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Direct production of dihydroxylated sesquiterpenoids by a maize terpene synthase

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Abstract
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Keywords
biosynthesis, maize, root, sesquiterpenoid, terpene synthase

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Direct production of di-hydroxylated sesquiterpenoids by a maize terpene synthase

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Summary

The astounding structural and biological diversity of the large class of terpenoid natural products are imparted by both their complex hydrocarbon backbones and further elaboration by the addition of multiple hydroxyl groups, which provide both solubility and specific binding properties. While the role of terpene synthases in generating hydrocarbons with complex backbones is well known, these also are known to generate (singly) hydroxylated products by the addition of water prior to terminating deprotonation. Here a maize sesquiterpene synthase was unexpectedly found to generate dually hydroxylated products directly from (*E,E*)-farnesyl diphosphate, primarily eudesmane-2,11-diol, along with two closely related structural isomers. The unprecedented formation of these diols was proposed to proceed via initial addition of water to a germacradienyl$^+$ intermediate, followed by protonation of the internal carbon-6,7-double-bond in the resulting hedycarol, with subsequent cyclization and further addition of water to an eudesmolyl$^+$ intermediate. Evidence for the proposed mechanism was provided by labeling studies, as well as site-directed mutagenesis, based on structural modeling, which identified an active site phenylalanine required for the protonation and further elaboration of hedycaryol. This di-hydroxylated sesquiterpenoid synthase was specifically expressed in maize roots and induced by pathogen infection, with its major enzymatic product only detected in root exudates or infected roots, suggesting a role in defense. Regardless of the ultimate metabolic fate or physiological role of these diols, this report not only reveals an unanticipated extension of the catalytic prowess of terpene synthases, but also provides insight into the underlying enzymatic mechanism.

Introduction

Terpenoids constitute the largest class of natural products, with almost 55,000 already known (Pateraki *et al.*, 2015), and a number are widely used in agriculture, as flavors and fragrances, or as pharmaceutical agents (Tholl, 2015). The structural diversity of these compounds is derived in large part from the complexity of the underlying hydrocarbon backbones, along

with their further elaboration that stems from the addition of hydroxyl groups, which can then undergo a variety of subsequent derivatization (Pateraki *et al.*, 2015). Indeed, the solubility of terpenoids generally relies on the presence of at least two spatially separated oxy groups, which also are important for biological activity, providing hydrogen-bonding capacity that enables specific binding to (macro)molecular targets (Zi *et al.*, 2014).

The role of terpene synthases (TPSs) in generating the complex hydrocarbon skeletons characteristic of terpenoid natural products is widely appreciated. These enzymes catalyze lysis of the allylic diphosphate ester bond in their substrates to initiate carbocationic cascades that mediate cyclization and/or rearrangement of relatively simple isoprenyl precursors, and can impart astounding structural complexity (Christianson, 2006). These electrophilic reactions are terminated by deprotonation, either directly or following the addition of water, leading to the usual TPS production of hydrocarbons, or less common production of (singly) hydroxylated, terpenes, respectively (Christianson, 2008). Nevertheless, the production of hydroxyl groups, particularly when present at multiple positions, is prototypically presumed to depend on the activity of oxygenases such as cytochromes P450 (Pateraki *et al.*, 2015).

Maize (*Zea mays*) is an important crop plant and, along with other cereal crop plants, produces numerous terpenoid natural products (Schmelz *et al.*, 2014). While some investigation of maize TPSs has been reported (Kollner *et al.*, 2009; Kollner *et al.*, 2008a; Kollner *et al.*, 2004; Lin *et al.*, 2008), a number remain uncharacterized. Here, in the course of investigating these maize TPSs it was discovered that one exhibits unprecedented activity, reacting with (*E,E*)-farnesyl diphosphate (FPP) to predominantly produce the di-hydroxylated sesquiterpenoid eudesmane-2,11-diol, along with mono- and other di-hydroxylated products. Support for a plausible mechanism involving protonation of a transiently formed hedycarol neutral intermediate was provided via labeling studies demonstrating the incorporation of a proton from bulk water into the hydrocarbon backbone. This hypothesis was further bolstered by site-directed mutagenesis, based on structural modeling, which led to identification of an

active site phenylalanine required for the protonation and further elaboration of hedycarol. Thus, not only are TPSs shown here to exhibit unanticipated catalytic prowess, with direct formation of dually hydroxylated terpenes, but some mechanistic insight is offered into the mechanism and underlying enzymatic structure-function relationship as well.

