-regulation patterns and the low product abundance when pairing ZmTPS42/KSL1 with the 8,13-CPP synthase, ZmTPS40/CPS4, or the (+)-CPP synthase, ZmTPS39/CPS3⁸⁰, support *ent*-CPP as the dominant ZmTPS42/KSL1 substrate. To current knowledge, the biosynthesis of 5-rosanol is unique to maize, although rosane-type diterpenoids have been identified in a range of, for example, liverworts as well as Araucariaceae, Linaceae, and Euphorbiaceae species.⁸⁷⁻⁹⁰

The dependence of rosalexin accumulation on the presence of a functional ZmTPS42/KSL1 gene sequence across the tested maize lines supports the hypothesis that ZmTPS42/KSL1 represents the core pathway node for the biosynthesis of rosalexins. Strikingly, similar to trihydroxydolabrene in dolabralalexin metabolism ¹⁸, epoxyrosanol can undergo spontaneous ring opening yielding the trihydroxy derivative, THR, as a major metabolite *in planta*. As similarly observed for dolabralalexins, epoxyrosanol, but to lesser extent 5-rosanol or THR, show potent antibiotic activity against all tested maize pathogens *F. verticillioides*, *A. parasiticus*, and *R. microsporus*, highlighting that the epoxide moiety is critical for bioactivity in both dolabralalexins and rosalexins. Notably, this finding aligns with the often high reactivity and antimicrobial potential of chemoenzymatically generated terpenoid epoxides ⁹¹. In maize, the functional relevance of these trihydroxy derivatives of epoxydolabrene and epoxyrosanol remains to be uncovered, given their lack of antimicrobial efficacy but substantial abundance *in planta*. Despite the pathogen-elicited accumulation of rosalexins in stem tissue and the potent antimicrobial activity of epoxyrosanol *in vitro*, the absence of a distinctive resistance phenotype in rosalexin-producing *versus* non-functional lines upon *F. verticillioides* infection suggests that rosalexins play a distinct context-dependent or lesser role in the dynamic network of maize chemical defense layers. For example, consistent ZmTPS42/KSL1 transcript accumulation patterns in aerial tissues have been observed in response to infection by foliar pathogens such as *Cochliobolus heterostrophus* and *Colletotrichum graminicola* ^{19,92}.

The discovery of the rosalexin pathway completes a triad of bioactive diterpenoid groups that evolved from ancestral GA metabolism in maize and predictably contribute to above- (kauralexins, rosalexins) and below-ground (dolabralalexins) chemical defenses and other environmental interactions. Knowledge of these enzyme functions and their role in the modular maize diterpenoid-metabolic network forms the foundation to comprehensively understand what drives the substantial variation in the abundance, composition, and stress-inducibility of specialized

diterpenoids across genetically diverse maize lines and commercial hybrids with direct impact on stress resilience ^{18,82}. Ultimately, identifying maize lines with diterpenoid profiles promoting stress resilience provides resources for optimizing resistance traits.

Chapter 3: Expanding the Knowledge of Terpenoid Biosynthesis in Maize

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Author statement: FY conducted gene discovery and SS performed all biochemical assays and transcriptomic analyses.

Introduction

As described in **Chapter 2**, substantial research has advanced our understanding of diterpenoid networks in maize. While the class II diTPSs ZmTPS39/CPS3 and ZmTPS40/CPS4 have been characterized and shown to produce (+)-CPP and 8,13-CPP respectively, the downstream products derived from these pathway branches have yet to be identified.⁸⁰ By contrast, maize produces several inducible diterpenoids from *ent*-CPP, a shared precursor in gibberellin (GA) and specialized diterpenoid metabolism. While both class II diTPSs ZmTPS38/AN2/CPS2 and ZmTPS37/CPS1/AN1 produce *ent*-CPP, only ZmTPS38/CPS2/AN2 shows an inducibility pattern consistent with a role in specialized diterpenoid metabolism beyond plant growth.

Class I diterpene synthases, known as kaurene synthase-like (KSL) enzymes, originated from a duplication of the GA pathway, and were later neofunctionalized to give rise to genes with roles in specialized metabolism.⁴ To date, KSL1-5 and 7 have been functionally characterized in maize. ZmTPS44/KSL3 and ZmTPS46/KSL5 act with ZmTPS37/CPS1/AN1 to produce *ent*-kaurene and are required to produce the conserved phytohormone GA. However, crosstalk between growth and defense metabolism has been observed, as *ent*-kaurene from this pathway can also be funneled into kauralexin biosynthesis.^{19,93} ZmTPS43/KSL2, ZmTPS45/KSL4 and ZmTPS42/KSL1 act with ZmTPS38/CPS2/AN2 in an inducible manner and produce kauralexins, dolabrallexins, and rosalexins, respectively. These compounds show distinct tissue and stress-specific accumulation patterns, including accumulation in above- and below-ground tissues in response to drought and/or fungal stress, supporting roles in stress adaptation.^{18,19,94} However, ZmTPS47/KSL6 remains uncharacterized in maize, potentially due to the high structural variation reported across maize lines, which reportedly affects gene expression.⁹⁵ Nevertheless, its presence across multiple maize lines suggests that it may have an important biological role that remains to be elucidated.

