A Maize (*E*)-β-Caryophyllene Synthase Implicated in Indirect Defense Responses against Herbivores Is Not Expressed in Most American Maize Varieties [W][OA]

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The sesquiterpene (*E*)-β-caryophyllene is emitted by maize (*Zea mays*) leaves in response to attack by lepidopteran larvae like *Spodoptera littoralis* and released from roots after damage by larvae of the coleopteran *Diabrotica virgifera virgifera*. We identified a maize terpene synthase, Terpene Synthase 23 (TPS23), that produces (*E*)-β-caryophyllene from farnesyl diphosphate. The expression of TPS23 is controlled at the transcript level and induced independently by *D. v. virgifera* damage in roots and *S. littoralis* damage in leaves. We demonstrate that (*E*)-β-caryophyllene can attract natural enemies of both herbivores: entomopathogenic nematodes below ground and parasitic wasps, after an initial learning experience, above ground. The biochemical properties of TPS23 are similar to those of (*E*)-β-caryophyllene synthases from dicotyledons but are the result of repeated evolution. The sequence of TPS23 is maintained by positive selection in maize and its closest wild relatives, teosinte (*Zea* sp) species. The gene encoding TPS23 is active in teosinte species and European maize lines, but decreased transcription in most North American lines resulted in the loss of (*E*)-β-caryophyllene production. We argue that the (*E*)-β-caryophyllene defense signal was lost during breeding of the North American lines and that its restoration might help to increase the resistance of these lines against agronomically important pests.

INTRODUCTION

In natural ecosystems, plants are usually part of a complex web of interactions with other organisms that may influence their growth and survival. To be successful in such an environment, plants have to respond correctly to a multitude of different herbivores, pathogens, competitors, and mutualists. Much plant biology research today is devoted to unraveling the molecular and biochemical processes that provide plants with flexible and appropriate responses to these various enemies and friends. Plant responses to herbivory often include the formation of secondary metabolites, especially phenolic and terpene compounds that act as toxins and feeding deterrents when ingested by the herbivore (Karban and Baldwin, 1999). Another defense tactic involves the recruitment of natural enemies of herbivores with induced volatiles. This so-called indirect defense has already been identified in >10 plant species (Dicke, 1999; Dicke and van Loon, 2000; Meiners and Hilker, 2000; Kessler and Baldwin, 2002).

A well-studied example of indirect defense is found in maize (*Zea mays*), in which foliar damage by lepidopteran larvae results

in the release of a complex volatile mixture containing indole, lipoxygenase pathway products, and a variety of monoterpene and sesquiterpene olefins. These volatiles attract parasitic wasps like *Cotesia marginiventris* to the site of damage, where they oviposit in the lepidopteran larvae (Turlings et al., 1990). Parasitized lepidopteran larvae feed less than unparasitized larvae and die upon emergence of the adult wasp, which can result in a considerable reduction in damage to the plant (Hoballah et al., 2002, 2004). The identification of the precise volatile compound(s) that attracts the wasp to the plant for oviposition is a complex and difficult task (Turlings et al., 1991; D'Alessandro and Turlings, 2005). We previously isolated the gene responsible for the biosynthesis of (*E*)-β-farnesene and (*E*)-α-bergamotene (Schnee et al., 2006), the predominant sesquiterpenes released upon caterpillar attack by maize (Köllner et al., 2004a). Overexpression of this gene in *Arabidopsis thaliana* demonstrated that the parasitic wasp *C. marginiventris* can use these sesquiterpenes for host finding after an initial learning experience (Schnee et al., 2006).

An additional sesquiterpene often emitted after herbivore damage is (*E*)-β-caryophyllene. This volatile compound has been found in response to herbivore damage in several wild relatives of maize (Gouinguené et al., 2001) and in cultivated maize lines from European breeding programs, but it is absent from maize lines originating from North American breeding programs (Degen et al., 2004). Below ground, (*E*)-β-caryophyllene has been found to serve as an important signal in the attraction of enemies to another maize herbivore, the root-feeding pest western maize rootworm (*Diabrotica virgifera virgifera*) (Rasmann
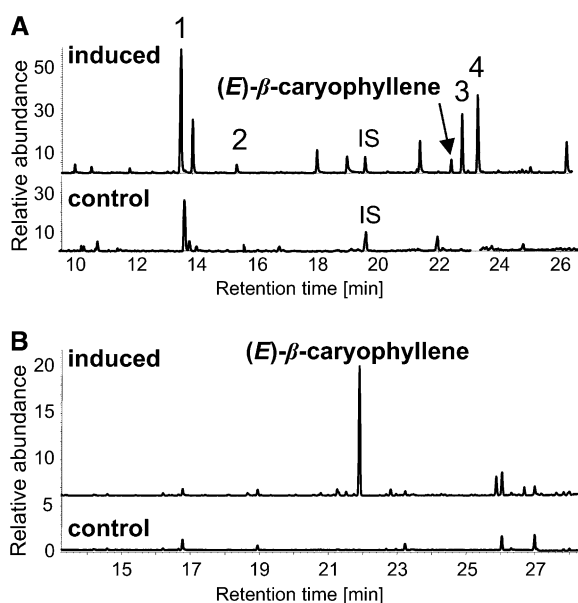
Figure 1. (*E*)-β-Caryophyllene Is Emitted in Response to Both Damage of the Leaves by *S. littoralis* and Attack of the Roots by *D. v. virgifera*.

(A) Volatiles from control leaves and leaves damaged by *S. littoralis* were collected and separated by gas chromatography. The major terpene compounds were identified as linalool (peak 1), 4,8-dimethylnona-1,3,7-triene (peak 2), (*E*)-α-bergamotene (peak 3), and (*E*)-β-farnesene (peak 4). Depicted are traces of the total ion current detector. IS, internal standard (nonylacetate).
(B) Volatiles from control roots and roots damaged by *D. v. virgifera*.

et al., 2005). In contrast with the complex volatile blend emitted by caterpillar-damaged leaves, maize roots only release (*E*)-β-caryophyllene upon damage by *D. v. virgifera*, which attracts entomopathogenic nematodes (Rasmann et al., 2005).

In the biosynthesis of terpenes, the large class of terpene synthase enzymes converts linear prenyl diphosphate precursors into the large diversity of terpene skeletons encountered in plants. For example, the sesquiterpene synthases convert the C_{15} farnesyl diphosphate to sesquiterpene olefin products. A characteristic feature of terpene synthases is the formation of multiple terpenes from a single substrate (Gershenzon and Kreis, 1999). In the course of our work on maize terpene biosynthesis, we have characterized the multiproduct sesquiterpene synthases TPS1, TPS4, TPS5, and TPS10, which contribute to the overall terpene composition of the aboveground plant parts (Schnee et al., 2002, 2006; Köllner et al., 2004b). However, no gene has been described yet for the biosynthesis of (*E*)-β-caryophyllene in maize.

The bicyclic sesquiterpene (*E*)-β-caryophyllene is of particular interest in maize interactions with other organisms since it is released both above and below ground (Turlings et al., 1998; Rasmann et al., 2005). A gene encoding its biosynthesis would provide a useful tool to study its regulation and function. Here, we describe the properties of maize Terpene Synthase 23 (TPS23), which catalyzes the cyclization of farnesyl diphosphate to (*E*)-β-caryophyllene and the complex regulation of the *tps23* gene in

leaves and roots in response to damage by different herbivores. We also show that the TPS23 product, (*E*)-β-caryophyllene, can function as a signal both above and below ground, thereby contributing to the plant's defense against herbivores with completely different sites and modes of attack. Finally, we demonstrate that the ability to produce (*E*)-β-caryophyllene is under positive selection pressure among the wild relatives of maize but was lost during the breeding of most North American maize varieties, not because of direct mutation of the *tps23* gene itself but due to alteration of the regulatory network that results in its transcription.

RESULTS

Caryophyllene Can Attract Two Types of Herbivore Enemies, Entomopathogenic Nematodes and Parasitic Wasps

In our attempt to identify the maize volatiles that are responsible for interactions with other organisms, we previously identified (*E*)-β-farnesene and (*E*)-α-bergamotene as major constituents of a blend used by parasitic wasps to find their lepidopteran hosts (Schnee et al., 2006). Many maize lines, especially those originating from European breeding programs, also emit the sesquiterpene (*E*)-β-caryophyllene after damage by lepidopterans (Degen et al., 2004). The hybrid line Delprim, which is of European origin, also emits high concentrations of this compound from leaves and roots after herbivore induction (Figure 1). In roots, (*E*)-β-caryophyllene is the sole compound released in significant amounts after damage by the herbivore *D. v. virgifera* (Rasmann et al., 2005). To test whether this amount of (*E*)-β-caryophyllene attracts the entomopathogenic nematode *Heterorhabditis megidis* Poinar, we applied (*E*)-β-caryophyllene from a volatile source in one arm of the six-arm olfactometer (Figure 2). Approximately twice as many nematodes were recovered on average from the arm of the olfactometer spiked with biologically
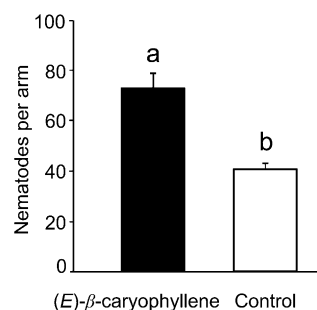
Figure 2. (*E*)-β-Caryophyllene Attracts Nematodes.