Results

Identification of a di-hydroxylating sesquiterpenoid synthase from maize

In the course of investigating uncharacterized maize TPSs by screening via a modular metabolic engineering system (Cyr *et al.*, 2007; Mao *et al.*, 2016), in which these were recombinantly expressed in *Escherichia coli* also engineered to produce FPP, it was found that the major product for one of these appeared to be a di-hydroxylated sesquiterpenoid, as indicated by its molecular weight (i.e., a molecular ion with *m/z* = 240), along with a number of other more minor products (Figures 1A, 1B and S1). The unprecedented production of this diol was further investigated by *in vitro* assays, which verified the surprising ability of this TPS to directly produce di-hydroxylated sesquiterpenoids from FPP (Figure 1A).

To more fully characterize this TPS, its products were investigated by scaling up the *E. coli* system, including incorporating further engineering that increases flux towards isoprenoid metabolism (Morrone *et al.*, 2010), to generate sufficient quantities for structural analysis by NMR. This revealed that the major product was eudesmane-2,11-diol (Figure 1C), with the NMR chemical shift data measured here (Supplemental Table S1) matching that previously reported for this sesquiterpenoid (Mathela *et al.*, 1989). Thus, this enzyme is termed here maize eudesmanediol synthase, ZmEDS. The second most abundant product also was investigated in this same manner (Figures 1C and Table S2), and identified to be 2-*epi*-α-eudesmol through comparison with the previously reported NMR data for α-eudesmol (Tebbaa *et al.*, 2011). One of the minor products, by comparison to a previously reported enzymatic product (Yu *et al.*, 2008), was found to be β-eudesmol (Figure S1).

Intriguingly, 2-*epi*-α-eudesmol and β-eudesmol are both mono-hydroxylated sequiterpenes, and bracket a group of products in the GC-MS chromatogram that all exhibit mass spectra indicative of a similar composition (i.e., apparent molecular ions of *m/z* = 222). Two other minor products have similar mass spectra, but elute closer to eudesmane-2,11-diol, suggesting that these might also be diols. Indeed, upon isolation and NMR analysis, these were found to be 2,3-*epi*-cryptomeridiol and 3-*epi*-cryptomeridiol, with the NMR chemical shift data measured here (Figure 1C and Table S2) also matching that already in the literature (Cornwell *et al.*, 2000).

Proposed catalytic mechanism for ZmEDS

Notably, all the ZmEDS products identified above exhibit the same overall hydrocarbon ring structure, and have a C11-hydroxy group, while the additional hydroxyl group in the diols can be found at either of two adjacent carbons (i.e., C2 in eudesmane-2,11-diol or C3 in the epimeric 2,3-*epi*-cryptomeridiol and 3-*epi*-cryptomeridiol, respectively; see Figure 1C). Consideration of the underlying hydrocarbon skeletal structure and hydroxylation pattern suggested a plausible mechanism for their production, particularly including that of the dihydroxylated sesquiterpenoids (Figure 2). In particular, by analogy to certain sesquiterpene TPSs that catalyze reactions proceeding via transient formation of a stable germacrene A olefin, with ensuing protonation of the internal C6,7-double-bond leading to further carbocationic cyclization and/or rearrangement (Degenhardt *et al.*, 2009). Accordingly, ZmEDS is hypothesized to initially produce the C11-hydroxy derivative of germacrene A, hedycarol. This is formed by the addition of water to the gremacren-11-yl^{+} carbocationic intermediate resulting from 1,10-(macro)cyclization, with subsequent deprotonation of the resulting oxocarbenium cation generating the neutral hedycarol. This transient intermediate would then be protonated at the internal 6,7-double-bond (specifically C6) to induce further internal 2,7-ring closure, forming a eudesmolyl^{+} intermediate. Direct deprotonation of this yields the observed 2-*epi*-α-eudesmol and β-eudesmol, while the diols are produced by prior addition of water, either following a 1,2-hydride shift (from C2 to C3) to produce

eudesmane-2,11-diol, or immediately to produce the epimeric 2,3-*epi*-cryptomeridiol and 3-*epi*-cryptomeridiol. Critically, while acid hydrolysis of hedycarol has been reported to form eudesmols such as 2-*epi*-α-eudesmol and α-eudesmol (Jones and Sutherland, 1968; Minnaard *et al.*, 1997), and the related diol 3-epi-cryptomeridiol also has been proposed to be generated by hydrolysis of hedycarol (Cornwell *et al.*, 2000), here the enzymatic products were directly extracted with hexanes, which were further not passed over silica gel, excluding the possibility that these were generated by acid hydrolysis of hedycarol or a cyclic ether (e.g., of a 2,11-epoxy to generate eudesmane-2,11-diol).

