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The Variability of Sesquiterpenes Emitted from Two *Zea mays* Cultivars Is Controlled by Allelic Variation of Two Terpene Synthase Genes Encoding Stereoselective Multiple Product Enzymes

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The mature leaves and husks of *Zea mays* release a complex blend of terpene volatiles after anthesis consisting predominantly of bisabolane-, sesquithujane-, and bergamotane-type sesquiterpenes. The varieties B73 and Delprim release the same volatile constituents but in significantly different proportions. To study the molecular genetic and biochemical mechanisms controlling terpene diversity and distribution in these varieties, we isolated the closely related terpene synthase genes *terpene synthase4* (*tps4*) and *tps5* from both varieties. The encoded enzymes, TPS4 and TPS5, each formed the same complex mixture of sesquiterpenes from the precursor farnesyl diphosphate but with different proportions of products. These mixtures correspond to the sesquiterpene blends observed in the varieties B73 and Delprim, respectively. The differences in the stereoselectivity of TPS4 and TPS5 are determined by four amino acid substitutions with the most important being a Gly instead of an Ala residue at position 409 at the catalytic site of the enzyme. Although both varieties contain *tps4* and *tps5* alleles, their differences in terpene composition result from the fact that B73 has only a single functional allele of *tps4* and no functional alleles of *tps5*, whereas Delprim has only a functional allele of *tps5* and no functional alleles of *tps4*. Lack of functionality was shown to be attributable to frame-shift mutations or amino acid substitutions that greatly reduce the activity of their encoded proteins. Therefore, the diversity of sesquiterpenes in these two maize cultivars is strongly influenced by single nucleotide changes in the alleles of two terpene synthase genes.

INTRODUCTION

Plant biologists and natural products chemists have long marveled over the huge variety of secondary metabolites produced by plants. At least 35,000 different secondary metabolites are now known based on recent estimates (Wink, 2003). Because individual secondary metabolites usually have a very restricted distribution among plant taxa (Giannasi and Crawford, 1986), nearly every species probably has a complement of secondary metabolites that is not only large and diverse but also unique. However, the molecular genetic and biochemical mechanisms responsible for generating this remarkable diversity and uniqueness are still largely unknown (Pichersky and Gang, 2000).

The largest group of plant secondary metabolites is the terpenes. With >20,000 representatives (Connolly and Hill, 1991), terpenes display an enormous range of diversity in carbon skeletons and functional groups, with individual plants containing as many as 100 different terpenes as components of complex oils, resins, or volatile mixtures (Harborne and Turner, 1984). In addition, terpene composition often shows substantial qualitative and quantitative differences among species and even among varieties of a single species. For example, within species of Perilla, Satureja, Thymus, and other genera of the Lamiaceae, numerous chemical races or chemotypes have been described based on genetically controlled differences in monoterpene profiles (Harborne and Turner, 1984; Giannasi and Crawford, 1986). Early efforts to understand this diversity and distribution employed crossing experiments among chemical races or species with different monoterpene compositions. This work revealed that monoterpene distribution is sometimes controlled by alleles that segregate independently, with particular loci affecting groups of monoterpenes rather than single compounds (Harborne and Turner, 1984; Vernet et al., 1986; Croteau and Gershenzon, 1994), but none of these loci have been functionally characterized.

Recent progress in terpene biosynthesis has offered new insights into the mechanisms governing terpene diversity and distribution. Especially interesting in this regard are studies of terpene synthases, a large class of enzymes that appears to be responsible for most of the structural variety of terpenes. Terpene synthases convert linear prenyl diphosphates of different chain lengths to the primary representatives of each terpene skeletal type (Cane, 1999; McMillan and Beale, 1999; Wise and Croteau, 1999). For example, the C_{10} intermediate, geranyl diphosphate (GPP), is converted to monoterpenes, and the C_{15} intermediate, farnesyl diphosphate (FPP), is converted to sesquiterpenes. The ability of terpene synthases to produce a diverse range of carbon skeletons from a few linear precursors results from their unusual

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reaction mechanism (Davis and Croteau, 2000) that involves the participation of carbocations that have a wide variety of metabolic fates. Often, individual intermediates have multiple fates in the reaction catalyzed by a single terpene synthase, leading to the formation of multiple products. For example, in *Salvia officinalis*, the three described terpene synthases each make 5 to 10 products (Wise et al., 1998).

Terpene synthases are thus a principal reason for the diversity of terpene products in the plant kingdom, and these catalysts have been the subjects of detailed study. Despite a very high sequence diversity, terpene synthases share several conserved amino acid residues, including an aspartate-rich motif (DDxxD) in the C-terminal half involved in the binding of a divalent metal cofactor (Cane, 1999; Wise and Croteau, 1999). The elucidation of the three-dimensional structures of two plant terpene synthases has allowed identification of the active site and determination of the amino acid residues that might be directly involved in the catalytic process (Starks et al., 1997; Whittington et al., 2002). To identify which regions of the protein control product specificity, domain swapping experiments have been performed that demonstrate that fragments of several hundred amino acids can determine catalytic activity (Back and Chappell, 1996; El Tamer et al., 2003; Peters and Croteau, 2003). Directed mutagenesis of single amino acids in terpene synthases showed that alterations of the DDxxD motif, a double Arg in the N terminus of the protein, and an active-site tyrosine residue dramatically altered both the kinetics and product specificity of the enzymes (Rising et al., 2000; Little and Croteau, 2002). Directed mutagenesis of terpene synthases of bacterial (Seemann et al., 1999, 2002) and fungal (Cane et al., 1996; Cane and Xue, 1996) origin yielded similar conclusions.

Terpene synthases may not only control the structural variety of terpenes present in plants but are also responsible for the unique terpene composition of each taxon. To learn more about

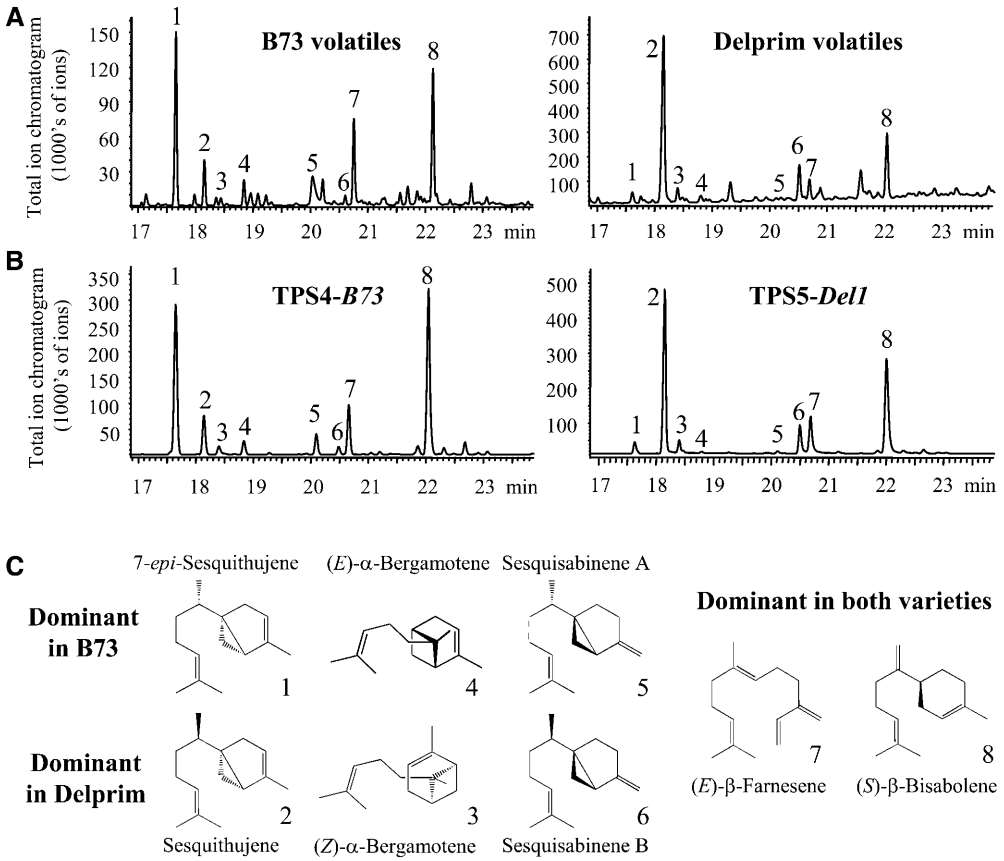

Figure 1. Volatile Sesquiterpene Products of Two Maize Varieties and the Corresponding Terpene Synthase Enzymes.

(A) Comparison of sesquiterpene volatiles of the mature leaves and husks of two maize varieties, Delprim and B73, collected by dynamic headspace sampling. Depicted is a portion of the total ion chromatogram of a gas chromatography–mass spectrometry analysis of each sample. Compounds were identified by mass spectrometry (Joulain and König, 1998) and comparison with authentic standards.

(B) Comparison of products of terpene synthases TPS4 and TPS5 expressed in *E. coli* and incubated with FPP. A portion of the gas chromatography–mass spectrometry analysis of each sample is shown.

(C) Structures of major sesquiterpene products. 1, 7-*epi*-sesquithujene; 2, sesquithujene; 3, (*Z*)-α-bergamotene; 4, (*E*)-α-bergamotene; 5, sesquisabinene B; 6, sesquisabinene A; 7, (*E*)-β-farnesene; 8, (*S*)-β-bisabolene.

the role of these enzymes in regulating terpene diversity and distribution, we have been investigating the terpene synthases of maize (*Zea mays*). The aerial parts of this crop species emit a blend of monoterpenes and sesquiterpenes after herbivore attack that, in combination with volatiles from the Trp and lipoxygenases pathways, attracts natural enemies of herbivores (Turlings et al., 1990; Hoballah and Turlings, 1999). We previously have characterized a terpene synthase regulated by herbivory TERPENE SYNTHASE1 (TPS1) that forms (*E*)-β-farnesene, (3*R*)-(*E*)-nerolidol, and (*E*,*E*)-farnesol (Schnee et al., 2002). The blend of terpenes emitted after damage can differ significantly among different maize cultivars and between cultivars and their wild ancestors (Gouinguene et al., 2001). Here, we report the differences in volatile terpene profiles between two maize cultivars that are attributable to differences among two terpene synthase genes. The encoded enzymes produce the same mixture of bisabolane-, sesquithujane-, and bergamotane-type sesquiterpenes but in different proportions. Site-directed mutagenesis demonstrated the role of individual amino acid residues in controlling product selectivity in the two enzymes, whereas mapping and functional analysis of alleles showed the contributions of gene duplication and allelic variation to the distribution and diversity of terpenes in these two maize varieties.

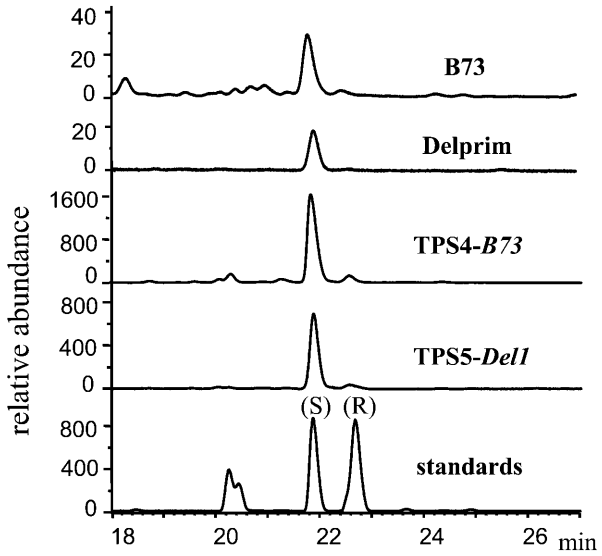

Figure 2. Identification of the Absolute Configuration of β-Bisabolene Produced by the Enzymes TPS4 and TPS5 and by the Mature Leaves and Husks of the Maize Varieties B73 and Delprim.