Additionally, P450s are central to the diversification of terpenoid metabolism because they mediate downstream oxidative modifications that expand chemical diversity. The large CYP71 clan is composed of several subfamilies that diversified to catalyze reactions across terpenoid metabolism in several different crop species. Members of the CYP701A subfamily act in both gibberellin and specialized diterpenoid production whereas the characterized CYP71Z genes in maize appear to act exclusively in specialized pathways in both diterpenoid and sesquiterpenoid biosynthesis. In other crops, the CYP99A and CYP76M subfamilies contribute to diterpenoid synthesis, including momilactone production in rice.⁹⁶ Beyond the CYP71 clan, the CYP88 clan mediates gibberellin metabolism, and the potential promiscuity of enzymes in this clan makes them candidates for the expansion of specialized pathways. Together, P450s from these subfamilies in maize may expand upon previously characterized defense pathways and reveal novel metabolites.

Results

ZmTPS47/KSL6 forms labdane-type compounds

To determine the biochemical function of ZmTPS47/KSL6, we used *Agrobacterium-mediated* co-expression in *Nicotiana benthamiana* as a platform to test the ZmTPS47/KSL6 gene sequence from the Zm-B73-REFERENCE-NAM-5.0 reference genome with each maize class II diTPS individually: *ent*-CPP synthase AN1/AN2, (+)-CPP synthase ZmTPS39/CPS3, and 8,13-CPP synthase ZmTPS40/CPS4. Combinations of ZmTPS47/KSL6 with AN1/AN2 and ZmTPS40/CPS4 did not yield any new compounds. By contrast, co-expression of ZmTPS47/KSL6 with ZmTPS39/CPS3 resulted in the formation of three distinct compounds (**Compounds 15-17**), although at lower concentrations in comparison to the (+)-CPP precursor (**Figure 10**). All three compounds featured ions with m/z 257 and m/z 272, consistent with labdane olefin scaffolds. In *Setaria italica* (foxtail millet), *ent*-CPP and SiTPS8 produce a host of characterized pimarane-type compounds including *ent*-pimara-8(14),15-diene, *ent*-

sandaracopimaradiene, and abietadiene.⁹⁷ Comparison of the (+)-CPP ZmTPS47/KSL6 products against these compounds found that **15** matched the retention time and mass spectra for *ent*-pimara-8(14),15-diene and **17** for *ent*-sandaracopimaradiene (**Figure 11a**). However, terpene synthases are not known to change a molecule's stereochemistry. Because *ent*- and normal stereochemistry cannot be distinguished using an Agilent DB-XLB column gas chromatography-mass spectrometry (GC-MS) column, compound **15** was identified as pimara-8(14),15-diene and compound **17** as sandaracopimaradiene consistent with their biosynthesis from a (+)-CPP precursor. While compound **16** possesses ions with similar *m/z* as miltiradiene⁹⁸, comparison against a miltiradiene standard, which was made from the combination of (+)-CPP and MvELS, revealed **16** was not miltiradiene (**Figure 11b**). Based on its similar mass spectra but distinct retention time, compound **16** is most likely a double bond stereoisomer of miltiradiene.

With the first committed pathway step determined, we performed additional co-expression assays with characterized P450s including members of the CYP71Z subfamily as well as uncharacterized maize P450s that are part of the larger CYP71 clan to discover downstream products from this pathway. Combinations with all tested P450s did not yield new compounds (**Table 1**). Curiously, when CYP71Z18, a promiscuous P450 involved in both di- and sesquiterpenoid production^{15,18,19}, was tested with ZmTPS39/CPS3 and ZmTPS47/KSL6, the previously seen pimarane compounds were either detectable at much lower abundances or no longer detectable, a pattern not observed for any of the other tested P450s (**Figure 12**). One explanation for the trend could be that ZmCYP71Z18 is responsible for the oxidation of these compounds resulting in carboxylic acids, similar to the zealexins and kauralexins.^{15,21} Because carboxylic acids are not easily detectable on GC-MS without derivatization,⁹⁹ future analysis using derivatization prior to GC-MS analysis or using liquid chromatography-mass spectrometry (LC-MS) might reveal maize terpenoids derived from ZmTPS47/KSL6 and ZmCYP71Z18.