To provide evidence for the proposed mechanism, specifically the proposed use of hedycarol as a transient neutral intermediate, the catalyzed reaction was probed by labeling. In particular, the incorporation of deuterium from deuterated water into the hydrocarbon backbone has been used to support a similar proposal that (*S*)-β-bisabolene served as a transient neutral intermediate in the production of (*S*)-β-macrocarpene by other maize sesquiterpene synthases (Kollner *et al.*, 2008b). Thus, *in vitro* enzymatic reactions with ZmEDS were carried out in normal versus deuterated water (i.e., H_2O vs ^2H_2O). Examination of the eudesmane-2,11-diol produced in these assays demonstrated the incorporation of a non-exchangeable deuterium (i.e., in the hydrocarbon backbone), with the molecular ion increasing from *m/z* = 240 to 241 (Figure S2A), consistent with the hypothesized olefin protonation. Moreover, examination of the MS fragmentation pattern for eudesmane-2,11-diol (Figure S2B), based on that reported for similar sesquiterpenoid alcohols (Rabe and Dickschat, 2016), indicates that the deuterium is incorporated at C6, consistent with the mechanism proposed above.

Identification of a key residue for reprotonation

The protonation of germacrene A as a transient neutral intermediate in the reaction catalyzed by the tobacco (*Nicotiana tabacum*) 5-*epi*-aristolochene synthase (5EAS) was suggested to critically depend on a key tyrosine residue, Y520, on the basis of the known crystal structure (Starks *et al.*, 1997). Indeed, a 5EAS:Y520F mutant has been reported to only generate germacrene A as its sole product (Rising *et al.*, 2000). Similarly, mutation of the equivalent tyrosine in a maize (*S*)-β-macrocarpene synthase led to predominant production of the putative transient neutral intermediate (*S*)-β-bisabolene (Kollner *et al.*, 2008b). Given that ZmEDS also contains an equivalent tyrosine, Y529 (Figure S3), this also was mutated to phenylalanine. However, while the ZmEDS:Y529F mutant produced an increased amount of hedycaryol, detected here by GC-MS as the thermally rearranged elemol [as previously reported (Jones and Sutherland, 1968), and found in characterization of a recently identified hedycarol synthase (Hattan *et al.*, 2016)], the other products, including the sesquiterpenoid diols, were still present (Figure 3B). Thus, this tyrosine is not absolutely required for the protonation of hedycarol by ZmEDS.

To provide further insight into the enzymatic structure-function relationship underlying the ability of ZmEDS to produce eudesmane-2,11-diol via hedycarol as a transient intermediate, a model was generated for ZmEDS using Swiss-Model, based on the known structure of 5EAS (32% amino acid sequence identity) as the template (Figure 3A). Intriguingly, examination of this structure, coupled to phylogenetic analysis (Figure S3), suggested that a phenylalanine, located at the bottom of the active site (Figure 3A), might be important in the use of hedycaryol as a transient intermediate. In particular, this phenylalanine is only found in ZmEDS and a previously reported sesquiterpene synthase from *Zingiber zerumbet* (ZzES) that largely produces β-eudesmol, presumably via reprotonation of hedycarol (Yu *et al.*, 2008). The importance of this residue was investigated by site-directed mutagenesis. Strikingly, substitution of alanine for this Phe in ZmEDS (F303A) led to predominant production of hedycarol (Figure 3B).

Indeed, expression of this ZmEDS:F303A mutant in *E. coli* also engineered to produce FPP enabled isolation and identification of hedycarol (Figure S4). This stable putative intermediate was fed to ZmEDS or ZzES in *in vitro* assays, however, no further transformation was observed. Nevertheless, this is consistent with previous studies of other sesquiterpene synthases whose products are hypothesized to be formed via germacrene A as a transient stable intermediate, as analogous negative results have been reported for feeding this as an exogenous substrate in *in vitro* assays (Rising *et al.*, 2000).

In planta activity of ZmEDS

To further explore the enzymatic activity of ZmEDS *in planta*, it was transiently expressed in leaves of *Nicotiana benthamiana* through agroinfiltration. Small amounts of eudesmane-2,11-diol were detected in the organic extract of these tobacco leaves (Figure 4), while the other minor products of ZmEDS were not detected. None of the ZmEDS products were detected in control plants infiltrated with the empty vector. While it has been reported that exogenous natural products in *N. benthamiana* often undergo glycosylation (Aharoni *et al.*, 2003; van Herpen *et al.*, 2010; Zeng *et al.*, 2016), deglycosylation did not lead to increased detection of any ZmEDS products. This may be due to alternative modification or lack of precursors, as has also been reported (Bruckner and Tissier, 2013; Reed *et al.*, 2017). Nevertheless, detection of eudesmane-2,11-diol indicates that ZmEDS exhibits the same enzymatic activity *in planta* as observed in *E. coli*.

