Characterization of CYP71Z18 indicates a role in maize zealexin biosynthesis

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Abstract
Maize (Zea mays) produces zealexins as phytoalexins, with the inducible production of these antibiotics providing biochemical protection against fungal infection. However, the biosynthesis of these sesquiterpenoids has remained unclear. In particular, it is unclear how the olefinic precursor, (S)-β-macrocarpene produced by the characterized maize sesquiterpene synthases TPS6/11, is further elaborated to form the bioactive zealexins. The first step is likely to be conversion of carbon-15 (C15) from a methyl group to a carboxylic acid by a cytochrome P450 mono-oxygenase (CYP). In this study, CYP71Z18, whose transcription is strongly induced by fungal infection, was found to catalyze oxidation of C15 in (S)-β-macrocarpene, forming zealexin A1. The inducible transcription of CYP71Z18 matches that observed for TPS6/11 and the accumulation of zealexins, which is consistent with a role for CYP71Z18 in sesquiterpenoid phytoalexin production. This completes identification of zealexin A1 biosynthesis, and represents the initial CYP identified for the production of maize terpenoid phytoalexins.

Keywords
Maize, Zea mays, Poaceae, Biosynthesis, Terpenoid, Phytoalexin, P450, Zealexin

Disciplines
Molecular Biology | Plant Biology | Plant Breeding and Genetics

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Characterization of CYP71Z18 indicates a role in maize zealexin biosynthesis

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Zealexin A1 production from β-macrocarpene by CYP71Z18 elucidates biosynthesis of this maize sesquiterpenoid phytoalexin
Highlights:

- Characterization of a maize cytochrome P450 monoxygenase, CYP71Z18
- CYP71Z18 catalyzes transformation of a methyl group (C15) in (S)-β-macrocarpene to a carboxylic acid, forming the phytoalexin zealexin A1
- Zealexin A1 production by CYP71Z18 represents the initial step in biosynthesis to more elaborated zealexin sesquiterpenoids
Characterization of CYP71Z18 indicates a role in maize zealexin biosynthesis

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ABSTRACT

Maize (*Zea mays*) produces zealexins as phytoalexins, with the inducible production of these antibiotics providing biochemical protection against fungal infection. However, the biosynthesis of these sesquiterpenoids has remained unclear. In particular, it is unclear how the olefinic precursor, (S)-β-macrocarpene produced by the characterized maize sesquiterpene synthases TPS6/11, is further elaborated to form the bioactive zealexins. The first step is likely to be conversion of carbon-15 (C15) from a methyl group to a carboxylic acid by a cytochrome P450 mono-oxygenase (CYP). In this study, CYP71Z18, whose transcription is strongly induced by fungal infection, was found to catalyze oxidation of C15 in (S)-β-macrocarpene, forming zealexin A1. The inducible transcription of *CYP71Z18* matches that observed for *TPS6/11* and the accumulation of zealexins, which is consistent with a role for CYP71Z18 in sesquiterpenoid phytoalexin production. This completes identification of zealexin A1 biosynthesis, and represents the initial CYP identified for the production of maize terpenoid phytoalexins.

**Keywords:** Maize, *Zea mays*, Poaceae, Biosynthesis, Terpenoid, Phytoalexin, P450, Zealexin
1. Introduction

Maize (Zea mays) is the most widely cultivated crop in the world, and is grown in particularly large quantities in the USA and China (FAOSTAT, 2014). As with any crop plant, biotic and abiotic stresses reduce yield and quality. The production of antibiotic natural products in response to infection provides phytoalexins that offer biochemical protection against diseases (VanEtten et al., 1994). This phenomenon has been widely explored in cereal crops, and largely consists of terpenoids (Schmelz et al., 2014). Antifungal terpenoids from rice (Oryza sativa) were identified in early studies (Cartwright et al., 1977), and rice has become a model system for study of such phytoalexin biosynthesis (Schmelz et al., 2014). There was a similarly early report of the production of various diterpenes by maize following infection with a variety of fungi (Mellon and West, 1979). However, these olefins do not exhibit antifungal activity, and the first terpenoid phytoalexins from maize were only recently identified, consisting of the diterpenoid kauralexins (Schmelz et al., 2011), and sesquiterpenoid zealexins (Huffaker et al., 2011).