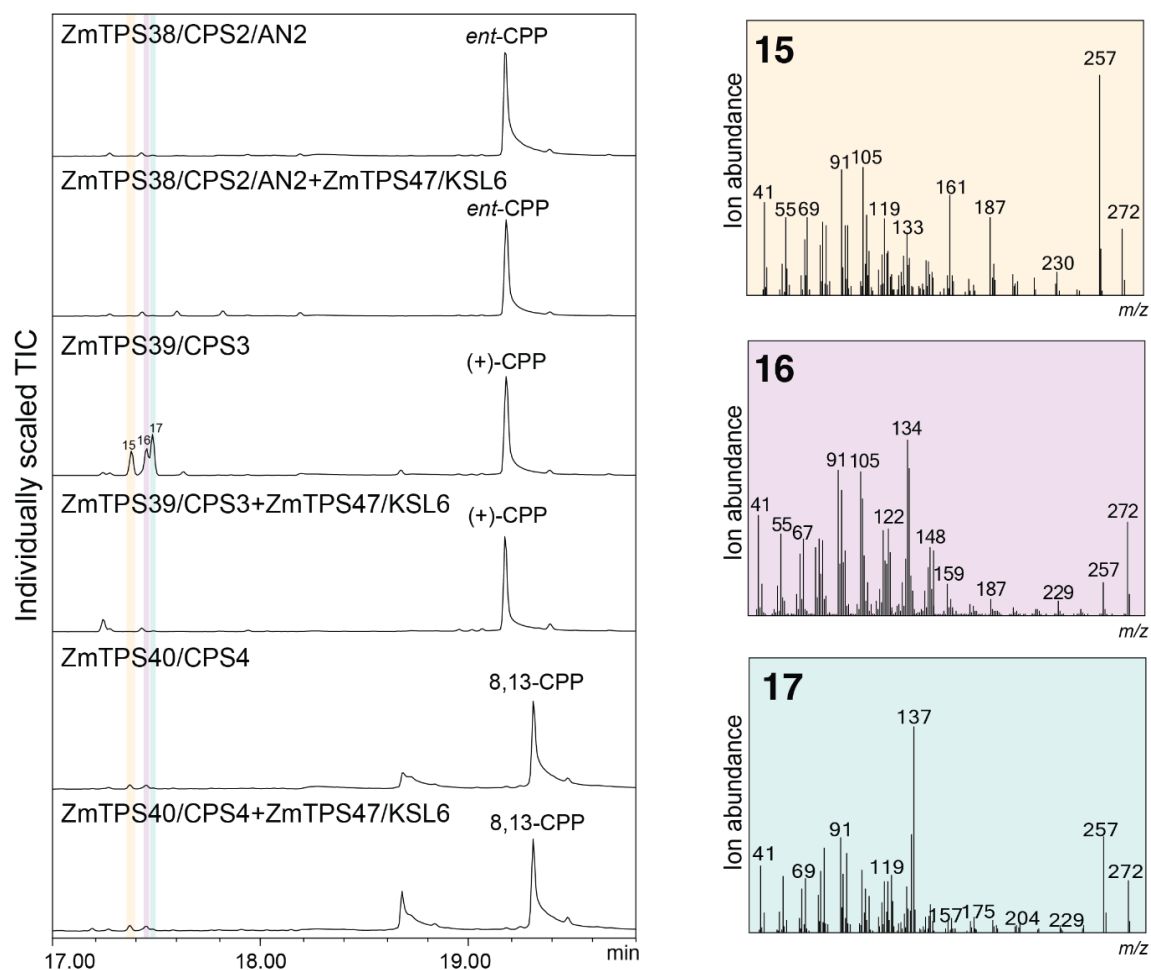


Figure 10. | Co-expression of ZmTPS47/KSL6 with class II diTPS. Total ion chromatograms (TIC) (left) and mass spectra (right) of reaction products resulting from *Agrobacterium*-mediated *Nicotiana benthamiana* co-expression assays of ZmTPS47/KSL6 with ZmTPS38/CPS2/AN2, ZmTPS39/CPS3, or ZmTPS40/CPS4.