The attractiveness of (*E*)-β-caryophyllene to the entomopathogenic nematode *H. megidis* was demonstrated in six-arm olfactometers filled with moist sand. Of the nematodes that were released in the centers of the olfactometers, a significantly larger number was recovered from the arm connected to a pot spiked with 0.2 μL of (*E*)-β-caryophyllene than from each of the five control arms (P < 0.0001; *n* = 12). Different letters above the bars indicate a significant difference. Means and SE are shown.
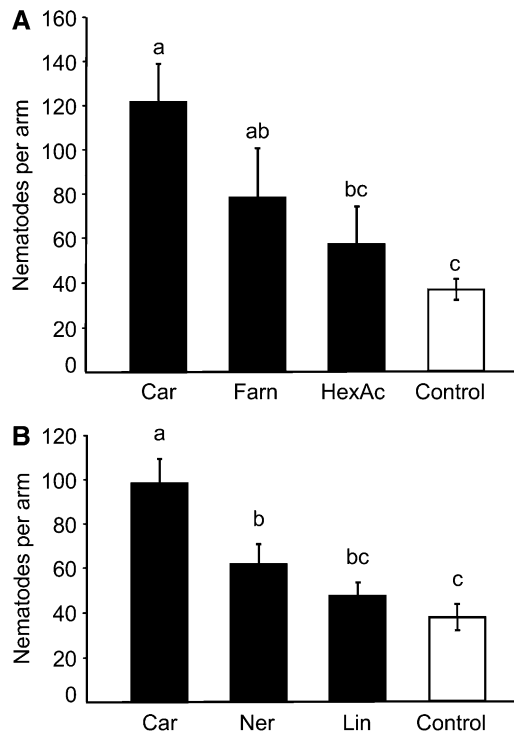
Figure 3. Nematode Attraction Is Specific to (*E*)-β-Caryophyllene.

The attractiveness of plant volatile compounds to the entomopathogenic nematode *H. megidis* was demonstrated in six-arm olfactometers filled with moist sand. The nematodes were released in the center of the olfactometer and chose between arms in which 0.2 μL of (*E*)-β-caryophyllene (Car), (*E*)-β-farnesene (Farn), and (*Z*)-3-hexenyl acetate (HexAc) **(A)** or (*E*)-β-caryophyllene, (*E*)-nerolidol (Ner), and linalool (Lin) **(B)** were added and a sand-only control. Different letters above the bars indicate significant differences at P < 0.05. Means and SE of *n* = 8 **(A)** or *n* = 12 **(B)** repetitions are shown.

relevant amounts of authentic caryophyllene compared with the average for the five remaining arms that were not spiked ($F_{1,34}$ = 13.13; P < 0.0001).

To test whether the attraction of nematodes is specific to (*E*)-β-caryophyllene, we compared it with another sesquiterpene olefin, (*E*)-β-farnesene, the sesquiterpene alcohol (*E*)-nerolidol, the monoterpene alcohol linalool, and a common volatile originating from the lipoxygenase pathway, (*Z*)-3-hexenyl acetate (Figures 3A and 3B). With the exception of (*E*)-β-farnesene, the remaining plant volatile compounds were less attractive [$F_{3,44}$ = 9.39; P < 0.001 for (*E*)-β-farnesene and (*Z*)-3-hexenyl acetate, $F_{3,68}$ = 8.26; P < 0.001 for (*E*)-nerolidol and linalool] than (*E*)-β-caryophyllene, indicating that (*E*)-β-caryophyllene is particularly effective at promoting plant–nematode interaction.

To test for a possible aboveground role of (*E*)-β-caryophyllene in attracting the parasitic wasp *C. marginiventris*, we used the pure compound for bioassays in a four-arm olfactometer. Naive wasps without any oviposition experience were not attracted ($F1.82$ = 0.98; P = 0.32). However, wasps preferred air containing (*E*)-β-caryophyllene to pure air ($F1.82$ = 52.06; P < 0.0001)

after they had experienced laying eggs in host larvae while perceiving (*E*)-β-caryophyllene (Figure 4). This effect of associative learning was reflected in a significant treatment effect ($F1.166$ = 34.04; P < 0.0001) and a significant treatment–experience interaction ($F1.164$ = 13.49; P < 0.001). These results confirm that (*E*)-β-caryophyllene by itself is a key attractant for the nematodes, whereas above ground this bicyclic sesquiterpene olefin is a component of a blend of leaf volatiles produced after herbivore damage that can be perceived and learned as a host location cue by herbivore parasitoids, as has been found for several other volatiles within this blend (D'Alessandro et al., 2006; Schnee et al., 2006).

Cloning of the Maize Terpene Synthase Gene *tps23*

To find the terpene synthase gene(s) producing (*E*)-β-caryophyllene from farnesyl diphosphate in maize, we screened a public maize genome database (http://maize.tigr.org/) for sequences with similarity to known terpene synthases. One of the resulting fragments, AZM4_53695, contained the two exons flanking the last intron of a putative sesquiterpene synthase gene. The 5′ end of this fragment was extended using a cDNA library from herbivore-induced leaves of the maize cultivars Graf and Delprim to obtain the complete open reading frame (ORF). Both cDNAs contained an identical ORF of 1644 bp designated as *tps23* that encodes a protein with a predicted molecular mass of 63.6 kD. Numerous amino acid motifs throughout its sequence are highly conserved among plant terpene synthases (Figure 5). The most characteristic element is an Asp-rich DDxxD motif in the C-terminal part that was implicated in the binding of the divalent metal
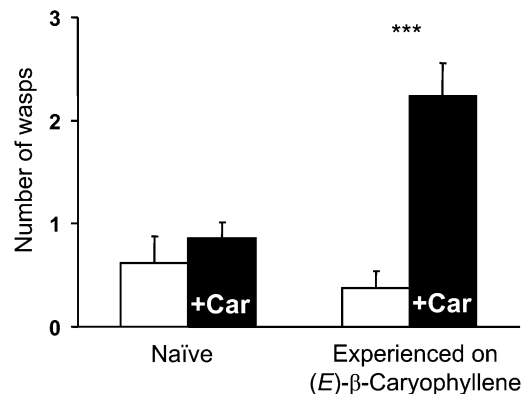
Figure 4. The Parasitic Wasp *C. marginiventris* Is Attracted by (*E*)-β-Caryophyllene after Previous Oviposition Experience.