Rice produces more than twenty diterpenoid phytoalexins, and the relevant terpene synthases (TPSs) have been identified by biochemical characterization of recombinantly expressed enzymes (Peters, 2006). In addition, functional clustering of the genes encoding these diterpene synthases (Prisic et al., 2004; Wilderman et al., 2004), along with similarly inducible genes for cytochromes P450 (CYPs)(Shimura et al., 2007; Swaminathan et al., 2009), has led to biochemical studies that provide evidence of roles for these mono-oxygenases in rice diterpenoid biosynthesis as well
(Kitaoka et al., 2015a). CYPs are grouped by homology into related families designated by numbering, with even more closely related sub-families designated by lettering (Nelson and Werck-Reichhart, 2011), and specific CYP sub-families have been associated with rice diterpenoid metabolism – namely CYP76M, CYP99A, CYP701A and CYP71Z (Swaminathan et al., 2009; Wang et al., 2012a; Wang et al., 2011; Wang et al., 2012b; Wu et al., 2011; Wu et al., 2013).

In maize, the diterpenes whose production is induced by fungal infection are uniformly derived from *ent*-copalyl diphosphate (*ent*-CPP), and include *ent*-kaurene and *ent*-isokaurene (Mellon and West, 1979), the precursors to kauralexins (Schmelz et al., 2011). It was later found that transcription of a gene encoding an *ent*-CPP synthase, An2, was induced by fungal infection (Harris et al., 2005). The role of An2 in kauralexin biosynthesis has recently been demonstrated by phytochemical analysis of an *an2* knock-out line (Vaughan et al., 2015). Similarly, the transcription of genes encoding ZmTPS6 and ZmTPS11, which convert (*E*, *E*)-farnesyl diphosphate (1) (see Figure 1) into (*S*)-β-macrocarpene (2) (Kollner et al., 2008), the sesquiterpene precursor of zealexins, also is induced by fungal infection (Huffaker et al., 2011), and a role for these in maize defense against fungi has been reported (van der Linde et al., 2011). While plausible pathways can be envisioned for both kauralexins and zealexins (Fig. 1), their biosynthesis requires the insertion of oxygen, presumably catalyzed by CYPs, but no CYPs involved in maize terpenoid phytoalexin biosynthesis have been identified to date.

While the maize genome sequence has been reported (Schnable et al., 2009), neither
An2, nor ZmTPS6/11, seem to be clustered with genes encoding CYPs (Boutanaev et al., 2015). Fortuitously, microarray analysis of the maize transcriptional response to infection with the fungal pathogens Ustilago maydis and Fusarium graminearum has been previously reported (Doehlemann et al., 2008; Huffaker et al., 2011). Thus, it was possible to use such transcriptional induction, along with homology to the CYP71Z6 and CYP71Z7 that act in rice (di)terpenoid phytoalexin biosynthesis (Fig. S1) (Kitaoka et al., 2015b; Wu et al., 2011), to pick out CYP71Z18 from the >350 CYPs in maize. The data presented here indicates that CYP71Z18 plays a role in zealexin biosynthesis, catalyzing multiple reactions to transform carbon-15 (C15) in (S)-β-macrocarpene (2) from a methyl to the characteristic carboxylic acid of the bioactive phytoalexin zealexins, directly forming zealexin A1 (3).
2. Results and discussion

2.1 Identification of CYP71Z18

To find candidate CYP genes for maize terpenoid phytoalexin biosynthesis, previously reported microarray analysis of the maize transcriptional response to infection by *F. graminearum* was searched (Huffaker et al., 2011). Seven CYP genes were found in this microarray data and six of them exhibited some increase in transcript level (Table S1). Particularly given that CYP71 family members have been shown to operate in rice (di)terpenoid phytoalexin biosynthesis (Kitaoka et al., 2015b; Wu et al., 2011), of particular interest was a CYP71 family member that exhibited the strongest induction (>200-fold). Moreover, phylogenetic analysis of the 46 CYP71 family members from maize indicated that this particular CYP falls into a sub-clade, along with two others, that is closely related to the CYP71Z6 and CYP71Z7 involved in rice (di)terpenoid phytoalexin biosynthesis (Fig S2). Indeed, this highly induced CYP was assigned to be CYP71Z18. The *CYP71Z18* gene corresponds to Genbank accession NM_001147894, and is located on the unmapped maize genomic scaffold_109. A cDNA encoding the full-length open reading frame was successfully cloned from infected maize tissue.