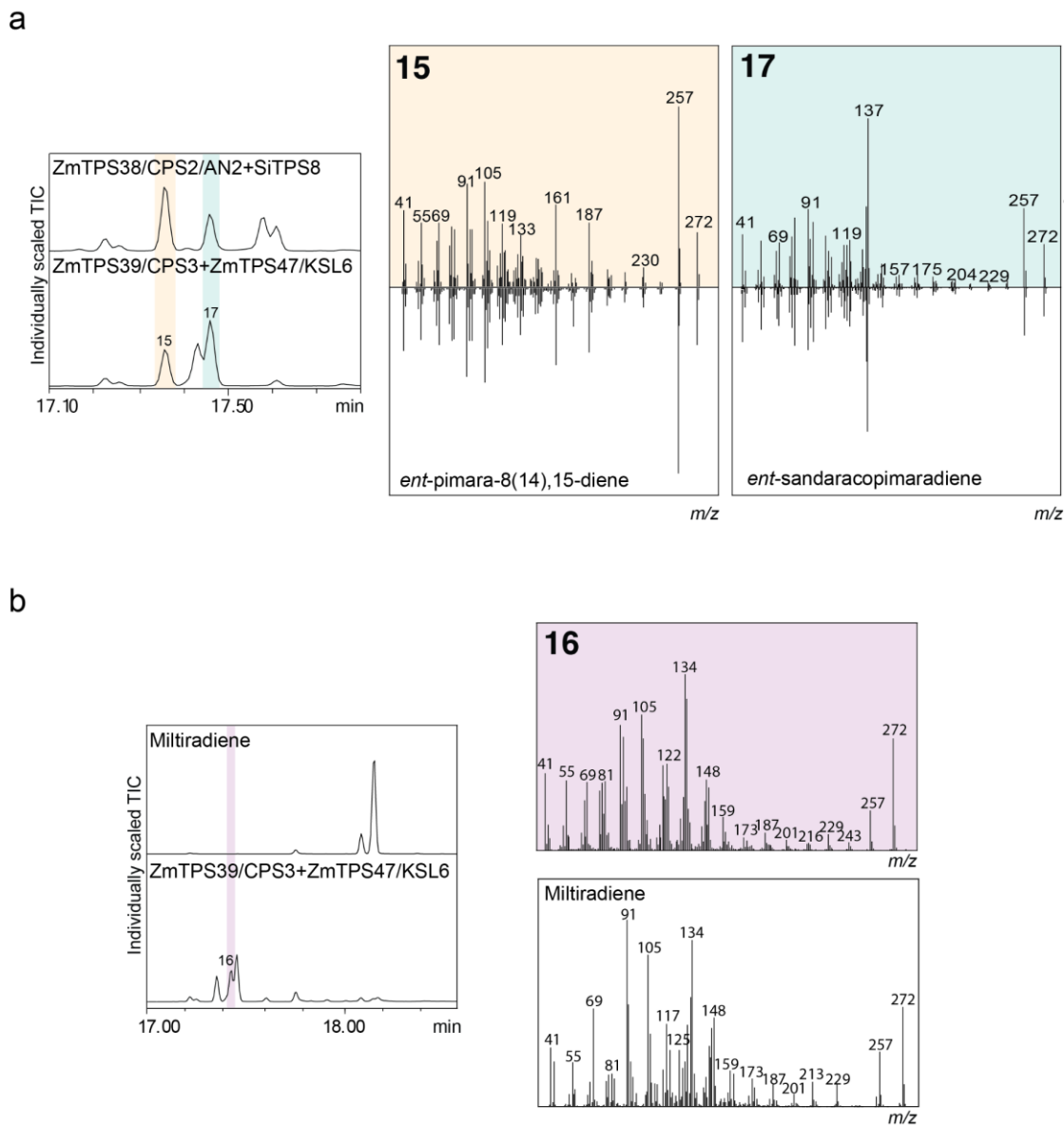


Figure 11. | (+)-CPP and TPS47/KSL6 products against internal standards. Total ion chromatograms and spectra of reaction products resulting from *Nicotiana benthamiana* co-expression assays of ZmTPS39/CPS3 and ZmTPS47/KSL6 compared against **a**, the total ion chromatogram and spectra of ZmTPS38/CPS2/AN2 and *Setaria italica* TPS8 which produces *ent*-pimara-8(14),15-diene (**15**) and *ent*-sandaracopimaradiene (**17**) and **b**, the total ion chromatogram and spectra of miltiradiene.

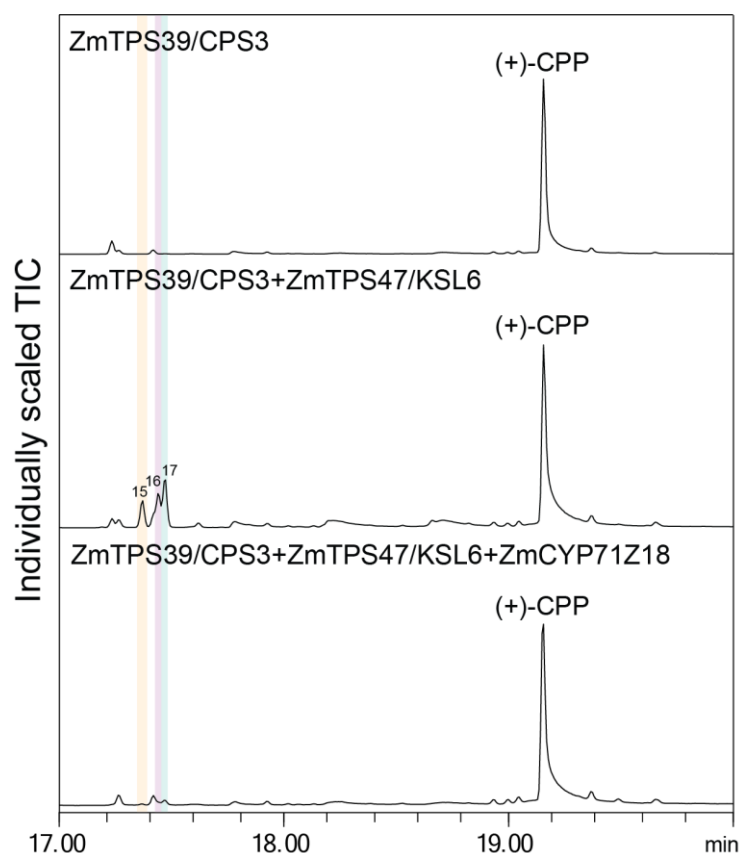


Figure 12. | Combination of KSL6 products with CYP71Z18. Total ion chromatograms of reaction products resulting from *Nicotiana benthamiana* co-expression assays of ZmTPS39/CPS3, ZmTPS39/CPS3 and ZmTPS47/KSL6 only, or with the addition of ZmCYP71Z18.

The maize genome possesses P450s predicted to be members of the CYP71 clan and CYP88A family

Beyond terpene synthases, several P450 genes remain uncharacterized in the maize genome and genome mining can be used to identify candidate enzymes that may expand known terpenoid networks. To identify P450 candidates, 72,539 coding sequences were mined from the Zm-B73-REFERENCE-NAM-5.0 genome from MaizeGDB¹⁰⁰. Sequences were BLAST against an in-house P450 database, and hits were filtered based on E-value with a cutoff of $\leq 1e-100$ and PFGxGRRxCxG, AGxDT, and KETLR motifs¹⁰¹ to yield 205 candidate genes.

From these, 15 enzymes belonging to subfamilies of interest were selected as target genes for *Agrobacterium*-mediated co-expression assays in *Nicotiana benthamiana* with class I diTPS (Table 1).