Root specific and inducible expression of *ZmEDS*

To elucidate the physiological role of ZmEDS, gene expression and product detection was carried out. Gene expression analysis showed that *ZmEDS* was specifically expressed in roots (Figure 5A, 5B and S5A), consistent with a previous RNA-Seq based study (Sekhon *et al.*, 2011). In addition, it was further found that infection with the common maize fungal pathogen *Fusarium verticillioides* significantly induced accumulation of *ZmEDS* transcripts

in roots (Figure 5C), which was also reported before (Lanubile *et al.*, 2014). Root infection by *F. verticillioides* further significantly increased the accumulation of eudesmane-2,11-diol (~5 ng g^{-1} FW) (Figure 5C). Additional analysis found eudesmane-2,11-diol mainly in the spent media from sterile seedlings or hydroponic culture (~250 ng g^{-1} FW of roots) but not in roots from aeroponic culture, indicating that this can be secreted from the roots (Figure 5C and S5B). *F. verticillioides* infection also induced eudesmane-2,11-diol accumulation in the roots of another inbred line HZ4 growing in aeroponic culture (~3 ng g^{-1} FW) (Figure S5B). However, eudesmane-2,11-diol did not exert any significant inhibitory effect on the growth of fungal pathogen spores (Figure S6). Given the inducible expression of *ZmEDS* in maize roots, it seems likely that the ZmEDS products are elaborated to further decorated sesquiterpenoid natural products that play a role in maize defense against fungi, although this remains to be determined.

Discussion

Although *ZmEDS* gene expression was observed in maize roots, only small amounts of the major product eudesmane-2,11-diol were found in root exudates, and this was only found in the roots following pathogen infection. Thus, it seems reasonable to propose that maize further elaborates eudesmane-2,11-diol, as well as other ZmEDS products, to form further elaborated sesquiterpenoids. Based on the induction of *ZmEDS* transcripts by the fungal pathogen *F. verticillioides*, these natural products presumably play a role in maize root defense, although these may also play a role in the rhizosphere.

Regardless of the physiological role of its products, characterization of ZmEDS as a sesquiterpene synthase capable of directly yielding di-hydroxylated products provides an unanticipated extension of TPS catalytic capabilities. The observed direct production of two hydroxyl groups was hypothesized to proceed via protonation of hedycarol, which then serves as a transient stable intermediate. While analogous protonation of the structurally

equivalent olefin germacrene A has been previously shown (Cane, 1990), it seemed prudent here to investigate the proposed protonation of hedycarol, particularly as this does not serve as a reactive substrate upon exogenous addition. Evidence for such protonation was provided by labeling studies from enzymatic assays carried out in deuterated water. This demonstrated incorporation of a non-exchangeable proton from bulk water (Figure S2), indicating a mechanism wherein an additional proton beyond those in the hydroxyl groups is incorporated in the course of the catalyzed reaction, consistent with the postulated protonation of hedycarol. Accordingly, ZmEDS catalyzes a reaction that combines both indirect (cyclo)hydrolytic and (cyclo)hydratase mechanisms (Figure 2).

Further insight into the structure-function relationship underlying protonation of hedycarol was provided by modeling of ZmEDS. This led to identification of a key active site phenylalanine that appears to be required for such protonation (Figure 3). However, this residue obviously cannot act as the catalytic acid. Thus, the key phenylalanine identified here presumably serves to position and/or activate hedycarol for protonation. Regardless of exact role, the presence of a phenylalanine at this position in the phylogenetically distant ZmEDS and ZzES, which share less than 35% amino acid sequence identity, is consistent with the importance of this residue for the protonation of hedycarol catalyzed by both of these TPSs. While the relevant catalytic acid seems likely to be formed, at least in part, by a tyrosine residue (Y529 of ZmEDS) previously identified in a number of sesquiterpene synthases that similarly protonate a transient neutral, albeit olefin rather than hydroxylated, intermediate (Kollner *et al.*, 2008b; Rising *et al.*, 2000), the analogous substitution of phenylalanine had a less significant effect in ZmEDS than these other sesquiterpene synthases.

Given the importance of having two or more oxy groups for the bioactivity of terpenoid natural products (Zi *et al.*, 2014), recognition of the ability of ZmEDS and, by extension TPSs more generally, to produce diols not only enhances our understanding of terpenoid biosynthesis, but also potential for enzymatic engineering. This likely extends beyond the

production of various eudesmol derived diols shown here, as similar protonation of a transient neutral (olefin) intermediate has been shown for production of the structurally distinct (*S*)-β-macrocarpene (Kollner *et al.*, 2008b). Moreover, it can be hypothesized that similar protonation of transient neutral intermediates might be carried out by TPSs that act on longer isoprenyl precursors as well. In each case, it is possible that both the transient intermediate and final product can be generated with addition of water prior to deprotonation, which would lead to diols, as shown here for ZmEDS. It will be of significant interest to determine if such combined indirect (cyclo)hydrolytic and (cyclo)hydratase activity can, in fact, be found in other TPSs. In addition, further study of ZmEDS to more fully elucidate the structure-function relationship underlying its ability to catalyze this diol generating reaction seems warranted, as it may then lead to the ability to engineer such activity in other TPSs as well.