2.2 Oxidation of (S)-β-macrocarpene (2) by CYP71Z18

Based on previous experience (Kitaoka et al., 2015a), the N-terminal transmembrane helix sequence of CYP71Z18 was replaced with a leader peptide. The resulting modified construct was then incorporated into a previously reported modular metabolic
engineering system (Cyr et al., 2007). Thus, CYP71Z18 was co-expressed with a
cytochrome P450 reductase (CPR), as required for CYP activity, in a strain of *E. coli*
that also was engineered to produce (S)-β-macrocarpene (2) using ZmTPS11. Extracts
of the resulting culture contained three major oxidation products (Fig. 2), which
correspond to formation of an alcohol (12) (MW = 220 Da), further oxidation to an
aldehyde (13) (MW = 218 Da), and then the corresponding carboxylic acid (3)
(observed as the methyl ester 3’, MW = 248 Da, with methylation carried out to enable
analysis of such carboxylates by GC-MS). To test the substrate specificity of
CYP71Z18, a γ-bisabolene synthase in Arabidopsis (AtTPS12) and *ent-*cassadiene
synthase in rice (OsKSL7) (Ro et al., 2006; Xu et al., 2007), were (separately)
incorporated into the metabolic engineering system as above; however, no oxidative
products were observed (Fig. S3).

2.3 CYP71Z18 produces Zealexin A1 (3) from β-macrocarpene (2)

To identify the chemical structure of these oxidative products, the pMBIS plasmid
that increases metabolic flux to sesquiterpenoids (Martin et al., 2003), was incorporated
as previously described (Morrone et al., 2010b). The resulting recombinant *E. coli* also
was cultured in larger amounts (6 L altogether). The carboxylic acid containing
sesquiterpenoid was targeted for purification, but after extraction and separation via
silica gel column chromatography this product was observed to co-elute with fatty
acids. Based on the methods used in the original purification of zealexins (Huffaker et
al., 2011), methylation was carried out to enable further purification. Thus, the final
purified compound was the methyl ester (3’) of the carboxylate (3) product, which was subjected to NMR analysis. $^{13}$C and $^1$H chemical shift values were found to match those previously reported for the methyl ester of (S)-β-macrocarpen-15-oic acid (Huffaker et al., 2011), corresponding to zealexin A1 (3) (Table 1). The observed mass spectra also matches that reported for zealexin A1 (3). Given the reasonable assumption that the alcohol (12) and aldehyde (13) containing products are intermediates en route to carboxylic acid (3), as previously observed in the production of such terpenoids by CYPs (Morrone et al., 2010a; Wang et al., 2011), it seems that CYP71Z18 sequentially oxidizes (S)-β-macrocarpene (2) via alcohol (12) and aldehyde (13) intermediates to form zealexin A1 (3) (Fig. 2F). The alcohol and aldehyde products are then suggested to be (S)-β-macrocarpen-15-ol (12) and (S)-β-macrocarpen-15-al (13). Given that C15 is present as a carboxylic acid in all zealexins (Huffaker et al., 2011), this transformation should be an early step in zealexin biosynthesis (Fig. 1), consistent with the ability of CYP71Z18 to act directly on (S)-β-macrocarpene (2). Thus, CYP71Z18 is likely to play a role in maize zealexins biosynthesis more generally.

2.4 Enzymatic characterization of CYP71Z18

To further characterize the oxidation activity of CYP71Z18 against (S)-β-macrocarpene (2), in vitro assays were carried out using cell-free extracts from recombinant E. coli co-expressing CYP71Z18 with CPR. Although CO-binding difference spectra were not measured, in vitro activity was detected for CYP71Z18 – i.e., oxidation of (S)-β-macrocarpene (2) to zealexin A1 (3), albeit without detection of
intermediates 12 or 13 (Fig. S4), which is consistent with previous characterization of similarly multiply-reactive, carboxylic acid producing CYPs (Morrone et al., 2010a; Wang et al., 2011). Steady state kinetic characterization established that CYP71Z18 exhibited reasonable affinity for (S)-β-macrocarpene (2) \( (K_M \approx 8 \pm 2 \ \mu M, \text{ with } V_{max} \approx 0.02 \pm 0.001 \ \mu \text{moles product/mg protein/min}).