Gene ID	P450 subfamily
Zm00001eb225960	CYP88A
Zm00001eb224350	CYP701A
Zm00001eb406670	CYP701A
Zm00001eb020100	CYP714B
Zm00001eb020890	CYP714B
Zm00001eb234470	CYP714B
Zm00001eb074170	CYP76M
Zm00001eb082030	CYP99A
Zm00001eb311630	CYP99A
Zm00001eb173980	CYP99A
Zm00001eb043630	CYP81A
Zm00001eb183820	CYP88A
Zm00001eb089130	CYP88A
Zm00001eb131350	CYP71Z
Zm00001eb219330	CYP99A

Table 1. | Target list of P450 candidates. The Zm-B73-REFERENCE-NAM-5.0 gene identifiers (left column) and P450 subfamilies (right column) are listed for all the target P450 genes to be tested with class I diTPSs.

Target P450 genes do not show activity with class I diterpene synthase products

The same *Nicotiana benthamiana* co-expression platform was used to test the 15 target P450 genes with class I diTPS ZmTPS42/KSL1, ZmTPS43/KSL2, ZmTPS44/KSL3, ZmTPS45/KSL4, and ZmTPS47/KSL6. P450 genes were tested individually with the class I diTPS genes as well as in combination with each other and CYP71Z18. None of the tested combinations resulted in the production of new compounds, suggesting that these enzymes either do not act on the tested diterpenoid scaffolds or require additional pathway components not included in the assay.

Using the same in-house transcriptomic dataset as in **Chapter 2**, mutual rank and Pearson's correlation coefficients were calculated for the target P450s against all known maize terpene synthases and P450s involved in defense terpenoid production (**Figure 13a-b**). In particular, Zm00001eb224350 showed strong correlation with defense genes including diTPS ZmTPS43/KSL2 and ZmTPS45/KSL4 as well as genes involved in the zealexin pathway such as ZmTPS6/Zx1, CYP81A37/Zx8, CYP81A38/Zx9, and CYP71Z18 (**Figure 13a**).¹⁵ *In vitro* assays showed Zm00001eb224350 did not work in combination with ZmTPS43/KSL2 or ZmTPS45/KSL4, and its high correlation with these two genes may reflect involvement in the zealexin pathway which is likewise pathogen-inducible and shows similar expression patterns to specialized diterpenoid metabolism. Interestingly, Zm00001eb089130 and Zm00001eb020890 are highly correlated with ZmTPS17/ZmEDS and CYP71Z16 and Zm00001eb311630 shows high correlation with CYP71Z16 alone (**Figure 13b**). Both CYP71Z16 and ZmTPS17 have predicted roles in pathogen defense, with CYP71Z16 being involved in zealexin, dolabralexin, and kauralexin production^{15,18,19,94}, and ZmTPS17/ZmEDS producing eudesman-2,11-diol and related sesquiterpenoids under *F. verticillioides* stress¹⁰².

and kauralexin production^{15,18,19,94}, and ZmTPS17/ZmEDS producing eudesman-2,11-diol and related sesquiterpenoids under *F. verticillioides* stress¹⁰².