Gas chromatography performed on a chiral column with authentic standards, as described in Methods, allowed the separation and identification of the (*S*) and (*R*) enantiomers of β-bisabolene.

RESULTS

The Maize Varieties B73 and Delprim Emit Blends of Sesquiterpene Volatiles Containing Stereoisomers in Different Proportions

We compared the volatiles released by mature plants of two maize varieties, the inbred line B73, for which many genetic and molecular resources have been developed (Gai et al., 2000), and the commercial hybrid line, Delprim, which has been employed previously in parasitoid attraction experiments (Gouinguene et al., 2003). Differences in the terpene profiles of these varieties already have been detected at the juvenile stage (Turlings et al., 1990; Schnee et al., 2002). In this study, we found large differences after anthesis in the blends of sesquiterpene olefins released from the leaves and husks of the plant (Figures 1A and 1C). The two maize lines emitted the same compounds but in very different proportions. Among the major compounds released were three pairs of stereoisomers, with one member of each pair predominating in each variety. Whereas B73 emitted large amounts of 7-*epi*-sesquithujene, (*E*)-α-bergamotene, and sesquisabinene A, the cultivar Delprim released large amounts of the corresponding isomers sesquithujene, (*Z*)-α-bergamotene, and sesquisabinene B (Figures 1A and 1C). Both varieties also emitted substantial amounts of (*E*)-β-farnesene and β-bisabolene, which was shown to consist almost exclusively of the (*S*)-enantiomer (Figure 2).

The Blend of Terpenes Emitted by Mature B73 Plants Is Formed by the Terpene Synthase TPS4

To discover the genes controlling sesquiterpene formation in these varieties after anthesis, we employed an EST database of line B73 assembled by Pioneer HiBred, which had been used previously to clone *tps1* (Schnee et al., 2002). A sequence fragment displaying similarity to other terpene synthases was isolated and extended by PCR with rapid amplification of cDNA ends, using a cDNA library made from leaves of B73 plants, to obtain the full-length cDNA clone. The deduced amino acid sequence of the open reading frame contained highly conserved elements of terpene synthases like the DDxxD motif (residues 308 to 312) and the RxR motif (residues 271 to 273) but had an amino acid identity of only 25% to *tps1* (Schnee et al., 2002) and 40% to *sesquiterpene cyclase1* (Shen et al., 2001), another previously identified maize terpene synthase (Figure 3). The gene was named *terpene synthase4* (*tps4*) and designated *tps4-B73* to indicate the line that it originates from. (The descriptions of *tps2* and *tps3* are in preparation.)

The gene *tps4-B73* was overexpressed in *Escherichia coli*, and the encoded protein purified and incubated with FPP because the absence of a transit peptide suggested that the native enzyme was not present in the plastids and was therefore involved in sesquiterpene, rather than monoterpene or diterpene, formation (Gershenzon and Kreis, 1999). (*S*)-β-bisabolene and 7-*epi*-sesquithujene were the major products identified by gas chromatography–mass spectrometry (GC-MS), along with a complex mixture of >20 other minor sesquiterpene olefins (Table 1, Figures 1B, 1C, and 2). An identical distribution of sesquiterpene products was obtained in assays with either the highly purified protein containing a C-terminal 6xHis tag or a lesser purified fraction containing an untagged enzyme, indicating that the addition of the 6xHis tag or the presence of residual *E. coli* proteins did not alter product formation.

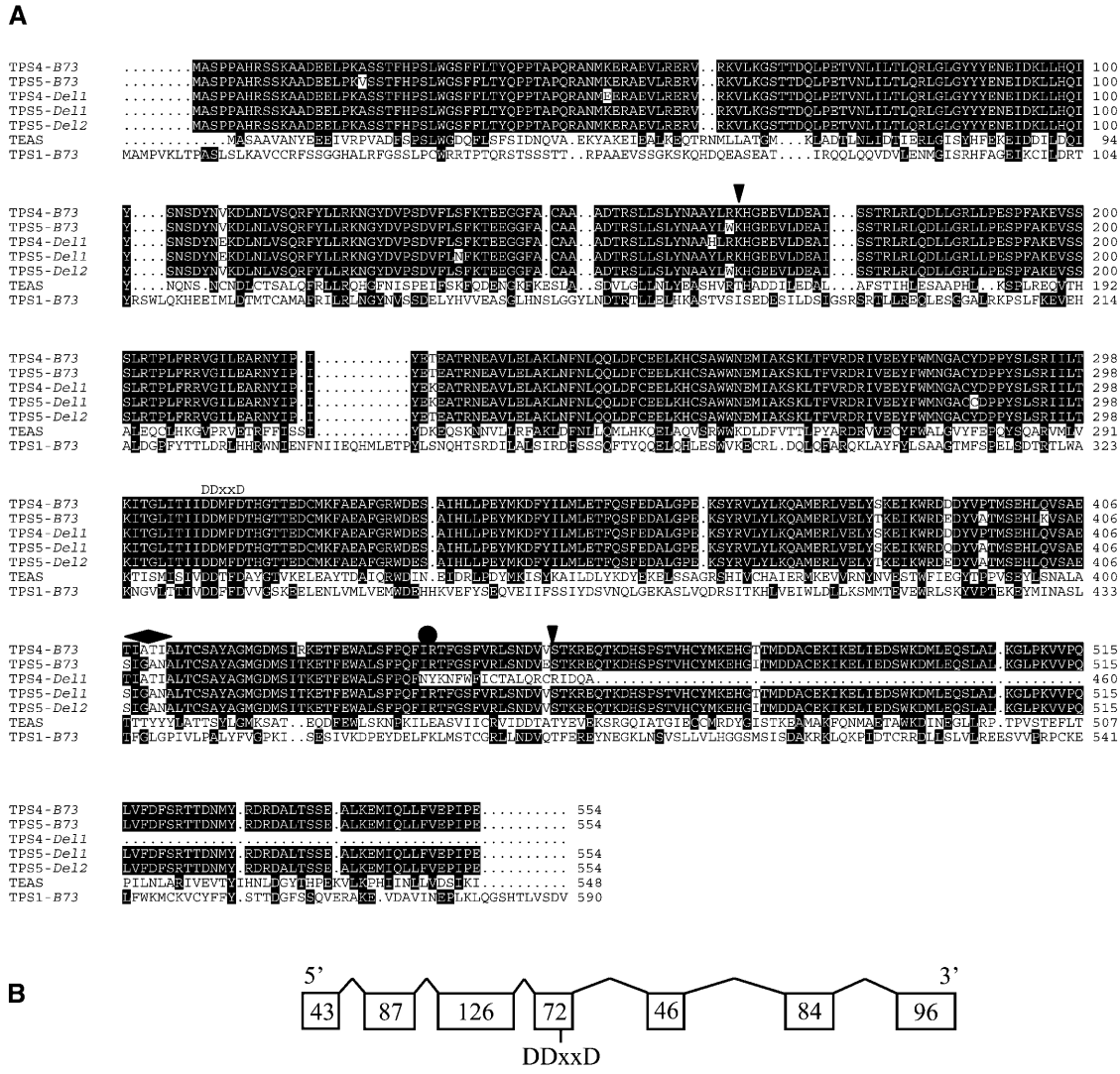

Figure 3. cDNA Sequences of the Maize Terpene Synthases *tps4* and *tps5* and the Genomic Sequence of *tps5*.

(A) Comparison of the deduced amino acid sequences of *tps4* and *tps5* cDNAs isolated from the varieties B73 and Delprim with the sequence of *tps1-B73* from maize (Schnee et al., 2002) and 5-*epi*-aristolochene synthase from *Nicotiana tabacum* (TEAS). Amino acids identical in the six genes are marked by black boxes. The highly conserved metal cofactor binding region is labeled DDxxD. The position of the frame-shift mutation in *tps4-Del1* is marked by a closed circle. The amino acids targeted by site-directed mutagenesis to investigate the stereoselectivity of TPS4 and TPS5 are highlighted by a rhombus, and the amino acids whose alteration causes inactivation of *tps5-B73* are marked with arrowheads.
(B) The exon–intron structure of *tps5*. The seven exons are represented by boxes showing the number of amino acids they contain.

To confirm the activity of the *tps4-B73*–encoded protein in the plant, we looked for the products of the heterologously expressed protein in different tissues of maize and in the headspace. The products of the TPS4-*B73* protein were found in extracts and headspace of the leaves and husks of mature plants after anthesis, but only traces of these substances were found in extracts and headspace collections from other developmental stages. The composition and relative proportion of sesquiterpenes volatilized from mature B73 leaves and husks was almost identical to that produced by TPS4 in vitro (Figure 1B), suggesting that *tps4-B73* is indeed a sesquiterpene synthase gene and that the encoded protein is essentially the only sesquiterpene synthase active in these organs.

TPS5 Produces the Blend of Terpenes Emitted by Mature Delprim Plants

To investigate the molecular basis for differences in sesquiterpene production between the varieties Delprim and B73, we searched for genes similar to *tps4-B73* in a cDNA library of Delprim. One of these clones, designated *tps5-Del1*, was shown after overexpression in *E. coli* to convert FPP to sesquithujene,

Table 1. Sesquiterpene Olefin Products of TPS4-*B73* and TPS5-*Del1*

Sesquiterpene Olefin	TPS4-*B73*	TPS5-*Del1*
(*S*)-β-bisabolene	29.1[a]	26.6[a]
7-*epi*-sesquithujene	24.4	2.0
(*E*)-β-farnesene	9.2	13.2
(7*S*)-β-curcumene[c]	5.7	0.6
Sesquithujene	5.6	28.2
(7*S*)-γ-curcumene[c]	3.3	0.8
(*E*)-α-bergamotene	2.6	0.5
α-Farnesene[b]	2.6	2.0
(*Z*)-γ-bisabolene	2.3	2.3
Sesquisabinene A (7*S*)[c]	2.1	0.4
(*Z*)-α-bisabolene[b]	2.1	1.3
(*E*)-γ-bisabolene	1.7	1.5
(*Z*)-α-bergamotene	1.5	2.3
Zingiberene[d]	1.2	1.4
β-Sesquiphellandrene[d]	1.0	1.1
Unknown	1.0	0.3
Sesquisabinene B (7*R*)[c]	0.9	4.5
Unknown	0.9	0.5
(7*R*)-γ-curcumene[c]	0.8	3.6
Unknown	0.7	0.3
(7*S*)-β-curcumene[c]	0.6	5.8
Unknown	0.4	0.4
Unknown	0.3	0.4

[a] Each compound is listed with its relative abundance expressed as a percentage of the total amount of products formed from FPP. Products were identified by matching their gas chromatographic retention times and mass spectra to those of authentic standards. In addition to the olefins, these enzymes also produced minor amounts (<1% each) of several sesquiterpene alcohols, of which the major product was (3*R*)-(*E*)-nerolidol.