Responses of the parasitic wasp *C. marginiventris* to (*E*)-β-caryophyllene. The attraction of parasitoid females to a pure standard of (*E*)-β-caryophyllene was tested in a four-arm olfactometer. Two groups of parasitoids were tested: naive wasps and wasps with a previous oviposition experience on host larvae in the presence of (*E*)-β-caryophyllene. The parasitoids were tested in groups of six (*n* = 14). The asterisks indicate a significant preference (P < 0.0001) of experienced wasps for the odor of (*E*)-β-caryophyllene (black bars) versus pure air (white bars show average preference for one arm). Naive wasps did not show this preference. Means and SE are shown.
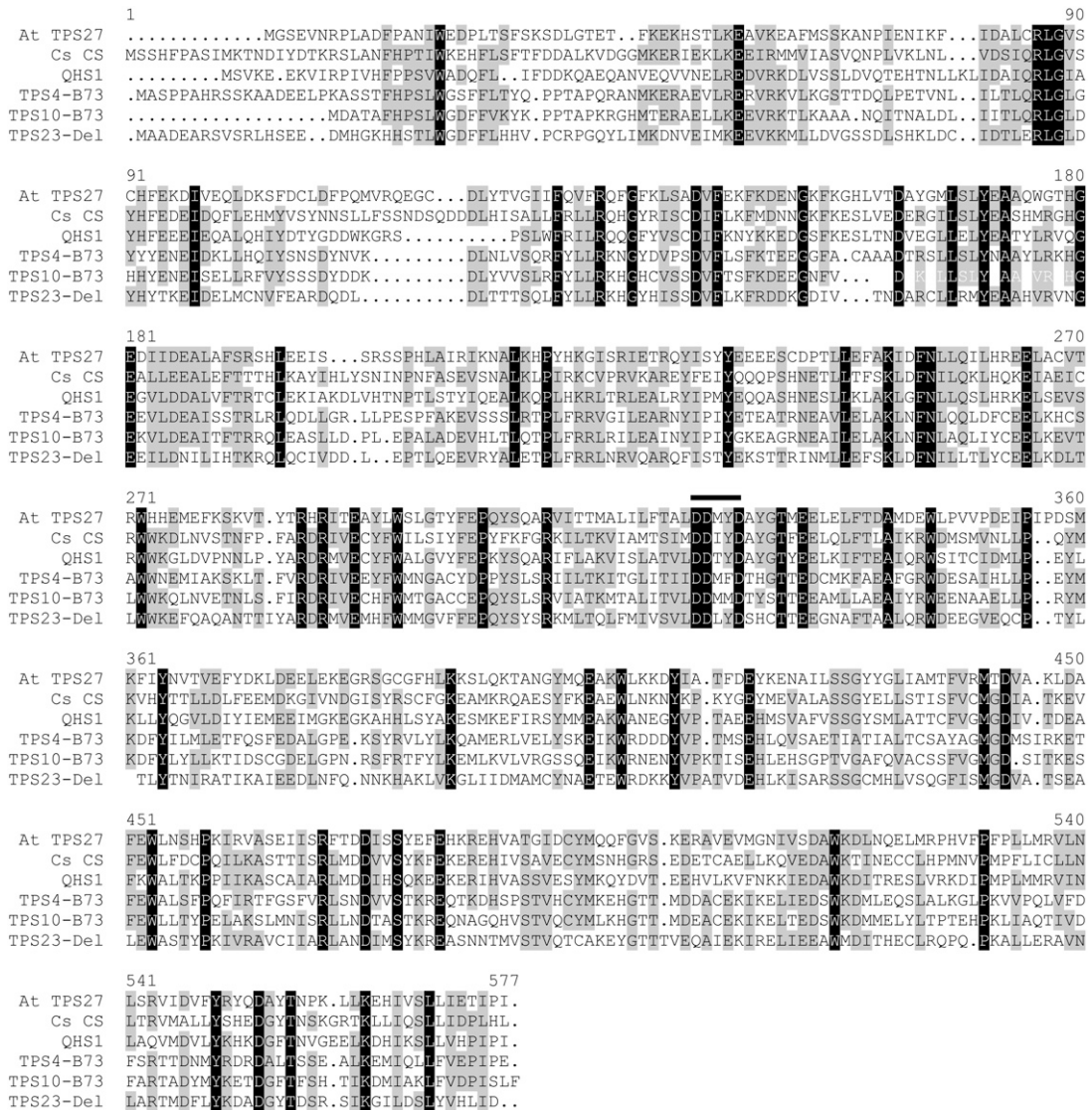
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              1                                                                          90
  At TPS27   .............MGSEVNRPLADFPANIWEDPLTSFSKSDLGTET..FKEKHSTLKEAVKEAFMSSKANPIENIKF...IDALCRLGVS
     Cs CS   MSSHFPASIMKTNDIYDTKRSLANFHPTIWKEHFLSFTFDDALKVDGGMKERIEKLKEEIRMMVIASVQNFVKLNL...VDSIQRLGVS
      QHS1   .........MSVKE.EKVIRPIVHFPPSVWADQFL..IFDDKQAEQANVEQVVNELREDVRKDLVSSLDVQTEHTNLLKLIDAIQRLGIA
   TPS4-B73  .MASPPAHRSSKAADEELPKASSTFHPSLWGSFFLTYQ.PPTAPQRANMKERAEVLRERVRKVLKGSTTDQLPETVNL..ILTLQRLGLG
  TPS10-B73  .................MDATAFHPSLWGDFFVKYK.PPTAPKRGHMTERAELLKEEVRKTLKAAA.NQITNALDL..IITLQRLGLD
  TPS23-Del  .MAADEARSVSRLHSEE..DMHGKHHSTLWGDFFLHHV.PCRPGQYLIMKDNVEIMKEEVKKMLLDVGSSDLSHKLDC..IDTLERLGLD

              91                                                                         180
  At TPS27   CHFEKDIVEQLDKSFDCLDFPQMVRQEGC...DLYTVGIIEQVFRQFGFKLSADVFEKFKDENGKFKGHLVTDAYGMLSIYEAAQWGTHG
     Cs CS   YHFEDEIDQFLEHMYVSYNNSLLFSSNDSQDDDLHISALLERLLRQHGYRISCDIFLKFMDNNGKFKESLVEDERGILSIYEASHMRGHG
      QHS1   YHFEEEIEQALQHIYDTYGDDWKGRS..........PSLWFRILRQQGFYVSCDIFKNYKKEDGSFKESLTNDVEGLLELYEATYLRVQG
   TPS4-B73  YYYENETDKLLHQIYSNSDYNVK........DLNLVSQREYLLRKNGYDVPSDVFLSFKTEEGGFA.CAAADTRSLLSIYNAAYLRKHG
  TPS10-B73  HHYENETSELLRFVYSSSDYDDK.........DLYVVSLREYLLRKHGHCVSSDVFTSFKDEEGNFV...D.....LSIYRAA...IYD
  TPS23-Del  YHYTKETDELMCNVFEARDQDL..........DLTTTSQLEYLLRKHGYHISSDVFLKFRDDKGDIV...TNDARCLLRMYEAAHVRVNG

              181                                                                        270
  At TPS27   EDIIDEALAFSRSHLEEIS...SRSSPHLAIRIKNALKHPYHKGISRIETRQYISYYEEEESCDPTILEFAKIDFNLLQILHREELACVT
     Cs CS   EALLEEALEFTTTHLKAYIHLYSNINPNFASEVSNALKLPIRKCVPRVKAREYFEIYQQQPSHNETLLTFSKLDFNILQKLHQKEIAEIC
      QHS1   EGVLDDALVFTRTCLEKIAKDLVHTNPTLSTYIQEALKQPLHKRLTRLEALRYIPMYEQQASHNESILKLAKLGFNLLQSLHRKELSEVS
   TPS4-B73  EEVLDEAISSTRLRLQDLLGR.LLPESPFAKEVSSSLRTPLFRRVGILEARNYIPIYETEATRNEAVLELAKLNFNLLQQLDFCEELKHCS
  TPS10-B73  EKVLDEAITFTRRQLEASLLD.PL.EPALADEVHLTLQTPLFRRLRILEAINYIPIYGKEAGRNEAILELAKLNFNLAQLIYCEELKEVT
  TPS23-Del  EEILDNILIHTKRQLQCIVDD.L...EPTLQEEVRYALETPLFRRLNRVQARQFISTYEKSTTRINMLLEFSKLDFNILLTLYCEELKDLT

              271                                                           360
  At TPS27   RWHHEMEFKSKVT.YTRHRITEAYLWSLGTYFEPQYSQARVITTMALILFTALDDMYDAYGTMEELELFTDAMDEVLPVVPDEIEIPDSM
     Cs CS   RWWKDLNVSTNFP.FARDRIVECYFWILSIYFEPYFKFGRKILTKVIAMTSIMDDIYDAYGTFEELQLFTIAIKRQDMSMVNLLE..QYM
      QHS1   RWWKGLDVPNNLP.YARDRMVECYFWMSVALGVYFEPKYSQARIFLAKVISLATVLDDIYDAYGTYEELKIFTEAIQRSITCIDMLP..EYL
   TPS4-B73  AWWNEMIAKSKLT.FVRDRIVEEYFWMNGACYDPYYSLSRIILTKITGLITIIDDMFDTHGTTEDCMKFABAFGRWDESAIHLLE..EYM
  TPS10-B73  LWWKQLNVETNLS.FIRDRIVECHFWMTGACCEPQYSLSRVIATKMTALITVLDDMMDTYSTTEEAMLLAEAIYRMEENAAELLE..RYM
  TPS23-Del  LWWKEFQAQANTTIYARDRMVEMHFWMMGVFFEPQYSYSRKMLTQLFMIVSVLDDLYDSHCTTEEGNAFTAALQRWDEEGVEQCE..TYL

              361                                                                        450
  At TPS27   KFIYNVTVEFYDKLDEELEKEGRSGCGFHLKKSLQKTANGYMQEAKWLKKDYIA.TFDEYKENAILSSGYYGLIAMTFVRMTDVA.KLDA
     Cs CS   KVHYTTLLDLFEEMDKGIVNDGISYRSCFGKEAMKRQAESYFKEABWLNKNYKP.KYGEYMEVALASSGYELLSTISFVCMGDIA.TKEV
      QHS1   KLLYQGVLDIYIEMEEIMGKEGKAHHLSYAKESMKEFIRSYMMEAKWANEGYVP.TAEEHMSVAFVSSGYSMLATTCFVCMGDIV.TDEA
   TPS4-B73  KDFYILMLETFQSFEDALGPE.KSYRVLYLKQAMERLVELYSKEIKWRDDDYVP.TMSEPHLQVSAETIATIALTCSAYAGMDMSIRKET
  TPS10-B73  KDFYLYLLKTIDSCGDELGPN.RSFRTFYLKEMLKVLVRGSSQEIKWRNENYVPKTISEPHLEHSGPTVGAFQVACSSFVGMGD.SITKES
  TPS23-Del  TILYTNIRATIKAIEEDLNFQ.NNKHAKLVKGLIIDMAMCYNAETEWRDKKYVPATVDEPHLKISARSSGCMHLVSQGFISMGDVA.TSEA