2.5 Constitutive and inducible gene expression of CYP71Z18

Zealexins not only accumulate following infection, but also are ubiquitously distributed in young seedlings (Huffaker et al., 2011; Vaughan et al., 2015; Vaughan et al., 2014). To examine the physiological relevance of CYP71Z18 to zealexin production, its gene expression pattern was analyzed. Soil grown maize seedlings were reported to constitutively produce zealexins, albeit with high variation proposed to result from unintentional elicitation by seed- and/or soil-derived fungi (Huffaker et al., 2011). To avoid any such elicitation and resulting variation, seedlings grown from surface sterilized seeds on sterile media were used for gene expression analysis in this study. CYP71Z18 was constitutively expressed throughout these seedlings, particularly in the root, and also exhibited inducible expression in leaves within 24 h of infection with *F. graminearum* (Fig. 3A), with quantitative RT-PCR analysis indicating steadily increasing accumulation for at least 48 h after inoculation (Fig. 3B). The ZmTPS6/11 responsible for (S)-β-macrocarpene (2) formation similarly exhibited inducible expression within 12 h after infection with *F. graminearum* (Fig. 3C), as previously reported (Huffaker et al., 2011; Kollner et al., 2008). Although transcripts of both
CYP71Z18 and TPS6/11 accumulate in response to fungal infection, those for CYP71Z18 continue to increase for at least 48 h, while those for TPS6/11 peak after 24 h. Given that zealexins have been shown to accumulate for up to 8 days following infection (Huffaker et al., 2011), and the proposed sequential action of TPS6/11 and then CYP71Z18 (Fig. 1), such lagging expression patterns are nevertheless consistent with roles in zealexin biosynthesis.

2.6 CYP71Z18 also reacts with a minor TPS11 product

CYPs involved in terpenoid biosynthesis exhibit a range of substrate specificity, as observed in rice, where CYP76M8 reacts with a broad range of diterpenes, while other CYP76M sub-family members are more specific (Swaminathan et al., 2009; Wang et al., 2012a). The CYP71Z6 and CYP71Z7 rice homologs of CYP71Z18 seem to be quite specific (Wu et al., 2013), although also multiply-reactive, able to act at different positions and carry out multiple oxidations at the same position (Kitaoka et al., 2015b), with this latter aspect of multiple reactivity evident with CYP71Z18 as well. Although CYP71Z18 does not react with the distinct sesquiterpene γ-bisabolene, nor the diterpene ent-cassadiene (Fig. S3), in addition to its predominant (S)-β-macrocarpene (2) product, ZmTPS11 produces minor amounts of a number of other sesquiterpenes, two of which have been previously identified as β-farnesene and (S)-β-bisabolene (Kollner et al., 2008), with nine others also observed here (Fig. S5). CYP71Z18 further appears to react with at least one of these minor products, as another series of oxidation products (putative alcohol, aldehyde and carboxylic acid) were observed here as well.
(Fig. S6), albeit in amounts so small as to preclude structural analysis. Nevertheless, CYP71Z18 does exhibit some substrate promiscuity. Moreover, the mass spectra observed for the minor carboxylic acid apparently product matches that for an unidentified acidic sesquiterpenoid (analyte 3) previously observed in maize (Huffaker et al., 2011), suggesting potential relevance for the (limited) CYP71Z18 promiscuity observed here.
3. Conclusions

In the present study, the biochemical characterization and inducible gene expression obtained provide strong evidence of a key role for CYP71Z18 in zealexin biosynthesis. Clearly, CYP71Z18 can react with \((S)-\beta\)-macrocarpene (2) to form the bioactive phytoalexin, zealexin A1 (3), which further seems to represent the initial CYP mediated step in zealexin biosynthesis. Moreover, although not proven, CYP71Z18 also may be involved in the production of other maize sesquiterpenes. Notably, CYP71Z18 appears to be the first CYP identified in maize terpenoid phytoalexin biosynthesis, representing a significant step towards the elucidation of such metabolism in this critically important cereal crop plant. Other CYPs also exhibited inducible expression with infection of *F. graminearum* (Table S1), although to a lesser extent than *CYP71Z18*. Nevertheless, these might be involved in further elaboration of zealexin A1 to form further elaborated zealexins or in kauralexin biosynthesis (Fig. 1), which will be the subject of future studies.
4. Experimental

4.1 General procedure

All chemicals used were analytical grade, except hexanes and EtOAc, which were HPLC grade. GC-MS analysis was performed using an HP-5 GC column (30 m × 0.32 mm × 0.25 μm) on an Agilent 6890-5973 instrument with quadrupole mass spectrometer in positive EI mode. The carrier gas was He at rate of 1 mL/min. Samples (1 μL) were injected in splitless mode at 70 °C, with the oven temperature held at 70 °C for 2 min, then raised to 300 °C at a rate of 10 °C/min, which was held for 2 min. MS data was collected from m/z of 50 to 600 from a retention time of 10 min to the end of the run.