[b] Compounds identified by mass spectra alone.

[c] Because of the lack of available standards, the absolute configurations of these three stereoisomeric pairs were uncertain. However, based on the configurations of the other products of TPS4 and TPS5 that could be identified with standards, it was possible to propose differences in the reaction mechanisms between the two enzymes (Figure 11) that, in turn, suggested the assignment of the (7*R*)- and (7*S*)-isomers as shown.

[d] These stereoisomeric pairs could not be chromatographically resolved. For the zingiberenes, the lack of appropriate standards also prevented assignment of the configuration at C7.

(*S*)-β-bisabolene, (*Z*)-α-bergamotene, sesquisabinene B, and several minor products (Figure 1B, Table 1). The components of this blend and their relative proportions were identical to those emanating from husks and mature leaves of Delprim plants (Figure 1A), but the stereoisomeric distribution was different from that of the blend released by B73 plants or made by TPS4-*B73* (Figure 1B). For example, for TPS5, sesquithujene makes up 28.2% of the total products, whereas 7-*epi*-sesquithujene makes up only 2.0%. For TPS4 on the other hand, sesquithujene makes up only 5.6% of the total products, whereas 7-*epi*-sesquithujene makes up 24.4%. In addition to these stereoisomeric pairs, both enzymes form (*S*)-β-bisabolene at 25 to 30% of the total products. The sequence of TPS5-*Del1* differed from that of TPS4-*B73* at 11 amino acid residues (Figure 3A), of which four were between residues 407 and 411 in the C-terminal domain of the enzyme, changing the sequence from TIATI to SIGAN.

The Properties of TPS4 and TPS5 Are Typical of Other Sesquiterpene Synthases

The basic features of the *tps4*- and *tps5*-encoded proteins were determined with enzyme that was heterologously produced in *E. coli* and then purified. Catalysis of both TPS4 and TPS5 required a divalent metal ion with Mg^{2+} and Mn^{2+} being essentially the only effective species of those tested. Optimum activity was obtained with 5 mM Mg^{2+} or 0.25 mM Mn^{2+} (Figure 4A). Although the K_m for Mn^{2+} is significantly lower than that for Mg^{2+} (Table 2), the enzyme is more likely to operate with a Mg^{2+} cofactor in planta because the concentration of Mg^{2+} in plant cells is about two orders of magnitude higher than Mn^{2+} (Marschner, 1998). In the presence of Mn^{2+}, the product spectrum of both enzymes

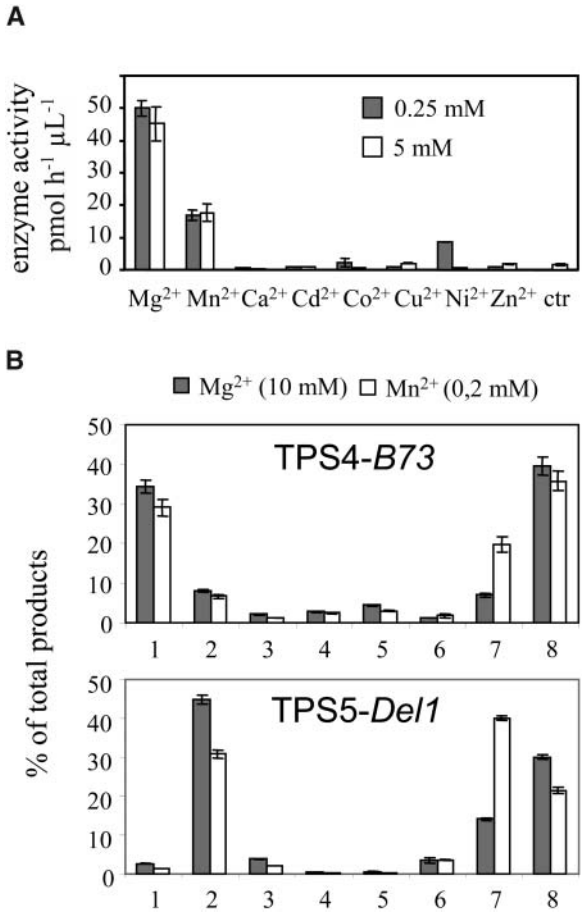

Figure 4. Divalent Metal Ion Cofactors Affect the Enzymatic Activity and Product Specificity of TPS4 and TPS5.

(A) The catalytic activity of TPS4, expressed in *E. coli* and purified, was measured in the presence of various divalent metal ions at 0.25 mM or 5.0 mM, with 1 mM EDTA as a control (ctr). Results with TPS5 were similar. Means and SE of triplicate assays are shown.

(B) Product formation differs in the presence of Mg^{2+} and Mn^{2+}. After incubation of enzyme and FPP substrate in the presence of 10 mM Mg^{2+} or 0.2 mM Mn^{2+}, the proportions of the major reaction products were determined. The compounds are numbered as in Figure 1. Means and SE of triplicate assays are shown.

Table 2. Kinetic Constants for TPS4-*B73* Heterologously Expressed in *E. coli*

K_m				V_{max} Relative	
FPP[a]	GPP[a]	Mg^{2+} [b]	Mn^{2+} [b]	FPP:GPP	Mg^{2+}:Mn^{2+}
1.1 μM	0.9 μM	170 μM	0.5 μM	100:20	100:25

[a] Values for FPP and GPP were measured in the presence of 10 mM Mg^{2+} and 1 mM Mn^{2+}.
[b] Values for Mg^{2+} and Mn^{2+} were measured with 10 μM FPP.

was shifted toward an increased production of (*E*)-β-farnesene (Figure 4B). Because the proportion of (*E*)-β-farnesene emitted from intact mature leaves is higher than the relative amount formed in vitro in the presence of Mg^{2+} (24.4% versus 9.3% for B73 plants and TPS4, respectively), it may be suggested that some Mn^{2+} also serves as a cofactor in planta. Gel permeation chromatography indicated that the mass of the active TPS4 enzyme was 66 kD ± 5 kD. This coincides with a mass of 63.96 kD predicted by the amino acid sequence, suggesting that the *E. coli*–expressed protein was full length, not significantly processed after translation, and that the active enzyme is a monomer. No dimer formation was observed, unlike with TPS1 (Schnee et al., 2002). The pH optimum of TPS4 was at 7.0, with half-maxima at 6.3 and 8.2.

The TPS4 and TPS5 enzymes not only accept the C_{15} substrate FPP but also accept the C_{10} analog GPP, the precursor of the monoterpenes. Catalysis with GPP and FPP has a similar K_m, but GPP is converted to products at a lower velocity than FPP (Table 2, data for TPS4). TPS4 and TPS5 produce the same 13 monoterpenes from GPP but in slightly different proportions (Figure 5). None of these monoterpene products were found in the tissues or the headspace of B73 or Delprim, suggesting that TPS4 and TPS5 do indeed function strictly as sesquiterpene synthases in planta. When all of the properties of TPS4 and TPS5, including molecular mass, subunit architecture, pH optimum, divalent metal ion requirement, and ability to use GPP, are taken together, these enzymes appear to be very similar to other sesquiterpene synthases previously characterized from angiosperms (Davis and Croteau, 2000).

The Stereoselectivity of TPS4 and TPS5 Is Determined by Four Amino Acids

The genes *tps4-B73* and *tps5-Del1* share 98% identity at the amino acid level and produce nearly the same end products but make dramatically different proportions of certain stereoisomer pairs. To pinpoint which amino acids determine this stereoselectivity, we modeled the encoded proteins TPS4 and TPS5 using the structural data available for 5-*epi*-aristolochene synthase, the only plant sesquiterpene synthase for which a three-dimensional structure has been obtained (Figure 3) (Starks et al., 1997). The structural models suggest that four of the amino acids different between the two enzymes (positions 407, 409, 410, and 411) are located in the bottom of the active site cavity (Figure 6). At positions 407 to 411, TPS5 has a SIGAN sequence, whereas TPS4 has a TIATI sequence. We used site-directed mutagenesis to exchange the four different residues of the TPS5 SIGAN sequence into those found in TPS4 and measured the enzyme activity after overexpression in *E. coli.* Alteration of the amino acid residues 407, 410, and 411, alone or together, did not affect product profile, but the change of Gly 409 to Ala resulted in a protein that produced mostly (*S*)-β-bisabolene with only minor amounts of the bicyclic

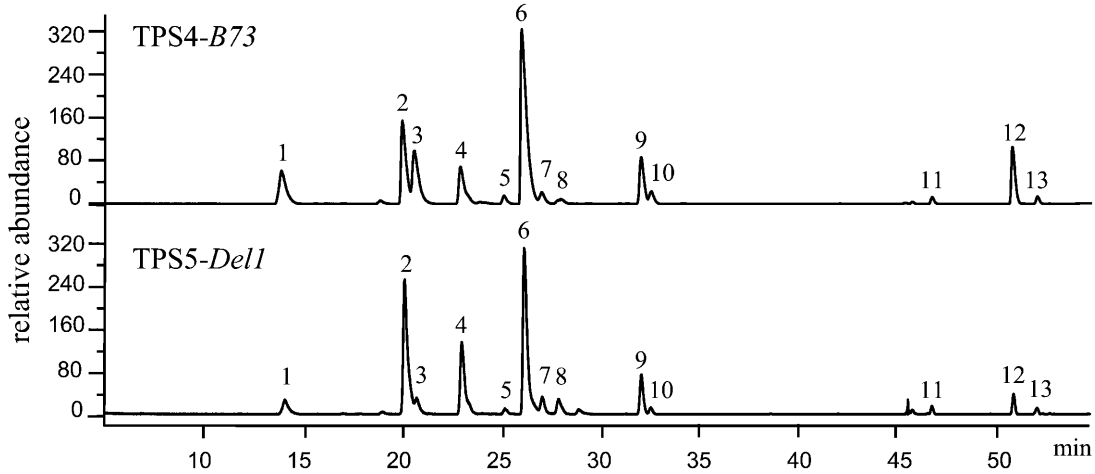

Figure 5. Monoterpene Olefin Products of TPS4 and TPS5.

The enzymes were expressed in *E. coli*, extracted, and incubated with the substrate GPP. The resulting products were separated by gas chromatography and identified by comparison of mass spectra and retention times with those of authentic standards on normal- and chiral-phase columns, except for compounds 7, 8, and 11, which were identified by comparison to reference spectra only. The traces of the total ion chromatogram are shown for both enzymes after separation on a chiral-phase column. 1, α-thujene; 2, β-myrcene; 3, (+)-sabinene; 4, (−)-sabinene; 5, α-terpinene; 6, (*S*)-(−)-limonene; 7, (*E*)-β-ocimene; 8, β-phellandrene; 9, α-terpinolene; 10, γ-terpinene; 11, sabinene hydrate; 12, (*R*)-(−)-linalool; 13, (*S*)-(+)-linalool.