              451                                                                        540
  At TPS27   FEWLNSHEKIRVASEIISRFTDDISSYEFEHKREHVATGIDCYMQQFGVS.KERAVEVMGNIVSDAWKDLNQELMRPHVFFFPLLMRVLN
     Cs CS   FEWLFDCEQILKASTTISRLMDDVVSYKFEKEREHIVSAVECYMSNHGRS.EDETCAELLKQVEDAWKTINECCLHPMNVEMPFLICLLN
      QHS1   FKWALTKPPIIKASCAIARLMDDIHSQKEPKERIHVASSVESYMKQYDVT.EEHVLKVFNKKIEDAWKDITRESLVRKDIPMPLMMRVIN
   TPS4-B73  FEWALSFPQFIRTFGSFVRLSNDVVSTKREQTKDHSPSTVHCYMKEHGTT.MDDACEKIKELIEDSWKDMLEQSLALKGLPKVVPQLVFD
  TPS10-B73  FEWLLTYPELAKSLMNISRLLNDTASTKREQNAGQHVSTVQCYMLKHGTT.MDEACEKIKELTEDSWKDMMELYLTPTEHPKLIAQTIVD
  TPS23-Del  LEWASTYPKIVRAVCIIARLANDIMSYKREASNNTMVSTVQTCAKEYGTTTVEQAIEKIRELIEEAWMDITHECLRQPQ.PKALLERAVN

              541          577
  At TPS27   LSRVIDVFYRYQDAYTNPK.LLKEHIVSLLIETIPI.
     Cs CS   LTRVMALLYSHEDGYTNSKGRTKLLIQSLLIDPLHL.
      QHS1   LAQVMDVLYKHKDGFTNVGEELKDHIKSLLVHPIPI.
   TPS4-B73  FSRTTDNMYRDRDALTSSE.ALKEMIQLLFVEPIPE.
  TPS10-B73  FARTADYMYKETDGFTFSH.TIKDMIAKLFVDPISLF
  TPS23-Del  LARTMDFLYKDADGYTDSR.SIKGILDSLYVHLID..
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**Figure 5.** Comparison of the Deduced Amino Acid Sequence of TPS23-Del with Other Terpene Synthases.

The sequence of TPS23 was compared with sequences of (*E*)-β-caryophyllene synthases from other plants (*Arabidopsis* At TPS27, *C. sativus* Cs CS, and *A. annua* QHS1) and two sesquiterpene synthases from maize (TPS10-B73 and TPS4-B73). Amino acids identical in all six proteins are indicated by black boxes. Amino acids identical in at least four proteins or representing conservative changes are highlighted with gray boxes. The highly conserved DDxxD region is marked with a bar.

cofactor in a sesquiterpene synthase from tobacco (*Nicotiana tabacum*) (Starks et al., 1997). The deduced amino acid sequence of TPS23 shows similarities to sequences of other terpene synthases from maize, for example, 40.5% amino acid identity with TPS10 (Schnee et al., 2006) and 37.8% amino acid identity with TPS4 (Köllner et al., 2004b). Among maize terpene synthases, the sequences are most highly conserved in the regions encoding the active site, which are situated toward the C terminus (Figure 5). Considerable effort was expended to search for maize terpene synthase sequences with higher similarity by repeated PCR with

maize cDNA and rapid amplification of cDNA ends (RACE) libraries as well as searching of all available maize genomic databases. However, no genes encoding proteins with a sequence identity of >50% were found, which was further supported by DNA hybridization analysis with *tps23* as a probe under low-stringency conditions (data not shown).

tps23 Encodes an (*E*)-β-Caryophyllene Synthase

Since the product specificity of a putative terpene synthase cannot be predicted from its amino acid sequence, we cloned
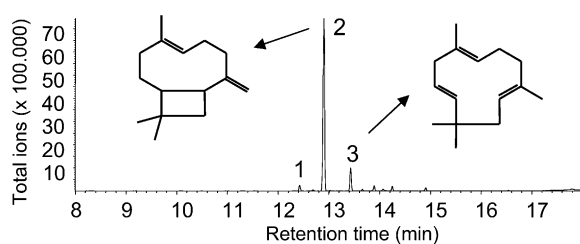
Figure 6. Sesquiterpene Products of TPS23.

The enzyme was expressed in *Escherichia coli*, extracted, partially purified, and incubated with the substrate (*E,E*)-FPP. The resulting terpene products were collected with a solid-phase microextraction (SPME) fiber and analyzed by gas chromatography–mass spectrometry. The products were identified as δ-elemene (peak 1), (*E*)-β-caryophyllene (peak 2), and α-humulene (peak 3) by comparison of their retention times and mass spectra with those of authentic standards.

tps23 into a bacterial expression system and incubated the recombinant protein with the potential substrates geranyl diphosphate (GPP; C_{10}), farnesyl diphosphate (FPP; C_{15}), and geranylgeranyl diphosphate (GGPP; C_{20}). The enzyme did not accept GPP or GGPP as a substrate (data not shown), converting only FPP to terpene products (Figure 6). The major product formed from FPP was identified as (*E*)-β-caryophyllene by mass spectrometry and cochromatography with an authentic standard, and the two minor products were α-humulene and δ-elemene. A similar spectrum of terpene products was observed from Os TPS3, an (*E*)-β-caryophyllene synthase from rice (*Oryza sativa*) (Cheng et al., 2007). Although this enzyme has 50% amino acid identity to TPS23, the present genomic information on rice and maize is not sufficient to establish homology between these two species. Caryophyllene synthases identified from dicotyledonous plants, including those from *Arabidopsis* (At TPS27), *Cucumis sativus* (Cs CS), and *Artemisia annua* (Aa QHS1), show only low amino acid identity to TPS23: 33.9, 30.3, and 35.7%, respectively. A dendrogram analysis demonstrates that TPS23 is much more closely related to the functionally unrelated terpene synthases of maize than to terpene synthases of similar function in other plant species, suggesting a repeated evolution of the ability to make (*E*)-β-caryophyllene in monocotyledonous grasses and dicotyledonous plants (Figure 7; see Supplemental Data Set 1 online).

Biochemical Characterization of TPS23

The biochemical properties of TPS23 were determined with purified enzyme and tritium-labeled FPP substrate. The enzyme exhibited a broad catalytic optimum from pH 8.0 to 9.5 but still showed substantial activity at typical cytoplasmic pH conditions (Figure 8). A divalent metal ion cofactor was required for enzyme activity (Figure 9). A range of potential cofactor species were tested at different concentrations, with Mg^{2+} ions at a concentration of 10 mM and Mn^{2+} ions at a concentration of 0.25 mM giving maximal activities. The K_m values were 183 ± 34 and 28 ± 6 μM for Mg^{2+} and Mn^{2+}, respectively (Table 1). Both the K_m and the k_{cat} values for the FPP substrate were similar to those of most

characterized plant sesquiterpene synthases identified to date (Chen et al., 1996; Crock et al., 1997; Picaud et al., 2005, 2006).

Herbivory above and below Ground Induces Transcript Levels of *tps23* Independently

To determine whether TPS23 is involved in the herbivore-induced synthesis of (*E*)-β-caryophyllene, we measured the transcript levels of *tps23* in response to feeding damage by *S. littoralis* and *D. v. virgifera* and compared them with the expression pattern of *tps10*, a gene known to be involved in aboveground, herbivore-induced sesquiterpene synthesis in maize (Schnee et al., 2006). The (*E*)-β-caryophyllene–producing line Graf accumulated transcripts of *tps23* in the leaves only after leaf damage by *S. littoralis*, but no transcripts were detectable in roots after *S. littoralis* leaf damage (Figure 10). Conversely, root damage by *D. v. virgifera* resulted in the accumulation of *tps23* transcripts in the roots but not in the shoots. The high transcript levels in the roots of Graf correlated with the production of more (*E*)-β-caryophyllene in this line (Rasmann et al., 2005). Simultaneous feeding by both herbivores resulted in the accumulation of *tps23* transcripts in both leaves and roots. The inbred line B73, which does not emit (*E*)-β-caryophyllene, had no detectable levels of *tps23* transcript throughout the plant, indicating the lack of *tps23* transcription or low transcript stability as the cause of the lack of (*E*)-β-caryophyllene production. As expected, the transcript of the leaf-specific terpene synthase *tps10* accumulated only in response to the aboveground damage by *S. littoralis* but was not induced by belowground attack by *D. v. virgifera*. Transcripts of *tps10* are also present in caryophyllene-producing and non-caryophyllene-producing plants alike, consistent with earlier reports on the emission of TPS10 volatiles by line B73 (Köllner et al., 2004a).

Maize *tps23* and Its Teosinte Orthologs Are Maintained by Positive Selection

The appearance of (*E*)-β-caryophyllene in herbivore-induced volatiles of many grasses related to maize suggests that this
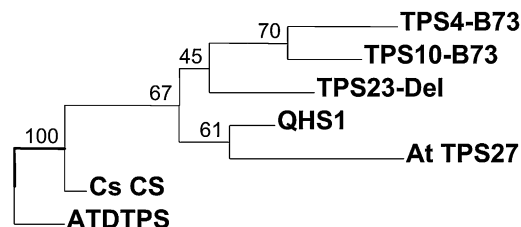
Figure 7. TPS23 Shows Low Sequence Identity to Other (*E*)-β-Caryophyllene Synthases.