4.2 Plant and fungal materials

Seeds of maize inbred line Mo17 were sterilized by immersion in 20% NaOCl solution for 20 min, and then germinated on sterilized H2O-agar medium. After 7 days, the sterile seedlings were used for fungal infection and RNA extraction. *F. graminearum* was grown on the potato dextrose agar medium, and then transferred to spore-production medium (sodium carboxymethyl cellulose 7.5 g/L, potassium phosphate monobasic 0.5 g/L, yeast extract 0.5 g/L, NH₄NO₃ 0.5 g/L, MgSO₄ 0.25 g/L), with spores collected after 5 days. Infection was carried out with solutions of 10⁶ mL⁻¹ spores in 0.1% Tween 20 after cutting the leaves, as described (Huffaker et al., 2011).
4.3 Gene identification and bioinformatics analysis

Previously reported microarray data from maize inoculated with *F. graminearum* (Huffaker et al., 2011) was analyzed to find candidate CYP genes for maize terpenoid phytoalexin biosynthesis. All CYP genes from the microarray data were used in BLAST searches at the cytochrome P450 Homepage (drnelson.uthsc.edu/cytochromeP450.html) to assign these to the relevant CYP family. The CYP with highest fold change in response to *F. graminearum* infection was assigned to be CYP71Z18 by Dr. David Nelson (Univ. Tennessee Health Science Center), and used as the probe sequence in BLAST searches to identify CYP71 family members (i.e., those CYPs with $\geq 40\%$ amino acid identity) from the maize genome at Phytozome. Sequence alignment and construction of the phylogenetic tree were carried out using the CLC sequence viewer 7.0 software package (CLC bio).

4.4 Gene cloning and recombinant constructs

Plant tissue inoculated with *F. graminearum* spores for 24 h was used for RNA extraction, and cDNA was obtained using reverse transcriptase M-MLV (Takara) following the manufacturers protocol. The full-length cDNA for CYP71Z18 was amplified using high fidelity PrimeSTAR HS DNA polymerase (Takara), cloned into a pGM-T vector (Tiangen, Beijing), and confirmed by complete sequencing. N-terminal modification of CYP71Z18 was carried out as described before (Swaminathan et al., 2009), with the first 32 amino acids (aa) replaced by the 10 aa lysine-rich sequence MAKKTSSKGK. The modified gene was sub-cloned into the second multiple cloning
site (MCS) of a previously described pCDF-Duet vector (Novagen) that contains a CPR from *Arabidopsis thaliana* (AtCPR1) (Mizutani and Ohta, 1998; Urban et al., 1997) in the first MCS (Swaminathan et al., 2009). The (S)-β-macrocarpene (2) synthase ZmTPS11 was cloned using the same procedure described above for CYP71Z18, and sub-cloned into pET28a (Novagen) (Kollner et al., 2008). The FPP (1) synthase *IspA* was cloned from *E. coli*, using primers designed from the genome sequence available at NCBI, and cloned into the second MCS of the pACYC-Duet vector (Novagen) (Fujisaki et al., 1990). A γ-bisabolene synthase, AtTPS12 was cloned from *A. thaliana* (Col-0) and sub-cloned into pET28a as described before (Ro et al., 2006). Cloning of the rice *ent*-cassadiene synthase, OsKSL7, has been previously reported (Xu et al., 2007).

### 4.5 Recombinant expression

CYP71Z18 was expressed in the C41 (DE3) OverExpress strain of *E. coli* (Lucigen) using a similar metabolic engineering approach as previously described for analysis of rice CYPs (Kitaoka et al., 2015a). *IspA* and TPS11 were co-expressed to provide the FPP (1) precursor and (S)-β-macrocarpene (2) sesquiterpene substrate, along with AtCPR1 to provide the requisite reducing equivalents. Liquid cultures were grown in TB media, with the appropriate antibiotics, to an A_{600} of ~0.6, then shifted to 16 °C for 1 hr prior to induction with 1 mM IPTG. Terpenoid products were extracted from these cultures after 72 h, using an equal volume of hexanes. All samples were methylated with CH$_2$N$_2$ to reduce the vaporization temperature of carboxylated products and
enable their detection by GC-MS analysis.