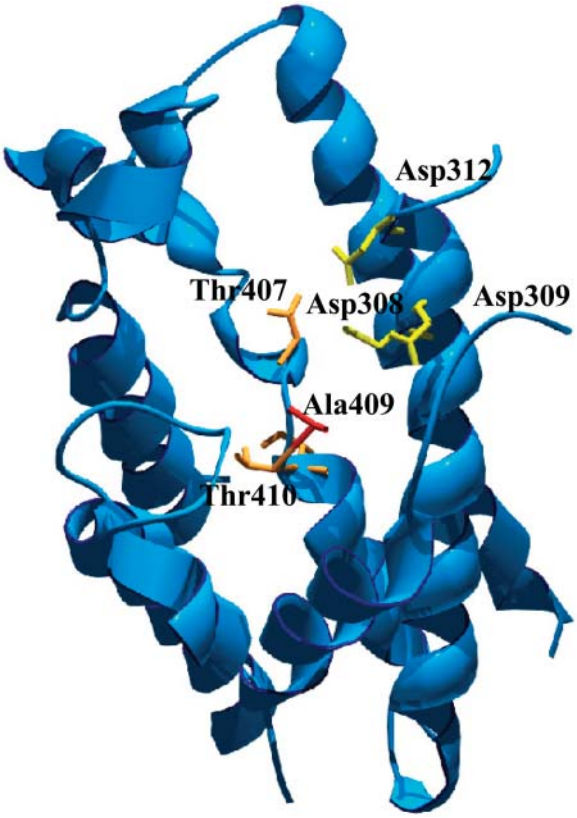

Figure 6. Model of TPS4-B73 Showing a Section of the C-Terminal Domain with a View into the Bottom of the Active Site.

Marked are the aspartate residues (308, 309, and 312) that are part of the DDxxD motif that binds the substrate and the residues responsible for the product differences between TPS4 and TPS5. The model was derived using the SWISS MODEL software (see Methods) employing the three-dimensional structure of *N. tabacum* 5-*epi*-aristolochene synthase as a template.

compounds, sesquithujene and sesquisabinene (Figure 7). In the presence of an Ala residue at position 409, only traces of bicyclic compounds were observed unless Ala 410 was changed to Thr. The exchange of Thr for Ala at position 410 resulted in the formation of 7-*epi*-sesquithujene and sesquisabinene A, the major bicyclic terpene products of *tps4-B73*. The simultaneous exchange of all four amino acids resulted in an activity identical to TPS4, demonstrating that these four amino acids determine the stereoselectivity of TPS4 and TPS5.

TPS4 and TPS5 Are Encoded by Separate Genes with Functional and Nonfunctional Alleles

We investigated the genetic basis of terpene variation between the maize inbred line B73 and hybrid variety Delprim by trying to discern whether *tps4* and *tps5* are alleles or separate genes. Utilizing the B73 cDNA library and EST databases of B73, a search was undertaken to look for further cDNA clones with similarity to *tps4* or *tps5*. One clone was isolated with a 98% amino acid identity to *tps5-Del1*, which also contained the characteristic SIGAN sequence at amino acids 407 to 411. No other sequences in these collections had a high enough identity to *tps4* or *tps5* to be considered alleles. Because no allelic variation should be present in an inbred line, the new sequence was considered to represent a gene separate from the *tps4* already isolated from B73 and designated *tps5-B73* because of its similarity to *tps5-Del1* (Figure 3). To eliminate the possibility that this sequence was a consequence of a residual heterozygosity in the inbred line B73, we obtained a line with a transposon insertion in *tps5* that disrupted the second exon at amino acid 47. After backcrossing into B73 and the selfing of a plant heterozygous for the insertion, the transposon was shown to segregate in the progeny, but a molecular marker targeted toward *tps4* was present in all individuals of the cross (data not shown). This result is a further indication that *tps4-B73* and the new sequence, *tps5-B73*, are separate genes and not alleles at the same locus. Surprisingly, the transposon insertion into *tps5-B73* did not have any measurable effect on the accumulation and emission of terpenes, demonstrating that this allele is not contributing significantly to terpene biosynthesis in B73 plants. In accordance with this observation, heterologous expression of *tps5-B73* in *E. coli* gave very low enzymatic activity when assayed with FPP in vitro, representing <1% of the activity obtained from expression of *tps5-Del1*, and the product spectrum had a much higher proportion of (*E*)-β-farnesene than that of the expressed *tps5-Del1*, 43.1% versus 14.3%.

We also searched for additional similar genes in the variety Delprim, which is likely to carry two alleles each for *tps4* and *tps5* because of its hybrid origin. A cDNA was cloned with high similarity to *tps4-B73* including the TIATI sequence and was designated *tps4-Del1*. However, this sequence had a frame-shift mutation at nucleotide 1321 that alters the amino acid sequence after amino acid 441 and leads to termination of the protein at amino acid 460 (Figure 3). There are no further nucleotide differences between tps4-Del1 and tps4-B73 located 3′ of the site of the frame-shift mutation. The truncated protein would not carry the complete catalytic site and is therefore very unlikely to be active. A second additional sequence found in Delprim displayed a SIGAN motif and a 98.6% similarity to *tps5-Del1* and was therefore designated *tps5-Del2*. When expressed in *E. coli*, this allele was found to have a residual catalytic activity measuring <1% of the activity of the protein encoded by *tps5-Del1* after in vitro expression.

The five isolated cDNA sequences of *tps4* and *tps5* appear to represent two genes based on the segregation in the *tps5-B73* transposon line cited above. Mapping of *tps4* and *tps5* with a population of T232xCM37 and CO159xTx303 inbred lines (Burr and Burr, 1991) positioned both genes on the short arm of chromosome 10 near the marker *npi105A* (Figure 8). No recombination between the loci was observed within the mapping population, suggesting that the genes are in close proximity to each other. To analyze their genomic structure, we isolated and sequenced the genomic clone of *tps5-B73*, which consists of seven exons separated by introns (Figure 3B). According to a classification of terpene synthase gene structure by Trapp and Croteau (2001), this gene belongs to class III.

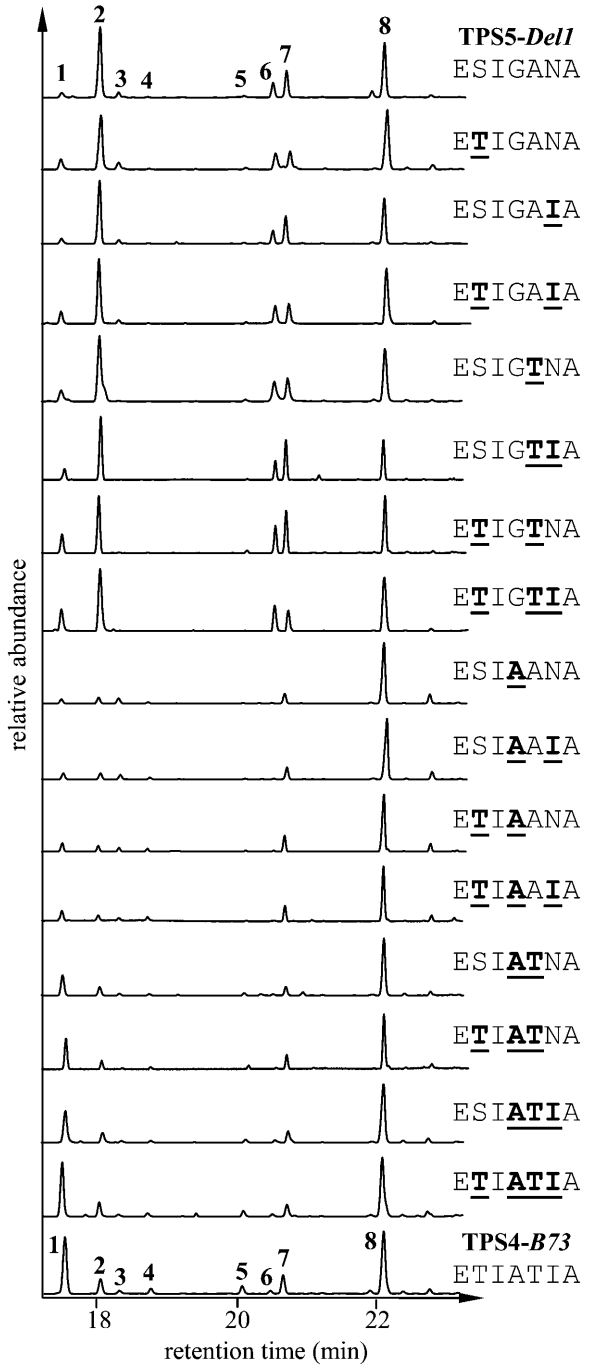

Figure 7. The Stereoselectivity of TPS4 and TPS5 Is Dependent on Four Amino Acids.

Amino acid changes were introduced sequentially into the active site of TPS5 to alter the active site sequence motif SIGAN into TIATI as found in TPS4. The 16 mutated enzymes that represent all possible combinations of the four amino acid exchanges between the two genes were expressed in *E. coli*, purified, and incubated with FPP. Total ion chromatograms of GC-MS analyses of the terpene products are shown. The sesquiterpene olefins were identified by mass spectrometry and comparison of mass spectra and retention times to those of authentic standards. 1, 7-epi-sesquithujene; 2, sesquithujene; 3, (*Z*)-α-bergamotene; 4, (*E*)-α-bergamotene; 5, sesquisabinene B; 6, sesquisabinene A; 7, (*E*)-β-farnesene; 8, (*S*)-β-bisabolene.

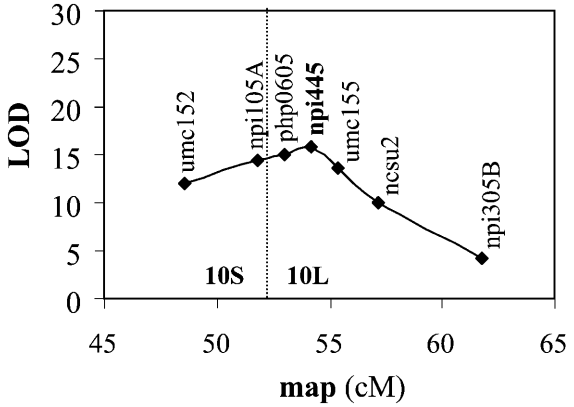

Figure 8. Mapping of the *tps4* and *tps5* Loci to Maize Chromosome 10 with Recombinant Inbred Lines.

Log of the odds (LOD) scores of the closest loci are shown. 10S and 10L indicate the short and long arms of chromosome 10, respectively. cM, centimorgan.

Two Amino Acid Changes Inactivate *tps5* in Variety B73

Alignment of the TPS5-*B73* and TPS5-*Del1* sequences revealed only 11 amino acid differences between them (Figure 3). Yet, when expressed in *E. coli*, TPS5-*B73* has <1% of the activity of its counterpart from Delprim. This difference is also reflected in planta with an almost complete lack of TPS5 products observed in B73. To determine which amino acid differences cause this drastic reduction in enzyme activity, we performed a mutational analysis on the inactive *tps5-B73* allele. Individual amino acid residues of *tps5-B73* were changed into those of the catalytically active *tps5-Del1* allele, and the activity of the encoded enzyme assessed after expression in *E. coli*. The mutations T383S, E391Q, K402Q, and I479T were located in the vicinity of the catalytic center (based on the modeling described above) but did not influence enzymatic activity either by themselves or in various combinations (data not shown). However, the mutation of Trp 163 to Arg restored much of the wild-type activity [albeit maintaining the increased concentration of (*E*)-β-farnesene relative to that of sesquithujene and (*S*)-β-bisabolene], although this residue is positioned far from the C-terminal catalytic domain (Figure 9). Conversely, the mutation of glutamate 455 to Val resulted in an increase of catalytic activity of only ~4% compared with that of the active *tps5* allele but resulted in a terpene blend that is indistinguishable from the active *TPS5-Del1* (Figure 1). The double mutation of both amino acid residues 163 and 455 gave the full activity and a terpene profile identical to *tps5-Del1*, indicating that these two amino acid changes are sufficient to transform the inactive *tps5-B73* allele into an active one.