Experimental procedures

Gene cloning, construction and site-directed mutagenesis

Seeds of the maize inbred lines Mo17 were germinated and grown on sterile water-agar medium. 10-days-old maize seedlings were utilized for total RNA extraction. cDNA was synthesized with M-MLV reverse transcriptase (Takara). The *ZmEDS* open reading frame was cloned from this cDNA based on the sequence from Phytozome (GRMZM2G010356), via PCR using the high fidelity PrimeSTAR (Takara) polymerase with the following primers: forward, 5'-ATGGCCCCGAGTAACATCGTC-3'; reverse, 5'-CTAGAGAGGGAGCACTTGCTTGAGG-3'. The amplified fragment was ligated into the pGM-T vector (Tiangen, Beijing) for sequencing. Alignment of the *ZmEDS* nucleotide sequence obtained here with that from Phytozome revealed a number of differences, which lead to five differences in amino acid sequence as shown in Supplemental Figures S7 and S8. The obtained *ZmEDS* was subcloned into the pET28a vector via restriction digestion with *Nde*I and *Eco*RI to construct pET28/*ZmEDS* for recombinant expression. The previously identified β-eudesmol synthase gene (Yu *et al.*, 2008), *ZzES* (UniProtKB ID: B1B1U4), was

synthesized with codon optimization for *E. coli* codon preference (GenScript) and similarly cloned into pET28a for recombinant expression. Site-directed mutagenesis was carried out using the Strategene Quikchange kit following the manufacturer's instructions. The mutants were confirmed by sequencing and the verified constructs directly used for recombinant expression.

Terpenoid production by microbial metabolic engineering

To determine product profile, pET28/*ZmEDS* was co-transformed into *E. coli* BL21 competent cells with pACYC/*IspA* harboring the endogenous FPP synthase gene for recombinant expression as previously described (Cyr *et al.*, 2007; Mao *et al.*, 2016). The resulting recombinant colonies were picked and grown in 5 mL NZY liquid media shaken (200 rpm) at 37 °C overnight. The starter culture was transferred into 50 mL NZY media and grown until $OD_{600} \approx 0.8$ under the same conditions. The culture was then induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), and the incubator/shaker temperature was shifted to 16 °C with continued shaking at 200 rpm overnight. Enzymatic products were extracted directly from the culture with an equal volume of hexanes, twice. The pooled organic extract was dried via rotary evaporation and the residue was dissolved in 0.5 mL hexanes for GC-MS analysis. ZzES was used to produce β-eudesmol as described above.

GC-MS analysis

GC-MS analysis was performed as before (Mao *et al.*, 2016). Briefly, samples were run over an HP-5 GC column on an Agilent 6890-5973 GC, which was coupled to a quadrupole mass spectrometer in EI mode. The carrier gas was He at 1 mL/min flow rate. 1 μL samples were injected in the splitless mode. The temperature program is as follows: 70 °C for 2 min, then increased to 250 °C at 10°C/min, where it was held for 2 min. β-eudesmol was identified by comparison to the previously reported ZzES product (Yu *et al.*, 2008). Other minor products were tentatively identified by using their mass spectra in searches of the NIST MS library.

Thermal rearrangement of hedycarol to elmol was verified by reducing the temperature of GC injection port from 250 to 150 °C, which led to almost complete disappearance of the elemol peak, with concurrent appearance of a new broad peak that has the same mass spectrum reported for hedycarol (Figure S4A). This matches a previous report (Jones and Sutherland, 1968), and findings from a more recently reported hedycaryol synthase (Hattan *et al.*, 2016). In addition, acidic treatment of purified hedycarol led to formation of three eudesmol isomers (Figure S4B), consistent with previous investigation (Jones and Sutherland, 1968). This was carried out as described before (Minnaard *et al.*, 1997). Specifically, hedycarol was dissolved in CH_2Cl_2 and stirred with adding small amount of $TsOH•H_2O$. After stirring at room temperature for 10 min, saturated $NaHCO_3$ solution was added to neutralize the acid. The organic phase was collected and concentrated for GC-MS analysis.

Terpenoid isolation

To obtain sufficient amounts of products for structural analysis, a previously described pMevT-MBIS vector harboring the MVA pathway genes and FPP synthase gene (Martin *et al.*, 2003), was co-transformed with pET28/*ZmEDS* to increase metabolic flux to sesquiterpenoids. 6 L of the resulting recombinant culture was grown and extracted as described above. The hexanes extract was concentrated and loaded on a silica gel column, which was washed with additional hexanes and then eluted with mixtures of hexanes with EtoAc. Eudesmane-2,11-diol was eluted with a 5:1 (v/v) mixture of hexanes with EtoAc. The other two minor diol products were eluted together, first by a 1:1 (v/v) mixture of hexanes with EtoAc, and then individually isolated via a second round of silica gel column chromatography using a 2:1 (v/v) mixture of CH_2Cl_2 and EtoAc for elution. 2-*epi*-α-eudesmol also was isolated by two rounds of silica gel column chromatography using mixed solvents for elution, first a 15:1 (v/v) mixture of hexanes with EtoAc, and then a 1:1 (v/v) mixture of hexanes with CH_2Cl_2. Hedycaryol was isolated from cultures co-transformed with

pMevT-MBIS and pET28/*ZmEDS:F303A* by silica gel column chromatography with elution via a 15:1 (v/v) mixture of hexanes and EtoAc.