Dendrogram analysis of TPS23 with two closely related maize sesquiterpene synthases (TPS10-B73 and TPS4-B73) and several (*E*)-β-caryophyllene synthases from other plants (*Arabidopsis* At TPS27, *C. sativus* Cs CS, and *A. annua* QHS1). The analysis was conducted using a neighbor-joining algorithm. Bootstrap values are shown in percentage and were generated with a sample of *n* = 1000. ATDTPS indicates ent-kaurene synthase from *Arabidopsis*, a functionally different diterpene synthase that was used as an outgroup.
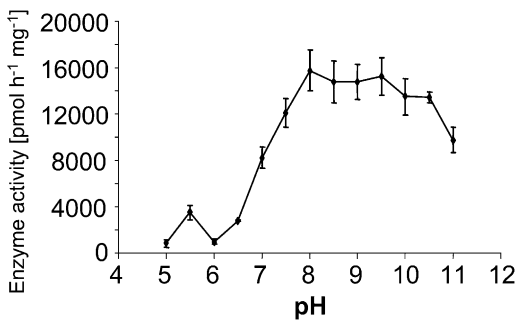
Figure 8. pH Dependence of the Enzymatic Activity of TPS23.

The catalytic activity of the purified enzyme was measured in the presence of 10 mM Mg^{2+}. The pH values were adjusted with the following buffers: pH 5.0 and 5.5, acetate buffer (100 mM); pH 6.0, MES buffer (100 mM); pH 6.5 to 9.5, bis-Tris-propane buffer (100 mM); pH 10.0 to 11, CAPS buffer (10 mM). Means ± SE of triplicate assays are shown.

compound has a widespread role in indirect defense (Gouinguené et al., 2001; Degen et al., 2004). To learn more about the evolution of (*E*)-β-caryophyllene formation, we isolated the apparent orthologs of *tps23* from six teosinte (*Zea* sp) taxa utilizing PCR (Figure 11A; see Supplemental Figure 1 and Supplemental Data Set 2 online). After expression in a bacterial system, all *tps23* apparent orthologs produced the (*E*)-β-caryophyllene main product as well as the characteristic by-products α-humulene and δ-elemene (Figure 11B; see Supplemental Figure 2 online), demonstrating complete functional conservation of *tps23* among maize and its close relatives. A dendrogram analysis of *tps23* apparent orthologs followed the phylogeny generally observed among the teosinte species (Buckler et al., 2006) and showed high levels of amino acid identity (Figure 11A). A positive selection pressure for the maintenance of (*E*)-β-caryophyllene synthase function is evident from the high average number of synonymous nucleotide changes relative to nonsynonymous changes among *tps23* from maize and its teosinte apparent orthologs (dS/dN = 6.88).

Reduced Transcription of *tps23* Prevents (*E*)-β-Caryophyllene Formation in Most North American Maize Lines

Initial studies by Degen et al. (2004) and Rasmann et al. (2005) suggested that maize lines originating from North American breeding programs have largely lost the ability to produce the (*E*)-β-caryophyllene signal. To determine the extent to which this defense trait was lost during domestication, we studied (*E*)-β-caryophyllene production in a set of 24 inbred founder lines assembled to reflect ~85% of the polymorphisms in North American maize (Liu et al., 2003). Of these 24 lines, only 2, NC358 and CML322, were found to produce (*E*)-β-caryophyllene in response to herbivore damage, suggesting that this trait is indeed largely absent from North American breeding lines (Figure 12A). The two (*E*)-β-caryophyllene–producing lines displayed high concentrations of transcripts of *tps23*, while very low levels

or no *tps23* transcripts were observed in all other lines except one (Figure 12B). This exceptional line, B97, accumulated high concentrations of *tps23* transcripts despite no (*E*)-β-caryophyllene production. Sequencing of the *tps23-B97* allele, however, showed a 2-bp insertion at position 315, which results in a frameshift that prevents the correct translation of the protein and thereby blocks (*E*)-β-caryophyllene production (Figure 12C).

To understand how domestication and breeding may have caused the loss of this defense signal, we compared the *tps23* alleles of six (*E*)-β-caryophyllene–producing lines (the hybrids Graf and Delprim and the inbred lines F2, F476, Du101, and W401) with four nonproducing lines (the hybrid Pactol and the inbred lines B73, F7001, and F670). All lines tested contained an active *tps23* allele, indicating that the lack of transcript in some of the lines is not due to differences in the ORF (Table 2). Next, the genomic structure of *tps23* was determined by analysis of the alleles *tps23-B73* and *tps23-F2*. The structure of both alleles consists of seven exons and is generally similar to that of other terpene synthases from maize (Shen et al., 2001; Köllner et al., 2004b) and class III terpene synthases from other plants (Trapp and Croteau, 2001). Unlike other terpene synthases, however, the first intron is very large and contains transposon sequences, indicating that this intron was enlarged by transposon insertion from a size of ~121 bp, usually observed in terpene synthases, to 5.6 kb (Figure 13). Since this insertion is observed in the *tps23* alleles of all lines, regardless of (*E*)-β-caryophyllene production, it too is not likely to be responsible for the inactivation of the gene in the nonproducing lines. Furthermore, we tested a 1.8-kb promoter fragment for specific differences that might regulate the transcriptional activity of *tps23* in the different maize lines. Two types of promoter sequence were found, which are distinguished by 18 single base pair changes throughout the fragment. A single base pair change that created an *Eco*RI restriction site was located 425 bp upstream of the transcription start. Among the hybrid lines, Pactol has only promoter type 1, Graf has only type 2, and Delprim has both. However, since all inbred lines had type 1 promoters regardless of their ability to produce (*E*)-β-caryophyllene, the changes in the 1.8-kb fragment did not account for the differences in the transcriptional activity of *tps23*. Similarly, the 880-bp 3′ untranslated region of all alleles was shown to be identical and therefore cannot cause differences in transcriptional activity or changes in mRNA stability.
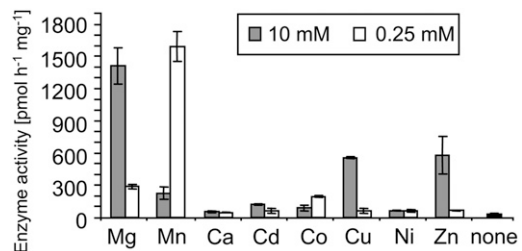
Figure 9. Metal Cofactors Affect the Enzymatic Activity of TPS23.

The catalytic activity of the purified enzyme was measured in the presence of various divalent metal ions at concentrations of 10 and 0.25 mM. Means ± SE of triplicate assays are shown.

Table 1. Kinetic Constants for TPS23-Del Heterologously Expressed in *E. coli*

Constant	FPP (10 mM MgCl$_2$)	FPP (0.25 mM MnCl$_2$)	Mg^{2+}	Mn^{2+}
K_m (μM)	3.7 ± 0.5	1.1 ± 0.3	183 ± 34	28 ± 5
k_{cat} (s^{-1})	$(1.91 \pm 0.09) \times 10^{-3}$	$(1.13 \pm 0.07) \times 10^{-3}$		

DISCUSSION

TPS23 Provides a Signal for the Attraction of Herbivore Enemies Both above and below Ground

Plants produce a large arsenal of terpenes, phenolics, and other presumed defensive metabolites whose exact functions are largely unknown. Here, we confirm that the sesquiterpene olefin (*E*)-β-caryophyllene can play a role in two spatially separate modes of induced defenses against herbivores: the attraction of parasitic wasps above ground that oviposit on lepidopteran larvae, such as *S. littoralis*, and the attraction of nematodes below ground that can attack larvae of the beetle *D. v. virgifera* (Rasmann et al., 2005). Both volatile signals are produced by a single enzyme, the terpene synthase TPS23. The attraction of the nematode to (*E*)-β-caryophyllene is innate (Rasmann et al., 2005) (Figure 2), whereas females of the parasitic wasp are attracted only after associative learning (Figure 4). (*E*)-β-Caryophyllene is one of many volatiles released by maize leaves that can serve as cues for parasitoids to find their herbivore hosts, but this specific compound is essential for attracting nematodes. A comparison with authentic versions of volatiles that are typically released from maize leaves has revealed that none diffuses as readily in sand and soil as (*E*)-β-caryophyllene (Hiltpold and Turlings, 2008), providing a possible reason why this substance is so much more attractive to the nematode than other typical maize volatiles (Rasmann et al., 2005).

(*E*)-β-Caryophyllene is also suitable for defense signaling above ground, as it diffuses rapidly in the air. However, compared with most other volatile monoterpenes and sesquiterpenes, (*E*)-β-caryophyllene is unstable in the atmosphere, reacting readily with ozone and other reactive oxygen species (Grosjean et al., 1993). Thus, (*E*)-β-caryophyllene may be diagnostic as a short-range cue for host or prey location. (*E*)-β-Caryophyllene also has antimicrobial activity (Sabulal et al., 2006) and might have been initially selected as a direct defense against pathogen attack. Its signaling function in indirect defense could have evolved secondarily.