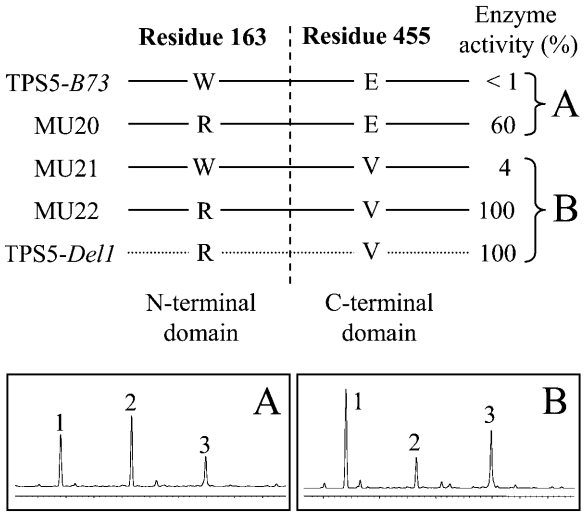

Figure 9. Differences in Enzyme Activity between Proteins Encoded by the Alleles *tps5-B73* and *tps5-Del1* Are Dependent on Two Amino Acids.

The amino acid changes W163R and E455V were introduced separately and simultaneously into *tps5-B73*. The mutated enzymes were expressed in *E. coli*, purified, and incubated with FPP. The approximate activities of the mutants were determined by measurement of total sesquiterpene products by gas chromatography with a flame-ionization detector and expressed as a percentage of the activity of the *tps5-Del1*–encoded protein. The terpene product spectra of these mutants fell into two groups depicted as A and B below. 1, sesquithujene; 2, (*E*)-β-farnesene; 3, (*S*)-β-bisabolene.

The Expression of *tps4* and *tps5* Is Highest in Husk Tissue

All *tps4* and *tps5* alleles in this study were isolated from cDNA libraries, indicating that sequences encoding catalytically inactive, as well as active, proteins were transcribed, at least to some extent. To learn more about the regulation of *tps4* and *tps5* expression, we measured transcript abundance in different tissues and after herbivore damage. In RNA hybridization assays with a probe that recognizes both *tps4* and *tps5* transcripts, the two maize lines exhibited high transcript levels in husk tissue (Figure 10A), which is also the site of the highest emission of TPS4 and TPS5 products (data not shown). A closer analysis of transcript abundance in the variety Delprim showed that the highest levels are within the outer husk tissue, which appears green. Lower levels were measured in the inner, more yellow husk leaves (Figure 10B). Still lower levels of transcript were found in the ear leaves, situated on the upper end of the green husks. Husk leaves are morphologically similar to other leaves of mature plants, which also show very low transcript levels at this developmental stage. High expression of *tps4* and *tps5* is therefore confined to the husk. In juvenile plants, *tps4* and *tps5* transcript levels were low throughout, except in leaf sheaths of B73 after herbivore damage. In general, there was good correspondence between *tps4* and *tps5* transcript levels in specific organs and emission of the specific terpene products formed by their encoded proteins.

DISCUSSION

TPS4 and TPS5 Each Produce the Same Mixture of Sesquiterpene Olefins but in Different Proportions

The large diversity of terpene natural products in plants can be attributed in large part to the catalytic versatility of terpene synthases. These enzymes not only convert prenyl diphosphates to a whole host of parent terpene skeletons, but often produce multiple products from a single substrate. For example, the previously described TPS1 from maize produces three products from FPP: (*E*)-β-farnesene (26%), (*E*)-nerolidol (29%), and (*E*,*E*)-farnesol (45%) (Schnee et al., 2002). The maize sesquiterpene synthases described here, TPS4 and TPS5, each make 21 identified products and are responsible for the great diversity of sesquiterpenes released from mature leaves and husks.

In addition to being multiple product enzymes, TPS4 and TPS5 exhibit a new dimension of variability for naturally occurring terpene synthases. Each makes the same mixture of sesquiterpenes, but the proportions of most products are significantly different. For the stereoisomeric pairs 7-*epi*-sesquithujene and

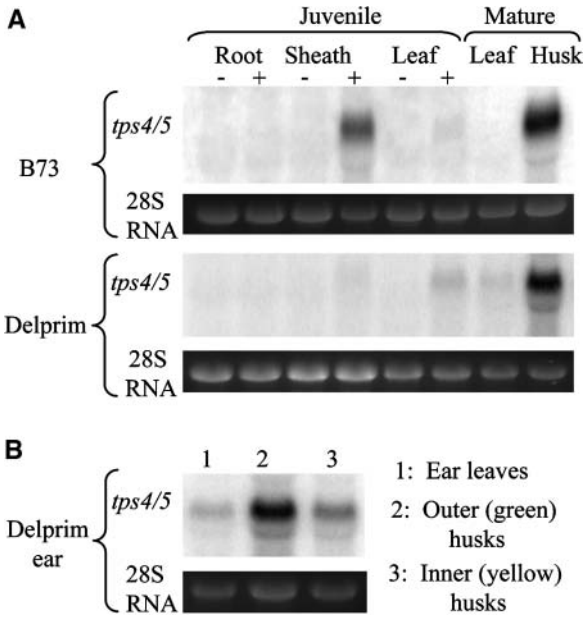

Figure 10. Transcript Levels of *tps4* and *tps5* in the Maize Varieties B73 and Delprim.

(A) Transcript levels of *tps4* and *tps5* in different organs and developmental stages of maize after herbivore damage (+) or in undamaged controls (−). RNA was isolated from plants of the cultivars B73 and Delprim that were 2 weeks old, except for the mature leaf and husk samples, which were isolated from plants after anthesis. The bottom panels show an ethidium bromide–stained agarose gel with (28S) rRNA as a control for equal RNA loading.

(B) Transcript levels of *tps4* and *tps5* are depicted in the outer (green) husk leaves, inner (yellow) husk leaves, and ear leaves, which develop on the tip of husks and are morphologically similar to vegetative leaves. The bottom panel shows an ethidium bromide–stained agarose gel with (28S) rRNA as a control for equal RNA loading.

sesquithujene, sesquisabinene A and sesquisabinene B, (*E*)-α-bergamotene and (*Z*)-α-bergamotene, (*S*)- and (*R*)-β-curcumene, and (*S*)- and (*R*)-γ-curcumene, the former is much more abundant in the product spectrum of TPS4 and the latter more abundant in the spectrum of TPS5. The presence of terpene synthases in a single species that produce products of opposite stereochemistry has been reported previously for *Pinus taeda* and *Solidago canadensis* (Schmidt et al., 1999; Phillips et al., 2003); however, these are predominantly single product enzymes with a rather low amino acid identity to each other.

The divergent product spectra of the two enzymes can be rationalized by reference to the proposed reaction mechanism in Figure 11. All identified products of TPS4 and TPS5 can be accounted for by a scheme based on the carbocationic mechanisms described for other sesquiterpene synthases (Cane, 1999). After the initial ionization of the diphosphate moiety from FPP, the resulting cation can be deprotonated to form (*E*)-β-farnesene or captured by water to form (*E*)-nerolidol. The remaining products are derived via nerolidyl diphosphate, formed by migration of the diphosphate moiety from C7 to C3. After C2-C3 bond rotation and a second ionization form the cisoid nerolidyl cation, intramolecular attack on the proximal double bond forms a new C-C bond between positions 1 and 6 (numbered as for FPP). The resulting cyclic intermediate, the bisabolyl cation, can exist in both (6*R*)- and (6*S*)-configurations. Intermediates of both configurations undergo further addition involving the C2-C3 double bond, leading to C2-C7 ring closure and formation of the bergamotyl cation. Alternatively, the bisabolyl cation can undergo 4,7- or 6,7-hydride shifts, with the latter followed by addition (C2-C6 ring closure) leading to the sesquithujyl cation. At every stage of this process, the intermediates formed may be deprotonated to yield the respective olefinic products. The final proportions of end products depend on the relative rate of deprotonation versus competing additions and hydride shifts.

In general, there is an extraordinary symmetry in the reaction mechanism. The (*R*)- and (*S*)-bisabolyl cations each form a nearly

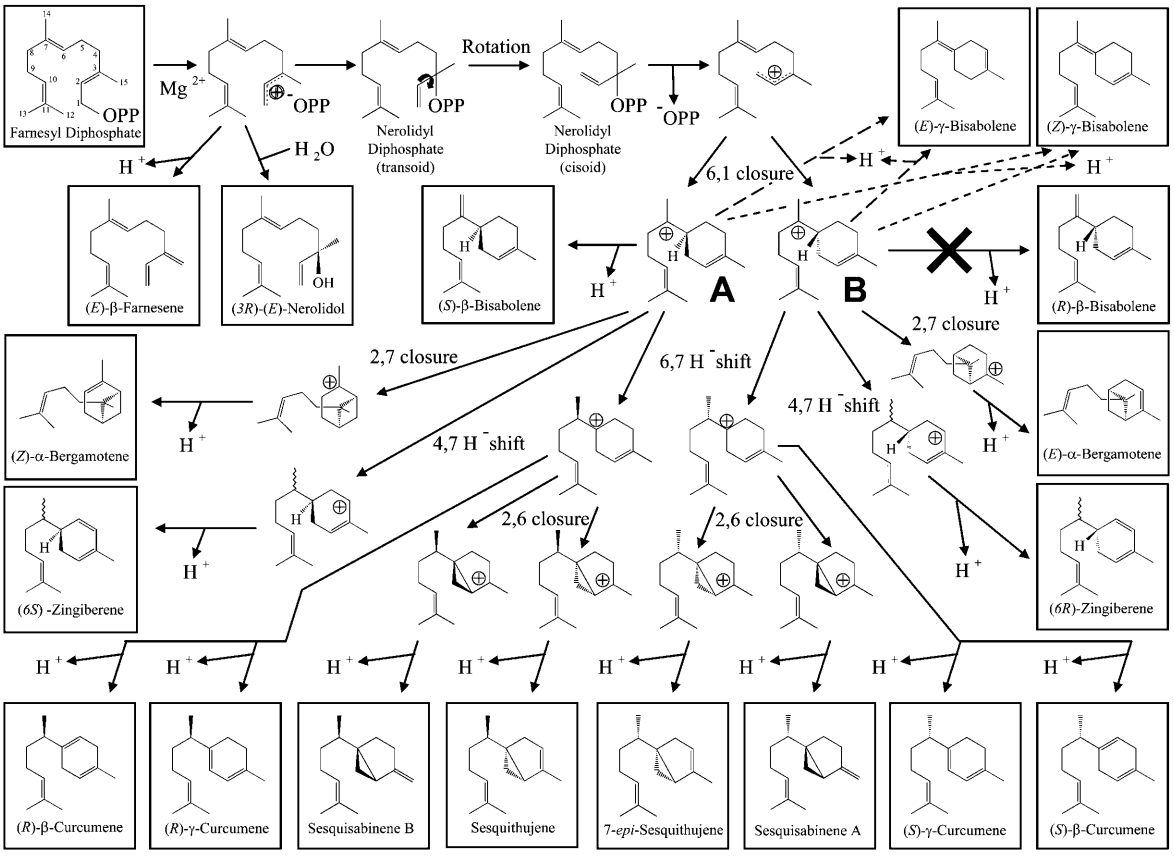

Figure 11. Proposed Reaction Mechanism for the Formation of Sesquiterpene Products by TPS4 and TPS5.