NMR analysis

The purified terpenoid products were dissolved in 0.5 mL $CDCl_3$ and transferred into a 2.5 mm × 100 mm NMR tube for NMR analysis, which was carried out on a Bruker Avance 600 MHz instrument with ^{1}H and ^{13}C spectroscopic analysis at 22 °C using a 5-mm TXI CryoProbe. The NMR shift data for the purified sesquiterpenoids (Tables S1 and 2) were compared with the NMR data in the literatures (Mathela *et al.*, 1989; Su *et al.*, 1995; Tebbaa *et al.*, 2011), which led to identification of the major sesquiterpenoid diol as eudesmane-2α,11-diol (Mathela *et al.*, 1989). One of the minor diols was identified as 3-*epi*-cryptomeridiol based on detailed spectroscopic analyses and in comparison with reported data (Cornwell *et al.*, 2000). The other minor diol had similar ^{13}C NMR data as 3-*epi*-cryptomeridiol, with the exception of the chemical shifts for C-10 and C-15 (Table S2), suggesting that this was a stereoisomer of 3-*epi*-cryptomeridiol. This was confirmed by the 2D NMR data, especially the heteronuclear multiple bond correlations (HMBC), which led to its identification as 2,3-*epi*-cryptomeridiol, as previously reported (Ando *et al.*, 1994). Similarly, the major sesquiterpenoid alcohol also was investigated by NMR, and its ^{13}C NMR data was similar to that reported for α-eudesmol (Tebbaa *et al.*, 2011), with the exception of the chemical shifts for C-8 and C-14 (Table S2). From the analogous shifts observed between 3-*epi*-cryptomeridiol and 2,3-*epi*-cryptomeridiol [as also previously reported (Ando *et al.*, 1994)], the differences in chemical shift data observed with this compound implied an αH-2. Thus, this was assigned to be 2-epi-α-eudesmol, with a systematic name of (2R,4aR,8aS)-1,2,3,4,4a,5,6,8a-octahydro-α,α,4a,8-tetramethyl-2-naphthalenemethanol.

In vitro assays

For enzyme isolation, pET28/*ZmEDS* was transformed alone into *E. coli* BL21 competent cells and initially grown as above. However, protein expression was induced by adding 1 mM IPTG at $OD_{600} \approx 0.6$. The culture was allowed to grow overnight at 16 °C, with shaking at 200 rpm, and the cells harvested by centrifugation. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.9], 500 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 0.1% [v/v] proteinase-inhibitor) and sonicated briefly. The cell lysates were centrifuged at 10,000 × *g* to remove the cell debris and ZmEDS recombinant protein was purified from the supernatant using a Ni-NTA column (CWbiotech, Beijing, China) following the manufacturer's instructions. The purified protein was loaded on an ultrafiltration column (Merck Millipore) for desalting and buffer substitution with the assay buffer (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 10 mM $MgCl_2$, 5 mM DTT, 10% glycerol). In vitro reactions were carried out in 300 μL assays containing 10 μg purified enzyme and 23 μM FPP (Sigma). Assays were kept at 30 °C for 1 h and then extracted with 1 mL hexanes. This organic extract was concentrated to 100 μL under a gentle N_2 flow for GC-MS analysis. Hedycaryol was tested as a potential substrate in assays using the same condition described above. Labeling studies were carried out using 0.6 mL assays composed of 540 μL D_2O, 60 μL 10× assay buffer, with addition of ZmEDS and FPP as above. These assays were extracted with 10% methanol (in hexane) to provide a source of unlabeled protons for exchange with the hydroxyl groups on the enzymatic products – i.e., such as eudesmane-2,11-diol.

Homology modeling

Homology modeling of ZmEDS was completed on the SWISS-MODEL server (www.swissmodel.expasy.org). 5-*epi*-aristolochene synthase from *Nicotiana tabacum* (PDB ID: 5EAS) was chosen as the template. The docking of ZmEDS and FPP was carried out using Autodock (autodock.scripps.edu), with a 3D structure of FPP generated by Chem3D. The results of this docking run were visualized with PyMOL.