### Maize (*E*)-β-Caryophyllene Synthase Is a Product of Convergent Evolution

The properties of maize TPS23 are similar to those of other plant caryophyllene synthases from *A. annua* (Cai et al., 2002), *C. sativus* (Mercke et al., 2004), and *Arabidopsis* (Tholl et al., 2005) in kinetic parameters and cofactor requirement. These enzymes also have a common reaction mechanism, as indicated by the formation of the same minor products, α-humulene and δ-elemene.

Despite these similarities, sequence comparisons indicate that the (*E*)-β-caryophyllene synthases are products of convergent or repeated evolution (Pichersky and Gang, 2000). TPS23 is more closely related to other maize terpene synthases than to (*E*)-β-caryophyllene synthases isolated from dicotyledons. One such closely related maize terpene synthase is TPS10, which might share with TPS23 a common ancestor involved in indirect defense. The mechanism to produce (*E*)-β-caryophyllene may subsequently have been acquired by TPS23. Such convergent evolution is probably facilitated by the ability of terpene synthases to alter product specificity in response to only a few amino acid changes (Köllner et al., 2004b, 2006; Yoshikuni et al., 2006).

Regulation of *tps23* Transcript Allows Independent Expression in Different Organs

Enzyme activities in plants are often controlled by differential regulation of the members of a gene family. In terpene biosynthesis, for example, enzymes catalyzing important regulatory steps, such as 3-hydroxy-3-methylglutaryl-CoA synthase (Enjuto et al., 1995; Daraselia et al., 1996; Korth et al., 1997), 1-deoxy-xylulose phosphate synthase (Walter et al., 2002), and isoprenyl diphosphate synthases (Cunillera et al., 1997; Okada et al., 2000), are encoded by small gene families with differential expression. However, *tps23* provides an example of a single gene with two distinct expression patterns in different organs. The aboveground induction of *tps23* is similar to that of *tps10*, a maize terpene synthase gene that is activated after herbivory by lepidopteran
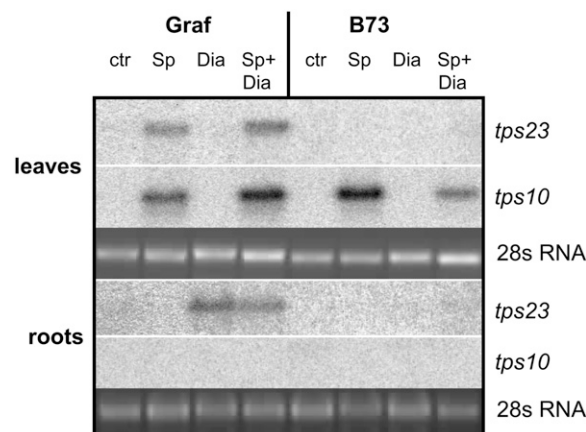
Figure 10. *tps23* Is Selectively Induced by Herbivory of *S. littoralis* and *D. v. virgifera*.

The transcript levels of *tps23* and *tps10* were determined in leaves and roots of the maize cultivars Graf and B73 after feeding of *S. littoralis* (Sp), *D. v. virgifera* (Dia), and *S. littorals* plus *D. v. virgifera* (Sp+Dia) or in undamaged controls (ctr). RNA isolated from 2-week-old plants was hybridized with probes specific for *tps23* or *tps10*. The bottom panels show the 28S RNA band of the ethidium bromide–stained RNA gels.

A

0.001 nt exchanges site^{-1}

B
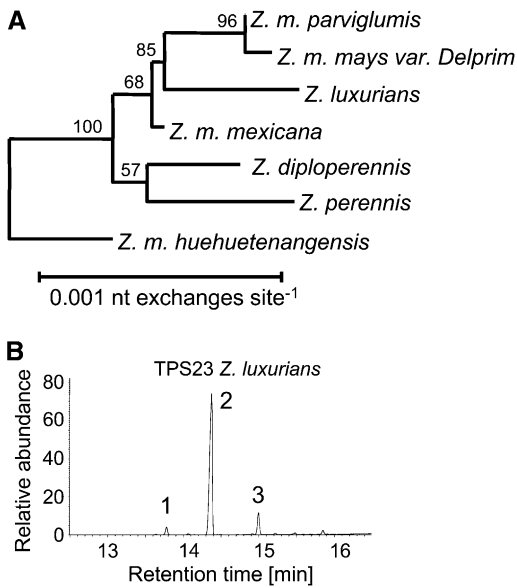
Figure 11. TPS23 Is Functionally Conserved among Relatives of Maize.

(A) Dendrogram analysis of TPS23 from maize (*Z. m. mays*) and its teosinte orthologs from *Z. m. parviglumis*, *Z. luxurians*, *Z. m. mexicana*, *Z. diploperennis*, *Z. perennis*, and *Z. m. huehuetenangensis*. The analysis was conducted as described in Methods. The teosinte *Z. m. huehuetenangensis* was the designated outgroup due to its overall distance from the other teosinte varieties used in the analysis.

(B) Sesquiterpene products of a putative TPS23 ortholog from *Z. luxurians*. The enzyme was expressed in *E. coli*, extracted, partially purified, and incubated with the substrate (*E,E*)-FPP. The resulting terpene products were collected with a SPME fiber and analyzed by gas chromatography–mass spectrometry. The products were identified as δ-elemene (peak 1), (*E*)-β-caryophyllene (peak 2), and α-humulene (peak 3) by comparison of their retention times and mass spectra with those of authentic standards.

larvae on leaves and produces most of the herbivore-induced sesquiterpene hydrocarbon volatiles of maize (Schnee et al., 2006). By contrast, only *tps23*, and not *tps10*, is activated below ground. It is conceivable that the *tps23* and *tps10* genes share the same regulatory mechanism for aboveground induction but that *tps23* contains an additional promoter element that activates the gene in the root after herbivory. We will test this hypothesis of a modular regulatory system by comparing the promoters of *tps23* and *tps10*.

(*E*)-β-Caryophyllene Emission Was Lost Due to a Decrease in *tps23* Transcription during the Breeding of North American Maize Lines

(*E*)-β-Caryophyllene is emitted from all tested maize lines from European breeding programs and from species of teosinte, the closest wild relative of maize. On the other hand, a range of inbred lines that represent ~85% of the genetic diversity of North American maize lines showed (*E*)-β-caryophyllene production in <10% of the lines. Therefore, we assume that this defensive trait was largely lost during the breeding of North American maize lines.

All lines that did not produce (*E*)-β-caryophyllene show very low or no *tps23* transcript (with one easily rationalized exception), indicating that the (*E*)-β-caryophyllene polymorphism results from differences in transcription. Differences in transcript stability can be ruled out, since the hypothetical *tps23* transcript is identical in all maize lines analyzed regardless of (*E*)-β-caryophyllene production. The strongly reduced transcription in most North American lines might be due to the inactivation of a transcription factor or the corruption of an enhancer element outside of the assayed promoter region. Whatever the identity of this factor or enhancer element, it is clearly not necessary for the activation of *tps10*, which has a very similar expression profile to that of *tps23* in leaves after *S. littoralis* attack.

The loss of defensive traits during crop domestication has frequently been postulated, but the genetic basis of this process, such as the inactivation of *tps23* expression described here, has rarely been elucidated (Sotelo, 1997). The loss of *tps23* expression might be ascribed to several causes. First, a null allele of a required transcription factor may be closely linked to a trait that is known to differ between North American and European maize lines, like flowering time. Breeding efforts to alter this trait could then have resulted in the accumulation of the null allele for (*E*)-β-caryophyllene production. Alternatively, the release of (*E*)-β-caryophyllene might be disadvantageous under conditions specific to North American agriculture and therefore may have been selected against. A possible disadvantage of (*E*)-β-caryophyllene release in this scenario could be its reported attractiveness to adult females of *D. v. virgifera* (Hammack, 2001).

The use of natural enemies, such as entomopathogenic nematodes, is an important component of many integrated pest control programs and could reduce damage by *D. v. virgifera*, an economically important maize pest that causes extensive yield losses. The failure of past efforts to control this pest with nematodes in North America (Ellsbury et al., 1996; Jackson, 1996) may be due to the lack of (*E*)-β-caryophyllene release from maize lines under cultivation. (*E*)-β-Caryophyllene release is correlated with increased nematode attraction to maize in the field (Rasmann et al., 2005). The identification of *tps23* provides a molecular tool to devise alternative strategies for *D. v. virgifera* control. The restoration of (*E*)-β-caryophyllene production in nonproducing maize lines should enhance their attractiveness to nematodes and thus increase *D. v. virgifera* mortality. We are currently transforming a non-(*E*)-β-caryophyllene–producing maize line with an (*E*)-β-caryophyllene synthase and will evaluate its performance in an agronomical setting.

Another strategy is the use of the *tps23* promoter to control the expression of toxins, such as the *Bacillus thuringensis* Cry3 Bb 1 protein, that are effective against *D. v. virgifera*. This could provide the plant with an efficient, timely, and well-localized defense against this pest.