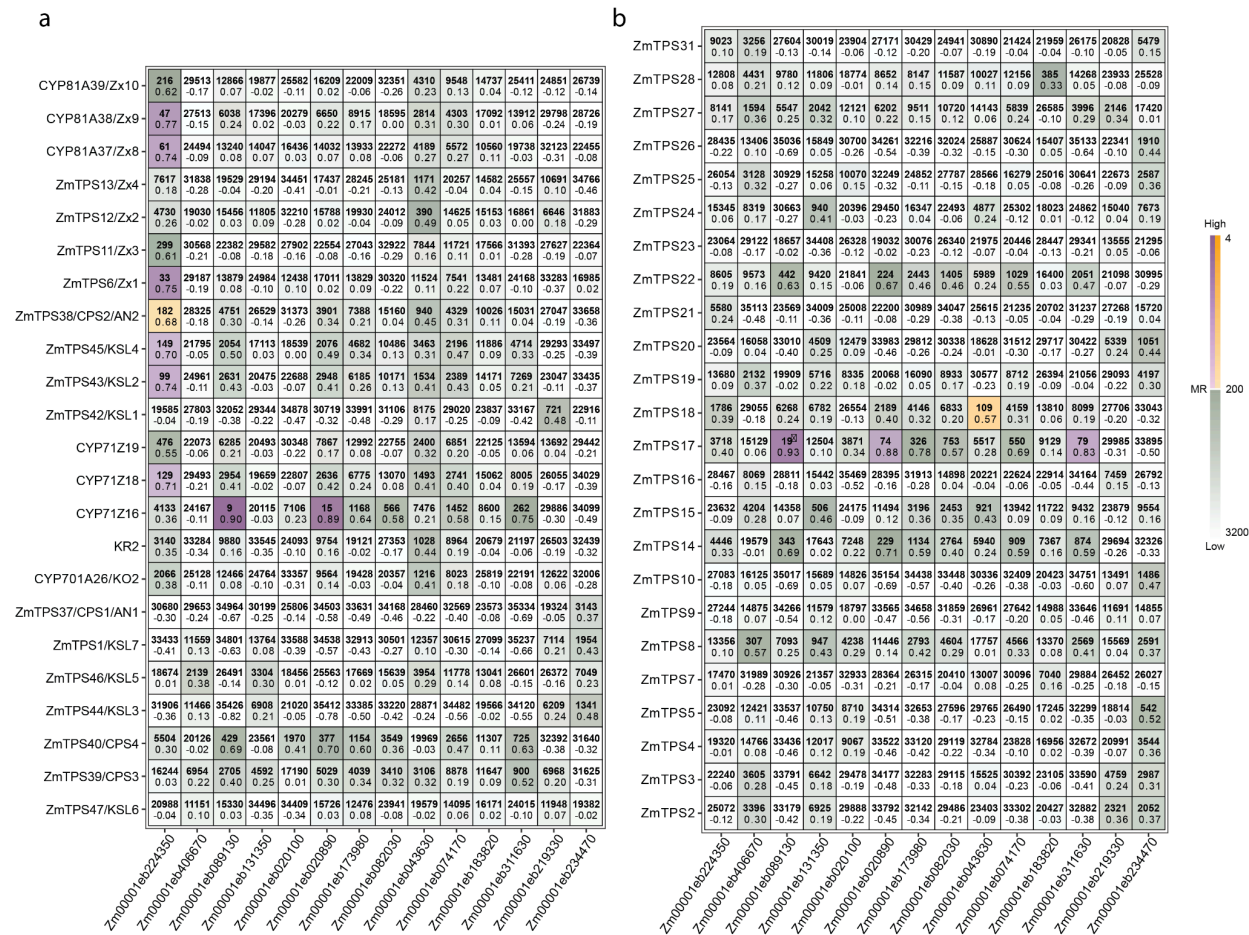


Figure 13. | Co-expression of target P450s with known TPS and P450 genes. Heat map illustrating the pairwise gene correlation for target P450 genes (**Table 1**) with **a**, maize terpene synthase genes and P450 genes involved in zealexin, kauralexin, dolabralalexin, and rosalexin metabolism and **b**, other terpene synthase genes. Strong co-expression was defined by PCC > 0.7 and MR of < 200. Strong pairs are shaded in purple (using $-\log_{10}(\text{MR})$), pairs with a MR < 200 but a PCC < 0.7 are shaded in orange, and weak correlations (PCC < 0.7 and MR > 200) in grey. MR and PCC values are printed on top and bottom respectively.

Conclusion

As the world's population continues to grow, food security is becoming an increasingly urgent issue. Significant crop losses are rising across the globe due to pest and pathogen damage, mycotoxin contamination, as well as drought and other environmental stressors.¹⁰³ One promising strategy to protect crops lies in understanding the biochemical pathways responsible for producing the small molecules that plants employ to adapt to stress, which directly impact crop fitness. Among these metabolites, terpenoids represent the largest class, playing crucial roles in plant growth, chemical defenses against microbial diseases, and response to drought and other environmental stressors.⁷⁹ Understanding the natural defense mechanisms associated with terpenoid biosynthesis could inform crop engineering to develop varieties better equipped to withstand environmental pressures.

In this study, we have shown that the combination of ZmTPS47/KSL6 with ZmTPS39/CPS3 yields diverse labdane-type products, furthering our understanding of the diterpenoid maize network. These compounds are found in other crop species including *Panicum virgatum* (switchgrass)¹⁰⁴, *Setaria italica* (foxtail millet)⁹⁷, and *Oryza sativa* (rice).¹⁰⁵ *Ent*-sandaracopimaradiene is especially important in the production of defensive oryzalexins in *Oryza sativa* (rice).¹⁰⁵ However, based on gene expression data from *Fusarium verticillioides* infected tissue, ZmTPS47/KSL6 is not upregulated in response to pathogen stress (**Figure 7**). Further analysis of publicly available transcriptomic datasets representing other environmental stimuli, such as nutrient stress, may help identify the biological role of this pathway in maize and identify candidate enzymes for downstream pathway production. ZmCYP71Z18 offers a potential target of interest based on co-expression with ZmTPS39/CPS3 and ZmTPS47/KSL6, but more research is needed (**Figure 12**). Additionally, TPS17, also known as ZmEDS, produces eudesman-2,11-diol, a dihydroxylated sesquiterpenoid that is specific to the roots and inducible under *F. verticillioides* infection.¹⁰²

Despite being pathogen-inducible, eudesman-2,11-diol did not display antifungal activity, most likely due to downstream products possessing antibiotic activity similar to the rosalexins and dolabralalexins. By mining the maize genome, we found target P450 genes with predicted roles in terpenoid biosynthesis based on subfamily classification (**Table 1**). Two of these target enzymes, Zm00001eb089130 and Zm00001eb020890, showed high co-expression with ZmTPS17 suggesting that these P450s might work downstream of TPS17. CYP71Z16, a promiscuous P450 involved in several pathogen-inducible terpenoid pathways, showed high co-expression with these genes. Gene expression data shows much higher gene expression for TPS17 and CYP71Z16 in roots compared to stem tissue, suggesting that Zm00001eb089130 and Zm00001eb020890 could work in a root-specific pathway for pathogen defense in maize (**Figure 8**). These results provide a foundation for expanding the current body of knowledge around maize terpenoid production and for the identification of novel metabolites with relevance to stress adaptation.

Materials and Methods

Plant and fungal material

Seeds of the tested maize genotypes were obtained from the USDA National Plant Germplasm System (<https://www.grin-global.org/>) and cultivated as outlined below. *Fusarium verticillioides* GL1093 was kindly provided by Dr. Eric Schmelz. Pathogens were grown on potato dextrose agar for 3 days before the quantification and use of spores in bioactivity assays.