The scheme is based on previous studies of monoterpene (Wise and Croteau, 1999) and other sesquiterpene synthases (Cane, 1999). The differences in the product spectra of TPS4 and TPS5 can be explained by the relative rate of formation of (*S*)- versus (*R*)-bisabolyl cations labeled as A and B, respectively. Of the reaction products not depicted here, α-farnesene is formed by an alternate deprotonation of the farnesyl cation, α-bisabolene is formed by an alternate deprotonation of the bisabolyl cation, and the β-sesquiphellandrenes are formed by 1,7 hydride shifts of the bisabolyl cation and subsequent deprotonation at C15. Dotted lines are used to indicate when more than one route for end product formation is possible. OPP indicates a diphosphate group.

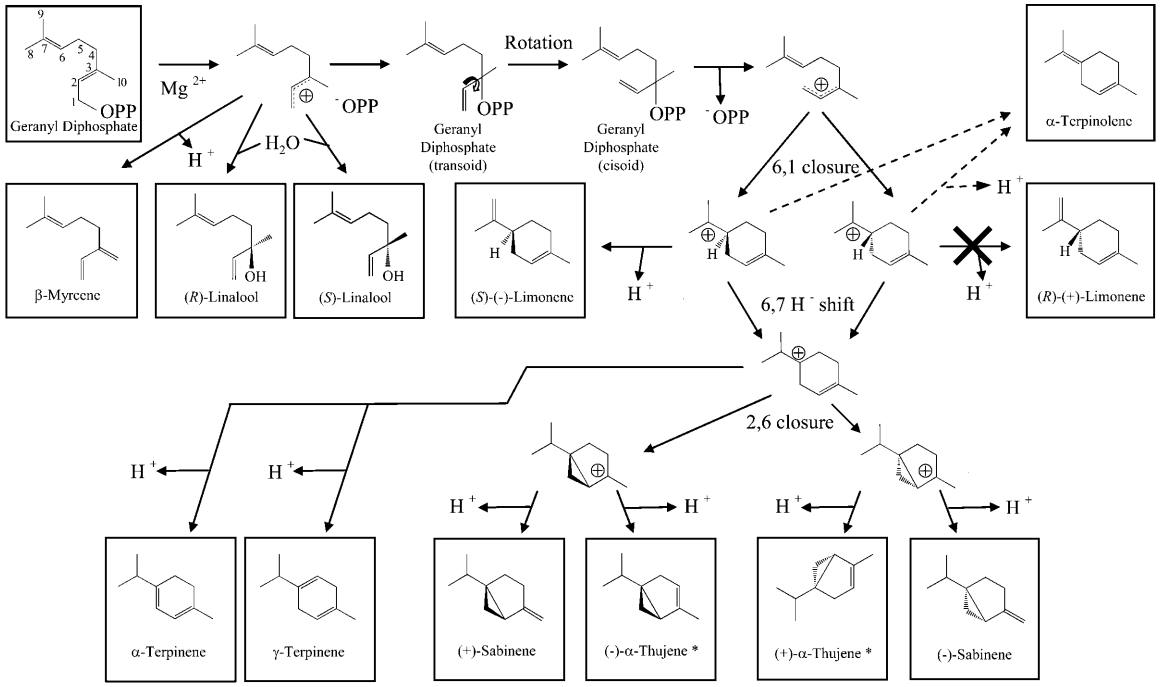

Figure 12. Proposed Reaction Mechanism for the Formation of Monoterpene Products by TPS4 and TPS5.

This scheme is analogous to the proposed reaction mechanism for the formation of sesquiterpene products (Figure 11), but because the (*S*)- and (*R*)-α-terpinyl carbocation intermediates are converted to a non-chiral product in the next step, the result is a single set of stereoisomeric compounds rather than the two sets seen in sesquiterpene formation. OPP indicates a diphosphate group.

analogous mixture of products by parallel routes. Each intermediate formed from the (*R*)-bisabolyl cation has a counterpart formed from the (*S*)-bisabolyl cation, with the exception of β-bisabolene, which is produced only as the (6*S*)-enantiomer. The differences in the product spectra of TPS4 and TPS5 can be explained by the relative rate of formation of (*S*)- versus (*R*)-bisabolyl cations. In TPS5, nearly 95% of the bisabolyl cation-derived products arise from reaction paths originating from the (*S*)-bisabolyl cation and only ~5% from the (*R*)-bisabolyl cation. While in TPS4, approximately half of the bisabolyl-derived products arise from the (*R*)-form, with the other half coming from the (*S*)-form, represented mostly by (*S*)-β-bisabolene.

The amount of monoterpene products formed by these enzymes was much lower than the amount of sesquiterpenes, and the rate of reaction with GPP was much less than that with FPP. Nevertheless, a reaction mechanism analogous to that proposed for sesquiterpene formation is sufficient to account for all of the monoterpenes formed by these enzymes. Although the reaction proceeds via both (*R*)- and (*S*)-α-terpinyl cations, both of these intermediates are converted to the terpinen-4-ol cation, a nonchiral intermediate. Thus, the two sets of stereoisomeric products occurring in sesquiterpene formation are not formed in monoterpene formation (Figure 12). Further reaction products are derived from the terpinen-4-ol carbocation by deprotonation or an additional 2,6-ring closure. These reactions are analogous to those postulated for sesquiterpene formation except for the absence of the products of a 2,7-cyclization.

The Different Stereoselectivities of TPS4 and TPS5 Are Determined by Only a Few Amino Acid Residues in the Active Site

The high sequence similarity between TPS4 and TPS5 facilitated the use of site-directed mutagenesis to ascertain which amino acid residues are responsible for the difference in product profiles. Changing a cluster of four residues in the active site

Maize variety	*tps4*	*tps5*
B73 (inbred line)	allele encodes an active enzyme	allele encodes an inactive enzyme
Delprim (hybrid line)	allele contains a frame shift mutation	two alleles encode an active and an inactive enzyme

Figure 13. Sesquiterpene Production in Mature Maize Plants of the Varieties B73 and Delprim Is Determined by the Presence of Alleles at Two Loci.

B73, which synthesizes TPS4 products, has a *tps4* sequence that is active when heterologously expressed in *E. coli* and a *tps5* sequence that is catalytically inactive. Conversely, Delprim, which synthesizes TPS5 products, has one *tps5* sequence that is catalytically active on heterologous expression, one *tps5* sequence that is inactive, and two *tps4* sequences that are inactive.

Table 3. Primer Sequences for Site-Directed Mutagenesis[a]

Name[b]	Sequence (5′/3′)	Mutation
MU1rev	CCAGCATATGCAGAACATGTTAGAGCATTGGCT**G**CGATGG	G409A
MU2rev	CCAGCATATGCAGAACATGTTAGAGCATTGG**T**TCCGATGG	A410T
MU4rev	CCAGCATATGCAGAACATGTTAGAGCA**A**TGGCTCCGATGG	N411I
MU7rev	GCATATGCAGAACATGTTAGAGCA**A**TGG**T**T**G**CGATGG**T**CTC	S407T, G409A, A410T, N411I
MU9rev	GCATATGCAGAACATGTTAGAGCA**A**TGG**T**TCCGATGG**T**CTC	S407T, A410T, N411I
MU10rev	GCATATGCAGAACATGTTAGAGCATTGGCTCCGATGG**T**CTC	S407T
MU14fwd	CGACAATGAGTGAACACCTC**C**AGGTTTCAGCAG	K402Q
MU14rev	CTGCTGAAACCT**G**GAGGTGTTCACTCATTGTCG	K402Q
MU15fwd	GTTGAGCTATACA**G**CAAGGAAATAAAATG	T383S
MU15rev	CATTTTATTTCCTTG**C**TGTATAGCTCAAC	T383S
MU16fwd	AAATGGCGTGAT**C**AGGATTATGTGG	E391Q
MU16rev	CCACATAATCCT**G**ATCACGCCATTT	E391Q
MU17fwd	GGAGCATGGAA**C**AACAATGGATGATG	I479T
MU17rev	CATCATCCATTGTT**G**TTCCATGCTCC	I479T
MU18fwd	GAGCTGCCAAAGG**C**TTCTTCGACCTTCCAC	V20A
MU18rev	GTGGAAGGTCGAAGAA**G**CCTTTGGCAGCTC	V20A
MU20fwd	GCAGCATATCTG**A**GGAAGCATGGAG	W163R
MU20rev	CTCCATGCTTCC**T**CAGATATGCTGC	W163R
MU21fwd	CTCTCCAACGATGTCG**T**ATCGACCAAGCGTG	E455V
MU21rev	CACGCTTGGTCGAT**A**CGACATCGTTGGAGAG	E455V
MU22fwd	GACCATCGCAACCA**A**TGCTCTAACATGTTCTG	S407T, G409A, A410T
MU22rev	CAGAACATGTTAGAGCA**T**TGGTTGCGATGGTC	S407T, G409A, A410T
MU23fwd	GCAGAGACCATCGCA**G**CCATTGCTCTAACATG	S407T, G409A, N411I
MU23rev	CATGTTAGAGCAATGG**C**TGCGATGGTCTCTGC	S407T, G409A, N411I
MU24fwd	GCAGAGACCATCGGA**A**CCAATGCTCTAACATG	S407T, A410T
MU24rev	CATGTTAGAGCATTGG**T**TCCGATGGTCTCTGC	S407T, A410T
MU25fwd	CAGCAGAGACCATCG**C**AGCCAATGCTCTAACA	S407T, G409A,
MU25rev	TGTTAGAGCATTGGCT**G**CGATGGTCTCTGCTG	S407T, G409A,
MU26fwd	GCAGAGTCCATCGGA**A**CCATTGCTCTAACATG	A410T, N411I
MU26rev	CATGTTAGAGCAATGG**T**TCCGATGGACTCTGC	A410T, N411I

[a] Underlined regions indicate the *Afl*III restriction site. The base changes are shown in boldface.
[b] fwd, forward; rev, reverse.

cavity (residues 407, 409, 410, and 411) by site-directed mutagenesis was sufficient to alter the product spectrum completely from that of TPS5 to that of TPS4. Hence, we could demonstrate that large differences in product specificity between naturally occurring terpene synthase genes can be the result of single amino acid exchanges. When the residues 407, 409, 410, and 411 were changed one by one, exchange at residue 409 (from Gly in TPS5 to Ala in TPS4) had the most significant effect on the products formed. Modeling suggests that residue 409 is located in a highly conserved kink of a helix, known as helix G in 5-*epi*-aristolochene synthase (Starks et al., 1997), which is located at the bottom of the active site. Here, the additional methyl group might alter the shape of the cavity wall or change the distance between the two α-helices that form the side of the cavity (Figure 6) in such a way that the relative rate of formation of the (*R*)- versus the (*S*)-bisabolyl cation is altered.