METHODS

Plant and Insect Material

Plants of the maize (*Zea mays*) varieties B73 (KWS Seeds), Graf (Landi), Delprim (Delley Samen und Pflanzen), and Pactol (Syngenta) were kindly provided by their respective breeders. The inbred lines F2, F476, Du101,
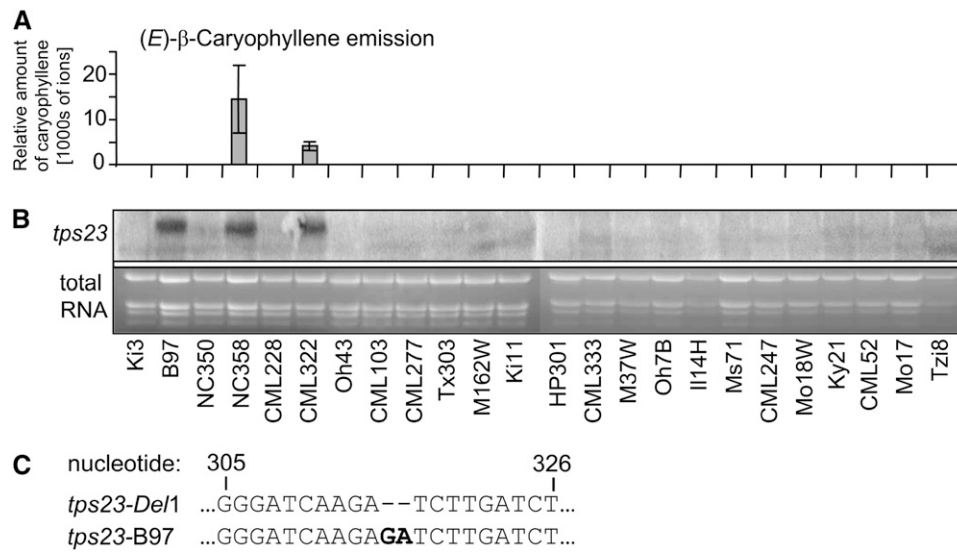
Figure 12. Most Maize Lines of North American Origin Do Not Produce (*E*)-β-Caryophyllene.

(A) A set of 24 inbred lines was tested for (*E*)-β-caryophyllene production in herbivore-damaged leaves. The averages ± SE of triplicate measurements of the (*E*)-β-caryophyllene amounts are shown.
(B) Accumulation of *tps23* transcript in herbivore-damaged leaves. The bottom panel shows the total RNA on the ethidium bromide–stained gel as a loading control.
**(C)** The *tps23* allele of the inbred line B97 contains a 2-bp insertion at nucleotide 315 compared with *tps23-Del*, which results in an inactive enzyme.

W401, F7001, and F670 and seeds of the teosinte (*Zea* sp) species were a gift from the Station de Génétique Végétale, Institut National de la Recherche Agronomique, and the 24 North American inbred lines (small diversity panel) was supplied by the National Germplasm System of the USDA Agricultural Research Service. The plants were grown in commercially available potting soil in a climate-controlled chamber with a 16-h photoperiod, 1 mmol·m^{-2}·s^{-1} photosynthetically active radiation, a temperature cycle of 22/18°C (day/night), and 65% RH. Twelve- to 15-d-old plants (20 to 30 cm high, four to five expanded leaves) were used in all experiments. Eggs of *Spodoptera littoralis* (Lepidoptera: Noctuidae) were obtained from Aventis and were reared on an artificial wheat germ diet (Heliothis mix; Stonefly Industries) for ∼10 to 15 d at 22°C under an illumination of 750 μmol·m^{-2}·s^{-1}. For the *S. littoralis* treatments, three third instar larvae were enclosed on the middle portion of each plant for 20 h 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 (Röse et al., 1996). Larvae of *Diabrotica virgifera virgifera* were obtained from CABI BioSience, and nematodes of the species *Heterorhabditis megidis* were supplied by Andermatt Biocontrol. For the *D. v. virgifera* treatment, each maize plant was subjected to four second instar or third instar larvae for 2 d. The solitary endoparasitoid *Cotesia marginiventris* that was used in the experiments originated from the USDA Agricultural Research Service, Biological Control and Mass Rearing Research Unit. For the rearing of parasitoids, 25 young caterpillars (3 to 4 d old) were offered to a single mated female (4 to 7 d old) for 3 h in a plastic box (9.5 cm diameter, 5 cm high). The caterpillars were further reared on artificial diet in an incubator (25°C, 16 h of light/8 h of dark) until cocoon formation. Cocoons were kept in Petri dishes until adult emergence. Emerging adults were sexed and kept in cages (30 × 30 × 30 cm) at a male:female ratio of 1:2, with distilled water on cotton and honey as a food

Table 2. Properties of the *tps23* Alleles and Their Promoters in the Hybrid Lines Delprim, Graf, and Pactol and the Inbred Lines B73, F2, F467, Du101, W401, F670, and F7001

Line	Caryophyllene	5.6-kb Intron	Promoter Allele 1	Promoter Allele 2	ORF Allele 1	3′ Untranslated Region Allele 1
Graf	+	+	−	+	+	+
Delprim	+	+	+	+	+	+
F2	+	+	+	−	+	+
F476	+	+	+	−	Not tested	+
Du101	+	+	+	−	Not tested	+
W401	+	+	+	−	Not tested	+
F670	−	+	+	−	Not tested	+
F7001	−	+	+	−	Not tested	+
B73	−	+	+	−	+	+
Pactol	−	+	+	−	+	+
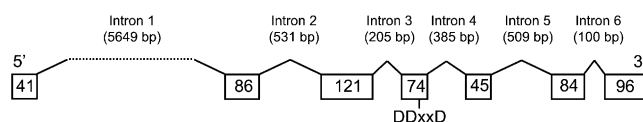
Figure 13. Exon–Intron Structure of *tps23*.

The seven exons are represented by boxes showing the number of amino acids they contain. The first intron is enlarged by the insertion of a transposon-like sequence element of ~5.4 kb. DDxxD marks the position of the Asp-rich region in the active center of the protein. The dotted line marks the transposon-like sequence included in the first intron.

source. The cages were kept in the laboratory under ambient light and temperature conditions.

Bioassays

Attraction of the nematode *H. megidis* toward (*E*)-β-caryophyllene was tested with belowground six-arm olfactometer assays (Rasmann et al., 2005). The apparatus consisted of a central glass chamber with six evenly distributed side arms that connect it to six glass pots. The entire system was filled with moist sand (10% water). To test the attractiveness of (*E*)-β-caryophyllene, amber glass vials (1.5 mL; Supelco and Sigma-Aldrich) were half filled with glass wool and 200 mL of authentic (*E*)-β-caryophyllene was added. Vials were closed with an open screw cap containing a septum, through which a 100-μL capillary (Hirschmann Laborgeräte) was inserted into the vial's saturated head space. The vial was then placed upside down with the capillary projecting into the sand in one of the outer pots of the six-arm olfactometer, ensuring a constant release of (*E*)-β-caryophyllene into the sand.

To compare the attractiveness of (*E*)-β-caryophyllene with that of other substances, an aliquot of 0.2 μL of authentic (*E*)-β-caryophyllene (98% pure; Sigma-Aldrich), (*E*)-β-farnesene (Bedoukian), linalool (95% pure; Sigma-Aldrich), nerolidol (98% pure; Sigma-Aldrich), or (*Z*)-3-hexenyl acetate (98% pure; Sigma-Aldrich) was injected into one of the glass pots, and the five untreated pots were used as controls. About 2000 *H. megidis* were released in a drop of water in the center of the central arena. Ultrafine screens at the end of each olfactometer arm prevented the nematodes from entering the pots. These arms consist of detachable parts from which nematodes can be recovered (for details, see Rasmann et al., 2005). Our study used six belowground olfactometers simultaneously. Twenty-four hours after *H. megidis* release, the belowground olfactometers were disassembled and the sand from each arm was placed on separate cotton filter discs (Hoeschele) in Bearmann extractors (Curran, 1992; Hass et al., 1999). The next day, recovered nematodes were counted.

To test the aboveground role of (*E*)-β-caryophyllene in attracting herbivore enemies, *S. littoralis* caterpillars and the solitary endoparasitoid wasp, *C. marginiventris*, were reared as described above. We tested mated, 2- to 5-d-old females, both naive and experienced individuals. Experienced females were obtained by placing them in a tube containing 20 *S. littoralis* larvae on top of a vessel that was connected via a glass capillary to a 2-mL glass vial filled with 300 μL of synthetic (*E*)-β-caryophyllene. The release rate was calibrated to the range of (*E*)-β-caryophyllene concentrations that are released by maize plants. The wasps were released in the tube one at a time and removed after three to five ovipositions. For each replicate, six wasps were provided with this oviposition experience.