Plant cultivation

For rosalexin pathway profiling of selected maize lines, plants were greenhouse grown at the University of California-Davis. In brief, maize seeds were sterilized using 70% EtOH followed by a 15% bleach solution before being washed with MilliQ water. Seeds were germinated in an autoclaved soil mixture of half BM7 35% Bark HP and half Turface Athletics MVP and supplemented with nutrient water (Ca(NO₃)₂, KNO₃, Fe-EDTA, Zn-EDTA, Cu-EDTA, Na₂MoO₄, KH₂PO₄, MgSO₄, and K₂SO₄).

For enzyme functional assays, *N. benthamiana* was grown from seed in Sunshine Mix (SUN GRO) and supplemented with nutrient water (Ca(NO₃)₂, KNO₃, Fe-EDTA, Zn-EDTA, Cu-EDTA, Na₂MoO₄, KH₂PO₄, MgSO₄, and K₂SO₄) in controlled environment growth chambers under a photoperiod of 12/12 h, 60% relative humidity, 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity, and a day/night temperature cycle of 26/25°C.

Fungal pathogen infection of maize tissues

Plants were 62 days old at the time of elicitation assays. For stem inoculations with *F. verticillioides*, 8-11 cm longitudinal incisions were made between nodes 3 and 4 using a surgical scalpel and treated with 1 mL of fungal spore solution ($1 \times 10^7 \text{ mL}^{-1}$) in MilliQ water or with water only as a control. For stem lesion imaging, a BD PrecisionGlide Needle (18G) was used to

create a 1.2 mm diameter hole under node 3 and inoculated with 1 mL of fungal spore solution (1×10^7 mL⁻¹) in MilliQ water. Wound sites were wrapped with clear packing tape to prevent desiccation.

For root elicitation assays, soil was gently brushed aside to reveal nodal roots, which were punctured at 1 cm intervals using a BD PrecisionGlide needle (27G1/2) and inoculated with 50 μ L fungal spore solution (1×10^7 mL⁻¹) in MilliQ water or water only (control) at each wound site. Plants were additionally watered with 600 mL of fungal spore solution (1×10^7 mL⁻¹) in MilliQ water or water (control) to increase root infection.

Plants (n=3-8 per genotype) were harvested 7-9 days post-infection over the course of 3 days in a random order to account for variation that may have occurred over the collection time period. Representative root sections were collected and washed gently with deionized water. Stem tissue was collected using sterile garden shears and a Zyliss 3.25 inch paring knife. Lesion images were taken immediately after harvest. Stem tissue from both wounding methods was pooled together for each plant for use in metabolomics and transcriptomics studies. All tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

Transcriptome sequencing and gene expression analyses

For transcriptome analysis, root and stem tissue (n=3) from control and fungal infected plants of maize genotypes selected for the pathogen elicitation assays described above was used. RNA was extracted using the Quick-RNA Plant Miniprep kit (Zymo Research). Gene expression analysis of the RNA-seq data was performed by Novogene. In brief, RNA sequencing libraries were prepared and sequenced using the Illumina NovaSeq X-Plus Sequencing platform with a 2x150 paired-end read configuration. Raw sequencing data were generated as FASTQ files. A total of 84 libraries were sequenced, yielding raw read counts ranging from 39,892,816 to

76,617,254 reads per sample. The obtained data were processed to remove low-quality sequences and adapter contamination using the Novogene QC pipeline. After filtering, the number of retained reads ranged from 38,794,843 to 67,827,953 reads per sample. Filtered reads from each library were aligned to the maize reference genome (Zm-B73-REFERENCE-NAM-5.0) using HISAT2 version 2.2.1.¹⁰⁶ FeatureCounts (2.06)¹⁰⁷ was used to count the number of reads mapped to each gene. The resulting averaged Fragments per Kilobase of transcript Million (FPKM) mapped reads were $\log_2[\text{FPKM}+0.001]$ transformed and analyzed in RStudio using the ComplexHeatmap package to generate the absolute gene expression heat map. To generate the differential gene expression heat map, $\log_{10}\left(\frac{\text{sample mean [FPKM+1]}}{\text{median across all samples (FPKM)}}\right)$ was used to calculate log fold change for each gene across samples for all known maize TPS genes¹⁰⁸ as well as P450 genes involved in the kauralexin and dolabrallexin pathways.^{18,19} RStudio was used to generate the heat map.

Mutual rank and Pearson correlation coefficient calculation of ZmTPS42/KSL1

RNA-seq datasets of infected and control (n=3) stem tissue from maize lines Oh7B, Ki3, CML103, and CML277 were used for analyzing Mutual Rank (MR) and Pearson correlation coefficients (PCC). Raw FPKM values were $\log_2[\text{FPKM}+0.001]$ transformed and zero variance genes were removed prior to analysis. ZmTPS38/CPS2/AN2 and ZmTPS42/KSL1 were used as bait genes and tested against kauralexin reductase 2 (KR2) as well as TPS and P450 genes involved in specialized metabolism and GA biosynthesis. PCC values were calculated using pairwise complete observations. To assess correlation strength and specificity, a MR analysis was performed for each gene pair. PCC values were generated for each gene in the gene pair against all expressed genes in the dataset, and the MR value was calculated from the geometric mean between these two ranks. Gene pair strength was visualized using heat maps. Strong correlations were determined by a $\text{MR} < 200$ and a $\text{PCC} > 0.7$ and pairs that met the cutoff were

shaded in orange using $-\log_{10}(\text{MR})$ whereas genes below the cutoff were characterized as weak correlations and shaded in grey using $-\log_{10}(\text{MR})$. Stars denote particularly strong correlations with a $\text{PCC} > 0.8$ and $\text{MR} < 50$.