4.6 Terpenoid production

To obtain sufficient amounts of enzymatic products for NMR analysis, the pMBIS plasmid was used to increase flux to towards FPP (1), with administration of 50 mM mevalonate. A total of 6 L cultures of this recombinant E. coli were grown, which were extracted with an equal volume of a 1:1 mixture of EtOAc and hexanes, with this organic extract separated and concentrated by rotary evaporation. The residue was resuspended in hexanes (5 mL) and separated using silica gel column chromatography (CC), with elution using a hexane- EtOAc mixture with 0.1% AcOH. Methylation was carried out using CH$_2$N$_2$ to enable further purification. Two additional rounds of fractionation via silica gel CC resulted in purification of the major carboxylic acid sesquiterpenoid product (3), as the methyl ester (3'), yielding ~5 mg (in over 95% purity as judged by GC-MS analysis) for structural characterization by NMR spectroscopy.

4.7 Zealexinn A1 (3) methyl ester

The purified sesquiterpenoid (3') was dissolved in CDCl$_3$ and transferred to a 2.5 mm × 100 mm NMR tube. NMR spectra was acquired at 22 °C using a 5-mm TXI CryoProbe and was measured on Bruker AVANCE 600 (1H at 600 MHz, 13C at 150 MHz) nuclear magnetic resonance spectrometer. The 1H and 13C chemical shift data for the purified sesquiterpenoid (Table 1) correspond to that previously reported for the
zealexin A1 (3) methyl ester (Huffaker et al., 2011), providing the basis for structural assignment.

4.8 Kinetic analysis

Kinetic analysis was carried out using CYP71Z18 expressed in E. coli strain C41 (DE3) with co-expression of AtCPR1 as above. The culture was centrifuged at 5000 × g and the cell pellet was resuspended in 100 mM Tris-HCl buffer (pH 7.5) with 20% glycerol and 1 mM DTT. After brief sonication, the lysate was centrifuged at 10,000 × g to pellet the cell debris, and the supernatant utilized for in vitro assays, with protein concentration determined by the Bradford method using a protein assay kit (Bio-Rad). Kinetic assays were performed in 1 mL volumes with 0.4 mM NADPH, 1 μM FAD and 1 μM FMN as before (Wang et al., 2011; Wu et al., 2011). Varying amounts of substrate, 0.4-100 μM (S)-β-macrocarpene (2), were added in DMSO (10 μL). The reaction was carried out at 25 °C for 30 min and stopped by adding 1 M HCL (5 μL). The enzymatic products were extracted from the reaction mixture using EtOAc (2 mL) and quantified on GC-MS with cembrene (10 ng) as an internal standard. To further verify quantification of the product, purified zealexin A1 (3) methyl ester was used to make a standard curve for comparison.

4.9 Gene expression analysis

Sterile seedlings of Mo17 lines were separated into root, leaf and scutella tissues for RNA extraction. Leaves inoculated with F. graminearum spores for 0, 12, 24 and 48
hours also were collected for RNA extraction. Semi-quantitative reverse transcriptional (RT)-PCR was carried out using gene-specific primers for CYP71Z18, with Actin as the endogenous control. PCR cycle number was tested to avoid over-amplification, with 25 cycles chosen for the analysis reported here. Quantitative RT-PCR was performed on a Bio-Rad CFX96 instrument using the SsoFast Eva Green Supermix (Bio-Rad). One pair primers (TPS6/11-F and R) were used to analyze gene expression of both TPS6 and TPS11 because their high sequence identity and similar inducible expression pattern reported before (Huffaker et al., 2011; Kollner et al., 2008). Ef-1α was used as endogenous control for qRT-PCR. All primers used in this study are listed in Table S2.
Acknowledgments

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Supplementary data

Fig. S1. Reactions catalyzed by CYP71Z6&7.

Fig. S2. Phylogenetic tree of maize CYPs from the CYP71 family.

Fig. S3. Negative catalytic activity of CYP71Z18 against γ-bisabolene and ent-cassadiene.

Fig. S4. In vitro assay of CYP71Z18 catalyzing zealexin A1 (3) formation from (S)-β-macrocarpene (2).

Fig. S5. Expression products of TPS11 in engineered E. coli.

Fig. S6. The mass spectra of CYP71Z18 minor oxidation products.