Based on their proposed reaction mechanism, TPS4 and TPS5 may be expected to contain other sequence elements that previously have been associated with features of terpene synthase catalysis. For example, TPS4 and TPS5 both contain the DDxxD motif (amino acid residues 308 to 312), which has been previously implicated in binding the substrate via divalent metal ion bridges (Cane et al., 1996) and the RxR motif 35 amino acids upstream thought to help direct the diphosphate anion away from the reactive carbocation after ionization (Starks et al., 1997). However, the N-terminal pair of Arg conserved in all monoterpene synthases and many sesquiterpene synthases that are thought to serve in isomerization of GPP to linalyl diphosphate (or FPP to nerolidyl diphosphate) (Williams et al., 1998) is not present. Neither are the additional DDxxD motifs found in some multiple product sesquiterpene synthases that are proposed to promote multiple product formation by allowing the substrate to bind to the active site in additional ways (Davis and Croteau, 2000).

Allelic Variation in *tps4* and *tps5* Controls the Distribution of Sesquiterpenes between Two Maize Varieties

Although terpene composition often varies among and within plant species, it has been difficult to attribute these differences to functionally defined genes (Croteau and Gershenzon, 1994). Here, we show that sesquiterpene variation among two maize varieties is under the control of the terpene synthases TPS4 and TPS5. The sesquiterpenes emitted from the mature leaves and husks of the inbred line B73 are products of TPS4, whereas sesquiterpenes emitted from the hybrid line Delprim are the

products of TPS5. Surprisingly, these two enzymes, which share >98% identity at the amino acid level, are not encoded for by alleles at the same locus. cDNA libraries from B73 and Delprim both contain *tps4* and *tps5* sequences, suggesting the presence of at least two *tps4*/*tps5* genes in these varieties. From the inbred line B73, which produces TPS4 products, a *tps5* sequence was isolated that is likely to be transcribed because it came from a cDNA library. Yet, when expressed in *E. coli*, this sequence had very low catalytic activity in contrast with the expression of *tps4-B73* or *tps5-Del1*. These results are consistent with the lack of TPS5 products emitted from intact B73 plants and suggest that in this case, catalytic activity upon heterologous expression accurately mirrors that in vivo. Thus, whether or not *tps5-B73* is translated in B73, it probably makes no contribution to terpene biosynthesis in this line.

For the Delprim variety, a hybrid line that produces TPS5 products, two *tps5* sequences and one *tps4* sequence were isolated. Heterologous expression showed that one of the *tps5* alleles encodes a catalytically active enzyme, but the second allele was not functional. The isolated *tps4* sequence contained a frame-shift mutation leading to an altered amino acid sequence after residue 441 and a stop codon at residue 460. The second *tps4* allele that is likely to be present in this hybrid variety could not be isolated from cDNA libraries or EST resources. Taken together with the behavior of the transposon insertion in *tps5-B73*, these results support the existence of separate *tps4* and *tps5* genes in maize. In both lines, only a single *tps4* or *tps5* was functional, leading to the differences in sesquiterpene composition observed (Figure 13).

Determining the Mutations That Inactivate TPS5 in Variety B73 Gives Additional Insights into the Reaction Mechanism of TPS4 and TPS5

The inactivation of *tps4* and *tps5* alleles results from frame-shift mutations that introduce premature stop codons and from mutations that give apparent full-length enzymes with very low catalytic activity. To learn more about the latter process, the *tps5* allele in variety B73 was subjected to site-directed mutagenesis and found to have undergone two critical mutations leading to loss of the normal activity of the *E. coli*–expressed protein of *tps5-Del1*. Mutation of amino acid residue 455 from Val to glutamate decreased enzyme activity by only 40% but significantly increased the proportion of the acyclic product (*E*)-β-farnesene. According to a model based on the structure of *N. tabacum* 5-*epi*-aristolochene synthase (Starks et al., 1997), residue 455 is located near the entrance of the active site where it is not in direct contact with the substrate. This area is close to the aspartate rich region that is responsible for the binding of the Mg^{2+} cofactors. Interestingly, both the exchange of amino acid 455 to glutamate and the replacement of Mg^{2+} ions by Mn^{2+} resulted in the increased formation of (*E*)-β-farnesene, suggesting that both of these changes increase the rate of deprotonation of the farnesyl cation relative to isomerization to nerolidyl diphosphate.

The second crucial mutation that reduced enzyme activity in *tps5-B73* is an exchange of Arg to Trp at amino acid 163 in the N-terminal region of the enzyme. The importance of the N-terminal domain of terpene synthases has been demonstrated previously by N-terminal deletions (Williams et al., 1998) and domain swapping experiments (Back and Chappell, 1996), but the actual function of this domain is still not known. Because modeling suggests that the side chain of amino acid 163 faces the C-terminal domain, it might play a role in the interaction between the two domains. This interaction could perhaps affect the correct folding or the shape of the active site in the C-terminal domain. The presence of a Trp residue at position 163 in the protein encoded by allele *tps5-Del2* is also very likely to be the cause of its catalytic inactivity.

Gene Duplication and Multiple Alleles Contribute to Terpene Diversity in Maize

The highly similar genes *tps4* and *tps5* map to the same location on chromosome 10 and are hence likely to be the result of a tandem duplication followed by divergence. Assuming an average rate of synonymous substitutions in maize of 6.5×10^{-9} substitutions per synonymous site per year (Gaut et al., 1996), *tps4* and *tps5* can be calculated to have diverged about three million years ago (Gaut and Doebley, 1997). Genes encoding terpene synthases might actually be expected to have more rapid rates of sequence divergence than other genes if secondary metabolism is under selection for increasing diversity. In this regard, gene duplication can be seen as an evolutionary process that can make an important contribution to generating diversity in terpene biosynthesis (Facchini and Chappell, 1992; Van der Hoeven et al., 2000; Aubourg et al., 2002). Many other genes of secondary metabolism also appear to have arisen from tandem duplication events (e.g., Pichersky and Gang, 2000; Kliebenstein et al., 2001; Kroymann et al., 2001; Reintanz et al., 2001; Clauss and Mitchell-Olds, 2003).

At the *tps4* locus, an inactive allele was characterized that had a frame-shift mutation and yet was still transcribed. At the *tps5* locus, several alleles were found to encode proteins that were full length but had extremely low enzyme activities. The significance of these nonfunctional alleles is hard to evaluate, particularly in a plant such as maize that has been bred artificially for at least 5000 years. Interestingly, some tomato (*Lycopersicon esculentum*) cultivars also have been reported to have nonfunctional terpene synthase alleles (Van der Hoeven et al., 2000). However, examples of secondary metabolism genes in which alleles encoding truncated mRNAs are still transcribed also are known from wild species (Kliebenstein et al., 2001), suggesting that these are not necessarily artifacts of artificial selection. Such alleles may be evolutionary relics that are being transcribed for only a limited period of time until sufficient mutations accumulate in the promoter region to abolish transcription. On the other hand, nonfunctional alleles also could represent a reservoir of genetic variability that may be of value under future selective regimes.

METHODS

Plant and Insect Material

Seeds of the maize (*Zea mays*) inbred line B73 were provided by KWS Seeds (Einbeck, Germany), and seeds of the maize hybrid variety Delprim

were obtained from Delley Samen und Pflanzen (Delley, Switzerland). Plants were grown in commercially available potting soil in a climate-controlled chamber with a 16-h photoperiod, 1 mmol $(m^2)^{-1}$ s^{-1} of photosynthetically active radiation, a temperature cycle of 22°C/18°C (day/night) and 65% relative humidity. To rear mature plants, seedlings were planted in large pots (∅ 35 cm) and grown in a greenhouse with 16-h supplemental lighting and a temperature of 25°C during the day and 22°C at night. Eggs of *Spodoptera littoralis* (Lepidoptera: Noctuidae) were obtained from Aventis (Frankfurt, Germany) and were reared on an artificial wheat germ diet (Heliothis mix; Stonefly Industries, Bryan, TX) for ~10 to 15 d at 22°C under an illumination of 750 μmol $(m^2)^{-1}$ sec^{-1}. For the herbivory treatments, three-thirds instar larvae were enclosed on the middle portion of each plant in a cage made out of two halves of a Petri dish (9 cm diameter) with a circle cut out of each side and covered with gauze to allow for ventilation (Degenhardt and Gershenzon, 2000).

Plant Volatile Collection and Analysis

An automated collection system (Analytical Research Systems, Gainesville, FL) based on the design of Heath and Manukian (1994) was employed to analyze plant headspace volatiles. In brief, whole juvenile plants or leaves and ears of mature plants were cut and placed in a glass of water in a large glass cylinder (50 × 20 cm) whose base was fitted with two adjustable blades. The blades closed around the lower part of the stem (of whole plants) or the lower parts of the leaves or ears (for collections from individual organs). Air that had been passed through a charcoal-infused medium for purification and moistened to a relative humidity of 65% entered the chamber from above at a rate of 5 liters min^{-1}. After sweeping over the plant material, the air exited the chamber through a collection trap, 150 × 5-mm-diameter glass tube containing 75 mg of Super Q (80/100 mesh; Alltech, Deerfield, IL) at the base of the chamber. Air was drawn through the trap at a rate of 1 liter min^{-1} by an automated flow controller. The remaining air escaped through the opening around the adjustable blades providing a positive pressure barrier against the entrance of ambient air. The entire volatile collection system was contained in a controlled environment chamber (Voetsch VB1014/S; Balingen, Germany) set at 25°C, 75% relative humidity, 16-h photoperiod, and 750 μmol $(m^2)^{-1}$ s^{-1} of photosynthetically active radiation. After the 3-h collection period, the trap was rinsed with 0.2 mL of dichloromethane, and the sample was analyzed by GC-MS.

A Hewlett-Packard model 6890 gas chromatograph (Palo Alto, CA) was employed with the carrier gas He at 1 mL min^{-1}, splitless injection (injector temperature, 220°C; injection volume, 2 μL), a DB-WAX column (polyethylene glycol, 300 m × 0.25 mm × 0.25-μm film; J and W Scientific, Folsom, CA), and a temperature program from 40°C (3-min hold) at 5°C min^{-1} to 240°C (3-min hold). The coupled mass spectrometer was a Hewlett-Packard model 5973 with a quadrupole mass selective detector, transfer line temperature of 230°C, source temperature of 230°C, quadrupole temperature of 150°C, ionization potential of 70 eV, and a scan range of 50 to 400 atomic mass units. Compounds were identified by comparison of retention times and mass spectra to those of authentic standards obtained from Fluka (Seelze, Germany), Roth (Karlsruhe, Germany), Sigma (St. Louis, MO), or Bedoukian (Danbury, CT) or by reference spectra in the Wiley and National Institute of Standards and Technology libraries and in the literature (Joulain and König, 1998). Many standards not commercially available were either obtained as described below or kindly supplied by Wilfried A. König, Hamburg, Germany. The enantiomers of β-bisabolene, β-curcumene, and γ-curcumene were separated and identified by GC-MS using a heptakis (2,3-di-*O*-methyl-6-*O*-*t*-butyldimethylsilyl)-β-cyclodextrin (35% in OV1701 [w/w]) column (30 m × 0.25 mm × 0.125 μm film; BGB Analytik, Adliswil, Switzerland) operated with H_2 (2 mL min^{-1}) as carrier gas, splitless injection (220°C, 1-μL volume), and a column temperature of 115°C. A racemic mixture of (*S*)- and (*R*)-β-bisabolene was prepared by dehydration of racemic α-bisabolol at 140°C with acidic aluminum oxide as catalyst (König et al., 1994; Joulain and König, 1998). The β-bisabolene enantiomers were identified by comparison with the Bergamot (*Citrus bergamia*) essential oil, which contains only the (*S*)-enantiomer. A zingiberene standard was obtained as an essential oil by a simultaneous steam distillation–pentane extraction) of ginger roots (*Zingiber officinale*). For accurate quantification, compounds were analyzed by gas chromatography with a flame ionization detector (250°C) using conditions as above with a DB-WAX column, except that carrier gas was H_2 at 2 mL min^{-1}. All analyses were performed at least twice.

cDNA Library Construction

Ten-day-old maize plants of the cultivar B73 and Delprim were subjected to herbivory by *Spodoptera littoralis* for 4 h. One gram of leaf material was ground in a mortar to a fine powder in liquid nitrogen and added to 10 mL of Trizol reagent (Gibco BRL, Rockville, MD). The mixture was treated with a Polytron (Kinematika, Kriens, Switzerland) for 1 min and incubated for 3 min on ice. Total RNA was isolated according to the manufacturer's instructions. From ~75 μg of total RNA, the mRNA was isolated using poly-T coated ferromagnetic beads (Dynal, Oslo, Sweden). The mRNA was transcribed into cDNA using a Marathon RACE library according to the manufacturer's instructions (BD Biosciences, Palo Alto, CA).