Mutual rank and Pearson correlation coefficient calculation for P450 gene discovery

RNA-seq datasets of infected and control ($n=3$) stem and root tissue from maize lines Oh7B, Ki3, CML103, CML277, Oh43, B73, B75, MoG, and PHW65 were used for analyzing Mutual Rank (MR) and Pearson correlation coefficients (PCC). Raw FPKM values were $\log_2[\text{FPKM} + 0.001]$ transformed and zero variance genes were removed prior to analysis. Target P450 genes were used as bait genes and tested against all known maize terpene synthases¹⁰⁸ as well as P450s involved in the kauralexin¹⁹, dolabralalexin¹⁸, and zealexin¹⁵ pathways. PCC values were calculated using pairwise complete observations. To assess correlation strength and specificity, a MR analysis was performed for each gene pair. PCC values were generated for each gene in the gene pair against all expressed genes in the dataset, and the MR value was calculated from the geometric mean between these two ranks. Gene pair strength was visualized using heat maps. Strong correlations were determined by a $\text{MR} < 200$ and a $\text{PCC} > 0.7$ and pairs that met the cutoff were shaded in purple using $-\log_{10}(\text{MR})$ whereas genes below the cutoff were characterized as weak correlations and shaded in grey using $-\log_{10}(\text{MR})$. Genes that had a $\text{MR} < 200$ but a $\text{PCC} < 0.7$ were shaded in orange.

Metabolite profiling of maize tissues via LC-MS/MS

To quantify plant metabolites, 100 mg of maize tissue was collected from the fungal elicitation experiment (see above). Metabolites were extracted using 80% Optima LC-MS grade MeOH (Fisher Scientific) containing 0.5 μM Telmisartan (Sigma-Aldrich) as internal standard for quantification. Liquid chromatography was performed on a Thermo Scientific Vanquish UHPLC with an Acquity UPLC BEH C18 column (2.1 $\times$ 100 mm, 1.7 μm particle size; Waters) using

Optima LC-MS grade water with 0.1% formic acid (v/v) as eluent A and Optima acetonitrile with 0.1% formic acid (v/v) as eluent B. The chromatography method used a flow rate of 0.6 mL min⁻¹: -1 to 0 min, 1% B, 0 to 0.5 min, 50% B, 0.5 to 7.5 min, 90% B, 7.5 to 9 min, 1% B, 9 to 10 min 1% B with the column compartment set to 40°C. Mass spectrometry was performed using a Thermo Scientific Q-Exactive equipped with an electrospray ionization source that was run in positive ionization mode (spray voltage, 3.50 [kV]; capillary temperature, 300°C; aux. gas heater, 350°C; sheath gas flow rate, 45; aux. gas flow rate, 10; sweep gas flow rate, 3). The MSDial software program¹⁰⁹ was used to generate and analyze the LC-MS/MS generated data. To quantify metabolites, peak area was autointegrated and extracted from Thermo Scientific Free Style and all calculations were performed in Excel.

In vivo transient co-expression assays in *Nicotiana benthamiana*

Full-length, codon optimized genes of the focal diTPSs and P450s were obtained by DNA synthesis (Twist Bioscience) with financial support by the DOE Joint Genome Institute (JGI) DNA Synthesis Science Program (grant no. 510448) and inserted into the binary pEAQ-HT vector system.¹¹⁰ For co-expression in *N. benthamiana*, constructs were transformed into *A. tumefaciens* strain GV3101 and cells were grown at 28°C for 48 hours in LB media supplemented with 10 µL mL⁻¹ of gentamicin and rifampicin and 50 µL mL⁻¹ of kanamycin. Cells were then adjusted to a final optical density (OD₆₀₀) of 0.5 in 10 mM MES buffer (pH 5.6) with 10 mM MgCl₂ and 150 µM acetosyringone (Acros Organics). For co-expression of the chosen diTPS and P450 combinations the respective *Agrobacterium* cultures were combined in equal volumes including the silencing suppressor strain p19¹¹¹ and infiltrated into the abaxial side of the leaves of 5-week-old *N. benthamiana* plants using a needleless syringe. Five days post-infiltration, metabolite extraction from transfected plants was performed via a 15-minute sonication using 5 mL 85:15 (v/v) hexane:ethyl acetate (Fisher Scientific) and analyzed via GC-MS.

GC-MS analysis of enzyme products

GC-MS analysis of enzyme products was conducted on an Agilent 7890B GC interfaced with a 5977 Extractor XL MS Detector at 70 eV and 1.2 mL min⁻¹ He flow, using an Agilent DB-XLB column (30 m, 250 µm i.d., 0.25 µm film) and the following GC parameters: 50°C for 3 min, 15°C min⁻¹ to 300°C, hold for 3 min with pulsed splitless injection at 250°C. MS data from 40–400 mass-to-charge ratio (m/z) were collected after a 13-min solvent delay.

Data availability

The RNA-sequencing data reported in this study were submitted to the Sequence Read Archive (SRA), accession no. PRJNA1454089.

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