Table S1. P450 genes were found in microarray analysis of maize inoculated with F. graminearum for 48 h.

Table S2. The primers for gene expression analysis.
References


Figure Legends

**Fig. 1.** Putative biosynthetic pathways for zealexins and kauralexins.

Dashed arrows indicate uncharacterized reactions.

**Fig. 2.** CYP71Z18 catalytic activity.

A, GC-MS chromatogram of extract from *E. coli* engineered for coexpression of TPS11 and CYP71Z18. Peaks are labeled with numbering corresponding to that used in the text. Unlabeled peaks are background and lipids from culture. The coexpression of TPS11 and empty vector was used as control, which only produced (S)-β-macrocarpene (2).

B-E, Mass spectra for substrate and enzymatic products of CYP71Z18: (B) (S)-β-macrocarpene (2); (C) (S)-β-macrocarpen-15-ol (12); (D) (S)-β-macrocarpen-15-al (13); (E) zealexin A1 (3), as its methyl ester derivative) (3'). More detailed analysis is provided in **Fig. S6**.

**Fig. 3.** Gene expression of CYP71Z18 in maize.

A, Semiquantitative RT-PCR analysis of CYP71Z18 gene expression in root (R), scutella (SC), leaf (L) and leaf with 24 hour inoculation of *F. graminearum* spore (In). Actin gene was used as endogenous control.
**B-C**, qRT-PCR analysis of CYP71Z18 (B) and TPS6/11 (C) gene expression in leaves after *F. graminearum* spore inoculation at 0, 12, 24 and 48 hours.

All qRT-PCR analysis was replicated in trice. The error bars indicate the standard deviation.
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<td>2</td>
<td>27.10</td>
<td>1.44 (1H, m), 1.80 (1H, m)</td>
</tr>
<tr>
<td>3</td>
<td>24.82</td>
<td>2.44 (1H, dm, J=12.0), 2.20 (1H, m)</td>
</tr>
<tr>
<td>4</td>
<td>130.05</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>139.80</td>
<td>6.99 (1H, m)</td>
</tr>
<tr>
<td>6</td>
<td>31.17</td>
<td>2.08 (1H, m), 2.24 (1H, m)</td>
</tr>
<tr>
<td>7</td>
<td>139.70</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>40.66</td>
<td>1.71 (1H,s)</td>
</tr>
<tr>
<td>9</td>
<td>29.17</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>35.42</td>
<td>1.30 (1H, t, J=6.0)</td>
</tr>
<tr>
<td>11</td>
<td>23.29</td>
<td>2.02 (2H, m)</td>
</tr>
<tr>
<td>12</td>
<td>118.86</td>
<td>5.38 (1H, brs)</td>
</tr>
<tr>
<td>13</td>
<td>28.15</td>
<td>0.89 (3H,s)</td>
</tr>
<tr>
<td>14</td>
<td>28.52</td>
<td>0.90 (3H,s)</td>
</tr>
<tr>
<td>15</td>
<td>168.11</td>
<td>-</td>
</tr>
<tr>
<td>CH₃-O-C15</td>
<td>51.63</td>
<td>3.73 (3H,s)</td>
</tr>
</tbody>
</table>

**Note:** Coupling constants (J in Hz) are given in parentheses; signals are designated as follows: s, singlet; brs, broad singlet; t, triplet; m, multiplet; dm, doublet of multiplet.
Figure 1

FPP (1) → TPS6/11 → (S)-β-macrocarpene (2) → zealexin A1 (3) → zealexin B1 (4)

zealexin A2 (5) → zealexin A3 (6) → zealexin C3 (7)

GGPP (8) → An2 → ent-(iso)kaurene (9) → kauralexin A1 (B1) (10) → kauralexin A3 (B3) (11)
Figure 2

A) Total ion abundance

TPS11 + CYP71Z18

TPS11 + empty vector

Retention Time (min.)

B) m/z

(S)-β-macrocarpene (2)

C) m/z

(S)-β-macrocarpen
-15-ol (12)

D) m/z

(S)-β-macrocarpen
-15-al (13)

E) m/z

zealexin A1 methyl ester (3')

F) (S)-β-macrocarpene (2) → (S)-β-macrocarpen
-15-ol (12) → (S)-β-macrocarpen
-15-al (13) → zealexin A1 (3)

CYP71Z18
Figure 3

A

CYP71Z18

Actin

B

CYP71Z18

C

TPS6/11