Isolation of the Maize Terpene Synthase cDNAs

Sequences with high similarity to plant terpene synthases were identified in BLAST searches of an EST database maintained by Pioneer Hi-Bred International. Two of these ESTs from the maize cultivar B73 contained fragments of 2049 bp and 2071 bp, respectively, and were designated *tps4-B73* and *tps5-B73*. The open reading frames of both genes had a length of 1665 bp. The corresponding genes of the Delprim variety, *tps5-Del1*, *tps5-Del2*, and *tps4-Del1*, were amplified from two independent cDNA libraries made from *S. littoralis*–induced 14-d-old seedlings of husk tissue. To avoid sequence errors, the proofreading enzymes Pfu Turbo (Stratagene, La Jolla, CA) and Advantage 2 (BD Biosciences) were used in the PCR. Each allelic cDNA clone was amplified at least twice independently and fully sequenced. Sequence alignments were performed with the DNASTAR suite of programs (Madison, WI).

Heterologous Expression of Terpene Synthases

The open reading frame of *tps4-B73* was cloned as an *Nde*I-*Bam*HI fragment and inserted into the bacterial expression vector pSBET using the sticky-end PCR method of Zeng (1998). Expression of *tps4-B73* in other bacterial expression vectors, such as pQE60 (Qiagen, Heidelberg, Germany), lead to an identical enzymatic activity. The open reading frame of *tps5-B73* was cloned as an *Nco*I-*Bam*HI fragment into the expression vector pET11d. Both pSBET and pET11d constructs were transformed into the *Escherichia coli* strain BL21 (DE) and fully sequenced to avoid errors introduced by DNA amplification. Site-directed mutagenesis of *tps5-B73* was performed with the open reading frame cloned into the expression vector pASKIBA7 as a *Bsp*MI fragment with an N-terminal *Strep*-tag. This vector was also used for cloning and expression of *tps4-Del1*, *tps5-Del1*, and *tps5-Del2* as *Bsp*MI fragments. The constructs were introduced into the *E. coli* strain TOP10 (Invitrogen, Carlsbad, CA) and fully sequenced. Liquid cultures of the bacteria harboring the expression constructs were grown at 28°C to an OD_{600} of 0.6. Expression of the recombinant proteins from pSBET or pET11d constructs in BL21 (DE) was induced by addition of isopropyl-β-thiogalactopyranoside to a final concentration of 1 mM, and the expression of pASKIBA7 constructs in TOP10 cells was induced with 200 μg/liter of anhydrotetracycline (IBA, Göttingen, Germany). After 20-h incubation at 18°C, the cells were

collected by centrifugation and disrupted by a 4 × 30 s treatment at 36 W with a sonicator (Bandelin UW2070; Berlin, Germany) in chilled extraction buffer [50 mM 3-(*N*)-2-hydroxypropane sulfonic acid (Mopso), pH 7.0, with 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM DTT, and 10% (v/v) glycerol]. The cell fragments were removed by centrifugation at 14,000*g*, and the supernatant was desalted into assay buffer (10 mM Mopso, pH 7.0, 1 mM DTT, and 10% [v/v] glycerol) by passage through a Econopac 10DG column (Bio-Rad, Hercules, CA). If required, the *Strep*-tagged enzymes were further purified on a *Strep*-Tactine affinity column (IBA) according to the manufacturer's instructions. The enzyme activity was stable for at least 1 year when stored at −80°C. The protein concentration of the purified extract was determined by the method of Bradford (1976) using the Bio-Rad reagent with BSA as standard.

Assay for Terpene Synthase Activity

Standard assays contained 200 μL of the bacterial extract in assay buffer with 10 μM GPP or (*E*,*E*)-FPP, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM $NaWO_4$, and 0.1 mM NaF in a Teflon-sealed, 7-mL screw-capped glass test tube. A solid phase microextraction fiber consisting of 100 μm Polydimethylsiloxane (SUPELCO, Belafonte, PA) was placed into the headspace of the tube during a 60-min incubation at 30°C followed by 15 min at 40°C. The solid phase microextraction fiber was then directly inserted into the injector of a gas chromatograph for product analysis. Assay results are reported as the mean of at least three independent replicate assays, and each experiment was repeated two to three times with similar results. For the determination of kinetic parameters and the effects of pH and divalent metal ions, a scaled down assay of 100 μL volume was used with [1-^{3}H] (*E*,*E*)-FPP as substrate [American Radiolabeled Chemicals, St. Louis, MO; specific activity of 37 GBq mol^{-1} containing 10 to 20% of the (*Z*,*E*)-isomer]. The assay was overlaid with 0.5 mL of pentane to trap volatile products and incubated for 3 h at 30°C. The reaction was stopped by mixing with 2 mL of diethyl ether, and the resulting pentane-ether layer was removed and passed through a short column of $NaSO_4$ in a Pasteur pipette. The volume of the extracted organic phase was adjusted to 2 mL. A 100-μL portion of the organic phase was taken for measurement of radioactivity by liquid scintillation counting in 5 mL of Lipoluma cocktail (Packard Bioscience, Groningen, The Netherlands) using a Packard Tricarb 2300TR liquid scintillation counter (^{3}H efficiency = 61%). The remaining organic phase was concentrated to ~40 μL for radio gas chromatography and GC-MS analysis. The K_m values were determined with seven to nine substrate concentrations with three repetitions each using a Lineweaver-Burke plot. For the analysis of minor enzyme products, a 1-mL assay described above was performed in a 7-mL screw-capped glass test tube. Product identification was performed by GC-MS as described above.

Radio gas chromatography was performed on a Hewlett-Packard 6890 gas chromatograph (H_2 carrier gas 2 mL min^{-1}; splitless injector temperature, 220°C; injection volume, 2 μL) with a Chrompack CP-SIL-5 CB-MS column [(5%-phenyl)-methylpolysiloxane, 25 m × 0.25-mm i.d. × 0.25-μm film thickness; Varian, Walnut Creek, CA] or a DB-WAX column (polyethylene glycol, 30 m × 0.25-mm i.d. × 0.25-μm film thickness; J and W Scientific). Temperature was programmed from 40°C (3-min hold) at 5°C min^{-1} to 240°C (3-min hold). The outlet of the column was connected to a thermal conductivity detector (TCD) followed by a radioactivity detector (Raga 92; Raytest, Straubenhardt, Germany). Within the radioactivity detector, the eluent (9 mL min^{-1}) was passed through a conversion reactor filled with platinum chips at 740°C. After supplementation with a quench gas, methane (12 mL min^{-1}), the eluent entered two proportional counting tubes (10- and 2-mL volume) connected in series. TCD and radioactivity detector outputs were processed with the Hewlett-Packard Chemstation data system. The amount of radioactivity in each peak was determined as a percentage of total sample radioactivity (measured by liquid scintillation counting) by comparing peak areas within each run. To correct for losses during sample concentration and injection, peak area was standardized by comparison to the area of the coinjected unlabeled standards detected with the TCD.

Protein Size Determination

Crude bacterial extract (0.5 mL) was separated by fast performance liquid chromatography on a Superdex-200 16/6 gel filtration column in assay buffer (10 mM Mopso, pH 7.0, 1 mM DTT, and 10% [v/v] glycerol). Fraction size was 5 mL. The calibration was performed with a mixture of dextran blue, sweet potato β-amylase, yeast alcohol dehydrogenase, BSA, bovine carbonic anhydrase, and horse cytochrome c.

Modeling

Protein structure modeling was performed with the SWISS-MODEL service (http://www.expasy.org/swissmod/SWISS-MODEL.html; Peitsch, 1995, 1996; Guex and Peitsch, 1997) using the previously determined structure of 5-*epi*-aristolochene synthase (Starks et al., 1997) as a modeling template. Models were visualized and analyzed using the Swiss-PdbViewer version 3.7 program (http://www.expasy.org/spdbv/).

Site-Directed Mutagenesis

The site-directed mutations of *tps5-Del1* in the vector pASK-IBA7 were introduced by insertion of PCR fragments with the altered sequence. These PCR fragments comprised the first 1257 bp or 1260 bp and were amplified with the forward primer 5′-TATGGCGTCTCMTCCAGCA-CATCGTTCC-3′ and the reverse primers MU1rev through MU10rev that contain the mutations and an *Afl*III restriction site that is present in the gene (Table 3). The PCR product containing the mutation was cloned as an *Afl*III fragment into the *Afl*III digested pASK-IBA7-tps5-Del1 construct. Because the two *Afl*III sites of the PCR fragment and pASK-IBA7-tps5-Del1 have a different recognition sequence, the cloning was directional. For site-directed mutagenesis of the pASK-IBA7-*tps5-B73* construct, the QuikChange site-directed mutagenesis method (Stratagene) with the primers MU14 through MU26 (Table 3) was used according to the manufacturer's instructions.

Mapping of Terpene Synthase Genes

The method of Burr and Burr (1991) was used to map the genes in a population of 48 CM37xT232 and 41 Tx303xCO159 recombinant inbred lines kindly provided by R. Burr. Genomic DNA gel blots from all individuals of the population were probed with a 284-bp fragment of *tps4-B73* that cross-reacted with *tps5* (for a detailed description of the probe, see the RNA gel blotting procedure below). Only one segregating band was visible in all individuals, indicating that *tps4* and *tps5* are in close proximity to each other.

RNA Gel Blotting

Plant RNA was prepared with the RNeasy plant mini kit (Qiagen) according the manufacturer's instructions. To verify an even loading of RNA, the gel was stained with ethidium bromide and visualized. A 1426-bp fragment of the *tps4-B73* coding sequence was generated by PCR with the primers 5′-TGGCGTCTCMTCCAGCACATCGTTCC-3′ and 5′-TCCTTCATGTAACAATGGACAGTGGA-3′. This fragment was used as a template for synthesis of a 284-bp probe by linear PCR with the primer 5′-CTTGTCTATCTCGTTCTCGTAG-3′ in the presence of ^{32}P-adenosine triphosphate according to the Strip-EZ PCR procedure (Ambion, Austin, TX). Blotting on a Nytran-Plus nylon membrane (Schleicher and Schuell, Düren, Germany), hybridization, and washing were performed following standard procedures. The blots were exposed to BioMax MS1 film (Kodak, Rochester, NY) with an intensifying screen

and scanned with a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) for quantification.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY518310, AY518311, AY518313, AY518314, and AY518312.

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