Bioinformatics analysis

Sequence alignment of ZmEDS and other plant sesquiterpene synthases was carried out using CLC Sequence Viewer 7.0. These sesquiterpene synthases are listed as follows: GaXC14 (*Gossypium arboretum*, (+)-δ-cadinene synthase, Q39760.1), GhTPS1 (*Gossypium hirsutum*, (-)-germacrene D synthase, NP_001314061.1), PgSTS (*Panax ginseng*, sesquiterpene synthase, AGS16741.1), VvVAS (*Vitis vinifera*, Valencene synthase, Q6Q3H2.1), SlGCS (*Solanum lycopersicum*, germacrene C synthase, AAC39432.1), CsTPS1 (*Citrus sinensis*, valencene synthase, AAQ04608.1), ScGAS (*Solidago canadensis*, germacrene A synthase, CAC36896.1), MrBBS (*Matricaria chamomilla* var. *recutita*, (-)-α-bisabolol synthase, AIG92848.1), AaBOS (*Artemisia annua*, α-bisabolol synthase, AFV40969.1), AaADS (*Artemisia annua*, amorpha -4,11-diene synthase, Q9AR04.2), Mg25 (*Magnolia grandiflora*, β-cubebene synthase, B3TPQ6.1), PatTpsBF2 (*Pogostemon cablin*, Patchoulol synthase, Q49SP3.1), Nt5EAS (*Nicotiana Tabacum*, 5-epi-aristolochene synthase, 5EAS_A), StVS (*Solanum tuberosum*, vetispiradiene synthase, NP_001275171.1), CsAFS (*Cucumis sativus*, α-farnesene synthase, NP_001267674.1), As-SesTPS1 (*Aquilaria sinensis*, sesquiterpene synthase, AIT75876.1), ZmTPS4 (*Zea mays*, NP_001292867.2), ZmTPS5 (*Zea mays*, Q6JD70.1), ZmTPS6/11 (*Zea mays*, (S)-β-macrocarpene synthase, NP_001105950.1), ZmTPS10 (*Zea mays*, (E)-β-farnesene synthase, NP_001105850.2), ZzES (*Zingiber zerumbet*, β-eudesmol synthase, B1B1U4.1), ZSS1 (*Zingiber zerumbet*, α-humulene synthase, B1B1U3.1), AtTPS21 (*Arabidopsis thaliana*, NP_197784.2), MxpSS2 (*Mentha piperita*, β-farnesene synthase, O48935.1), MxpSS1 (*Mentha piperita*, cis-muuroladiene synthase, Q5W283.1), LaCADS (*Lavandula angustifolia*, cadinol synthase, AGL98418.1), PatTpsA (*Pogostemon cablin*, γ-curcumene synthase, Q49SP7.1), AaGAS (*Artemisia annua*, germacrene A, ABE03980.1)

Transient overexpression of ZmEDS in *N. benthamiana*

Transient overexpression in *N. benthamiana* was carried out as previously described (Fu *et al.*, 2016). Specifically, *ZmEDS* was subcloned into pCAMBIA3301, placing it under control of the 35S promoter, and then transformed into *Agrobacterium tumefaciens* GV3301. The Agrobacterium line harboring pCAMBIA3301/*ZmEDS* was infiltrated into *N. benthamiana* leaves. An *A. tumefaciens* line carrying P19 was co-infiltrated to suppress gene silencing. Tobacco leaves were harvested on the 5^{th} days post infiltration and grounded in liquid N_2 to a fine powder for product extraction by hexanes. The resulting organic extract was concentrated by rotary evaporation and analyzed by GC-MS. Deglycosylation was performed as reported (Morikawa *et al.*, 2011). Specifically, the transgenic tobacco leaves were grounded in liquid N_2 as the fine powder and extracted with 95% methanol. The extract was evaporated to remove the organic phase and dissolved in 5% H_2SO_4 for heating under reflux at 80 °C for 1 h. After cooling, the reaction mixture was extracted with equal volumes of EtOAc. The organic phase was concentrated by rotary evaporation and dissolved in hexanes for GC-MS analysis.

Gene expression analysis and compound detection

Seeds of maize inbred lines Mo17 and Huangzao4 (HZ4) were germinated and grown in sterile water agar medium. Ten-day-old maize seedlings were used for RNA and compound extraction. Roots and aerial parts were collected separately for RNA and compound extraction. Hydroponic growth was conducted in a similar fashion with 40% Hoagland solution as the medium. Pathogen infection of maize roots was carried out as previously described (Vaughan *et al.*, 2015). Specifically, Mo17 seedlings were grown aeroponically, using pearlite as the supporting medium, and the roots of ten-day old seedlings were dipped in a solution of *F. verticillioides* spores (1×10^6 mL^{-1}) for 30 s. Roots were collected from the infected seedlings after 0 (CK), 12, 24 and 48 h for RNA extraction. Total RNA was isolated with the TRNzol reagent (Tiangen, Beijing), with cDNA synthesis carried out with M-MLV reverse transcriptase (Takara). Quantitative RT-PCR was performed on a CFX96 (Bio-Rad)

using the SsoFast Eva Green Supermix (Bio-Rad). *Ef1a* was used as the endogenous control. The primers of *ZmEDS* for qRT-PCR were designed by primer 3.0 as followed: forward, 5'-CTAAACAATACTCTGATCACAC-3', reverse, 5'-CCATCAATATGATAGAAACAGG-3'. For compound detection roots were ground to a fine powder in liquid N_2 and twice extracted with of hexanes (50 mL/g root fresh weight). In addition, the water agar or hydroponic media were extracted with an equal volume of hexanes. These organic extracts were dried by rotary evaporation and the residues resuspended in fresh hexanes for GC-MS analysis. Purified eudesmane-2,11-diol was used to develop the external standard cure for quantification analysis to estimate sesquiterpenoid diol accumulation.

Anti-fungal activity assay

The anti-fungal activity of eudesmane-2,11-diol was tested as described previously (Schmelz *et al.*, 2011). *F. verticillioides* spores (2×10^4 mL^{-1}) were added in the 96-well microtiter plate with 200 μL broth medium. Eudesmane-2,11-diol was dissolved in DMSO and added into the medium with the final concentration of 0, 50 or 100 μg mL^{-1}. The plate was kept at 28 °C for fungal growth and OD_{600} was monitored for 48 h with Tecan/M200Pro reader.

Author contributions

Q.W. conceived the project. J.L., J.L., R.B., M.J., and K.Z. performed the experiments and collected data. Q.W., J.L., J.L., and R.J.P. analyzed the data. Q.W. and R.J.P. wrote the paper.

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Short Supporting Information Legends

Figure S1. GC-MS analysis of ZmEDS products.

Figure S2. Mass spectra of eudesmane-2,11-diol produced from in vitro labeling assays and MS fragmentation analysis.

Figure S3. Alignment of ZmEDS with other sesquiterpene synthases.

Figure S4. Identification of hedycaryol.

Figure S5. Root specific expression of ZmEDS in HZ4 inbred line.

Figure S6. No antifungal activity was observed for eudesmane-2,11-diol.

Figure S7. Nucleotide sequence alignment between *ZmEDS* and GRMZM2G010356 from B73.

Figure S8. Amino acid sequence alignment between ZmEDS and GRMZM2G010356 from B73.

Table S1. ^{1}H and ^{13}C NMR chemical shift data of eudesmane-2,11-diol.

Table S2. ^{13}C NMR data of two minor diols and the major sesquiterpenoid alcohol.

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Figure Legends

Figure 1. GC-MS analysis of ZmEDS enzymatic products.

(A) Total ion GC chromatograms of ZmEDS products through recombinant co-expression with FPP synthase in *E.coli* or *in vitro* assay. All products were labeled consistently throughout the whole paper. (B) Mass spectrum of the predominant product (peak 1). Mass spectra of other products were listed in the Figure S1. (C) Chemical structures of three sequiterpenoid diols (peak 1, 4 and 5) and the major sesquiterpenoid alcohol (peak 2).

Figure 2. Plausible catalytic mechanism of ZmEDS.

Figure 3. Homology modeling of ZmEDS and site-directed mutagenesis analysis.

(A) Catalytic cavity of ZmEDS based on the predication of homology modeling. Key aspartates from the DDxxD motif that define the active site are shown in blue, with the unique F303 in red and the conserved Y529 in yellow. Bound (*E*,*E*)-FPP is shown with the carbon skeleton (green) and pyrophosphate group (red). (B) Total ion GC chromatograms of

ZmEDS:F303A and Y529F products. The peak of elemol (peak 6) transformed thermally from hedycaryol is labeled. The minor peaks were labeled as that in Figure1. Unlabeled peaks appear to be contaminating compounds.

Figure 4. Transient overexpression of ZmEDS in *N. benthamiana*.

Extracted ion chromatograms (EIC) of ZmEDS enzymatic products from transient overexpression in *N. benthamiana* leaves. EV indicates the empty vector control. The predominant ZmEDS product, eudesmane-2,11-diol is indicated by the arrow.

Figure 5. Root specific and inducible gene expression of *ZmEDS*.

(A-B) qRT-PCR analysis of *ZmEDS* gene expression in Mo17 growing in aeroponic (A) or hydroponic culture (B). R, roots; S, aerial parts. (C) gene expression of *ZmEDS* in response to *F. verticillioide* (F.v.) infection in Mo17 roots. *Ef1α* was used as the control gene. (D) Extracted ion chromatograms (EIC) of Mo17 root exudates from hydroponic culture or roots (aeroponic culture) inoculated with *F. verticillioide* spores for 12 h by GC-MS analysis. ZmEDS major product eudesmane-2,11-diol is indicated by the arrow.