The attractiveness of the (*E*)-β-caryophyllene to *C. marginiventris* females was tested in a four-arm olfactometer as described by D'Alessandro and Turlings (2005). In all experiments, the (*E*)-β-caryophyllene–releasing device was installed in the airflow of one of the four olfactometer arms. Cleaned and humidified air entered each vessel at 1.2 L/min carrying the volatiles via the arms to a central cylinder. Simultaneously, 0.6 L/min air

was pulled out through volatile collection traps containing the adsorbent Super Q (80/100 mesh; Alltech), which were connected to a port at the top of each vessel. Wasps of the same experience type were released in groups of six into the central glass cylinder and could choose to enter one of the four arms. After entering an arm, their passage was blocked by a stainless steel screen and eventually they oriented toward a light source into glass bulbs, where they were counted and removed. Wasps that did not enter an arm within 30 min were considered as having made no choice, while wasps that chose an arm were considered responsive. A total of four groups of six wasps were tested during a 3-h period, alternating between the two experience types [naive or experienced with (*E*)-β-caryophyllene]. The experiment was repeated seven times.

The behavioral responses of parasitoids and entomopathogenic nematodes to (*E*)-β-caryophyllene were analyzed with a log-linear model. The data did not conform to the simple variance assumptions implied in using the multinomial distribution. Therefore, we used quasi-likelihood functions to compensate for the overdispersion of wasps within the olfactometer and for the fact that not all of the wasps made a choice during the 30 min of the trial. The adequacy of the model was assessed through likelihood ratio statistics and examination of residuals in the software package R (R Foundation of Statistical Software, version 2.4.0; www.r-project.org) (Turlings et al., 2004; Ricard and Davison, 2007). We tested treatment effects (i.e., odor sources) for naive and experienced wasps individually. In addition, we tested for a significant effect of the experience and an interaction between treatment and experience.

Volatile Collection

For the analysis of volatile terpenes, leaf material was frozen in liquid nitrogen and pulverized in a mortar. An aliquot of 0.2 g of plant powder was placed in a glass vial with a septum in the lid. A 100-μm PDMS SPME fiber (Supelco) was inserted through the septum and exposed for 60 min at 40°C. The compounds adsorbed onto the fiber were analyzed by gas chromatography–mass spectrometry.

cDNA Library Construction

Ten-day-old maize plants of cv Delprim were subjected to herbivory by *S. 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). The mixture was treated with a Polytron (Kinematika) for 1 min and incubated for 3 min on ice. Total RNA was isolated according to the manufacturer's instructions. From ~80 μg of total RNA, the mRNA was isolated using poly(T)-coated ferromagnetic beads (Dynal). The mRNA was transcribed into cDNA, and a Marathon RACE library was constructed according to the manufacturer's instructions (Clontech).

Isolation of *tps23* cDNA from Maize and Teosinte

Sequences with high similarity to plant terpene synthases were identified in BLAST searches of The Institute for Genomic Research Maize Database (http://maize.tigr.org/). One of these fragments (AZM4_53695) was cloned, sequenced, and extended toward the 5′ end by the Marathon RACE procedure (Clontech) from a cDNA library of herbivore-induced leaves of the maize cultivars Graf and Delprim. The complete sequence, amplified with the primers BH3fwd (5′-ATGGCAGCTGATAGGCAAG-ATCCG-3′) and BH2rev (5′-TTAGTCTATTAGATGCACATACAATG-3′) from a cDNA and introduced into the sequencing vector pCR4-TOPO (Invitrogen), contains an ORF of 1644 bp. The apparent orthologs of *tps23* from the teosinte species were cloned using cDNA from herbivore-induced leaves of each of the teosinte species and the primers mentioned above. The genomic clones from *tps23* were cloned from genomic DNA of the respective maize varieties using the same set of primers. To avoid sequence errors, all putative orthologs were cloned from two independent

amplification reactions. No evidence for additional genes with high sequence identity was found in any of the teosinte species or maize varieties.

Phylogenetic Analysis

Sequence analysis was performed with the DNAstar suite of programs (Lasergene), and nucleotide substitution rates were determined with the program Syn-SCAN (http://hivdb.standford.edu/pages/synscan.html) according to the method of Nei and Gojobori (1986). For dendrogram analysis, the ORFs of (*E*)-β-caryophyllene synthases (Figure 5) and ORFs of putative *tps23* orthologs (see Supplemental Figure 1 online) were aligned with DNAstar utilizing a ClustalW algorithm (matrix, PAM250; gap penalty,10; gap length, 0.2; delay divergent sequence, 20; DNA transition weight, 0.5) with no additional adjustment. The dendrograms were created using the TREECON 1.3b software package (Van de Peer and De Wachter, 1994) using a neighbor-joining algorithm with bootstrap values from 1000 trials.

Isolation of the 5′ and 3′ Flanking Regions and Intron 1 of *tps23*

For each of the maize lines assayed, genomic DNA was prepared with the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. The Universal GenomeWalker kit (Clontech) was used to isolate a 1.8-kb DNA fragment upstream of the *tps23* open reading frame. To compare promoter alleles from different maize cultivars, the promoter fragments were amplified from genomic DNA by nested PCR using the primers BH13fwd (5′-GTTAGTCCAATATTTGTGTTGGGC-3′), BH12rev (5′-GACGGATCTTGCCTCATCAGCTGCC-3′) and BH14fwd (5′-TTCAA-CCACCAAAATTAATACTGGG-3′), BH11rev (5′-GTATACTAGCTAGCTA-CTCTCCTGC-3′), respectively, cloned into the sequencing vector pCR4-TOPO (Invitrogen), and fully sequenced. To analyze the downstream region of *tps23*, a 1.5-kb fragment containing 880 bp of the untranslated 3′ region was amplified with the primers BH31fwd (5′-GTGCTATAA-TGCCGAGACAGAATGGCGTGACAAG-3′) and BH32rev (5′-CAATTCAT-GTGGATTGGGTAGGATTGAGTGGGTTTC-3′), cloned, and sequenced as described above. To test for the presence of the unusually large intron 1, PCR was performed with the gene-specific primer BH3fwd (5′-ATGGC-AGCTGATAGGCAAGATCCG-3′) located on exon 1 and the intron-specific primer BH26rev (5′-GATCTAAGGCCGTGTTTTATTCGC-3′). The resulting 600-bp fragment was cloned and sequenced. The complete intron 1 was isolated from the inbred lines B73 and F2 using nested PCR with the primers BH3fwd, BH22rev (5′-AGTAACATTTTCTTCACCTCCTCC-3′) and BH27fwd (5′-CACAGTGAGGAGGACATGCATGGG-3′), BH21rev (5′-ATT-TCGACGTTATCCTTCATAATC-3′), respectively.

Heterologous Expression of Terpene Synthases

For expression with an N-terminal 8× His tag, the ORF of *tps23* was amplified with the primers BH8fwd (5′-ATTGCCATGGCGCAGCTGAT-GAGGCAAGATCC-3′) and BH9rev (5′-ATTAGAATTCTTAGTCTATTAGA-TGCACATAC-3′) and cloned as a *Nco*I-*Eco*RI fragment into the expression vector pHIS8-3. The construct was introduced into the *Escherichia coli* strain BL21 (DE3) and fully sequenced to avoid errors introduced by DNA amplification. Liquid cultures of the bacteria harboring the expression constructs were grown at 37°C to an OD_{600} of 0.6. Then, isopropyl-β-thiogalactopyranoside was added to a final concentration of 1 mM, and the cultures were incubated for 20 h at 18°C. The cells were collected by centrifugation and disrupted by a 4 × 30 s treatment with a sonicator (Bandelin UW2070) in chilled extraction buffer (50 mM 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 an Econopac 10DG column (Bio-Rad). For

kinetic studies, the His-tagged enzyme was further purified on a nickel-nitrilotriacetate agarose column (Qiagen) according to the manufacturer's instructions.

Assay for Terpene Synthase Activity

To determine the catalytic activity of the terpene synthase TPS23, enzyme assays containing 50 μL of the bacterial extract and 50 μL of assay buffer with 10 μM (*E*,*E*)-FPP, 10 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.2 mM $NaWO_4$, and 0.1 mM NaF in a Teflon-sealed, screw-capped 1-mL gas chromatograph glass vial were performed. A SPME fiber consisting of 100-μm polydimethylsiloxane (Supelco) was placed into the head space of the vial for a 1-h incubation at 30°C. For analysis of the adsorbed reaction products, the SPME fiber was inserted directly into the injector of the gas chromatograph.

For the determination of metal ion cofactors, K_m values, and effects of pH, an assay containing 1 μM purified TPS23 protein, 10 μM [1-^{3}H](*E*,*E*)-FPP (37 GBq/mol; American Radiolabeled Chemicals), and 10 mM $MgCl_2$ in 100 μL of assay buffer was used. The assay was overlaid with 1 mL of pentane to trap volatile products and incubated for 20 min at 30°C. The reaction was stopped by mixing, and 0.5 mL of the pentane layer was taken for the measurement of radioactivity by liquid scintillation counting in 2 mL of Lipoluma cocktail (Packard Bioscience) using a Packard Tricarb 2300TR liquid scintillation counter (^{3}H efficiency = 61%).

The pH optimum was determined in buffers from pH 5.0 to 11.0. Assay results are reported as means of three independent replicate assays, and each experiment was repeated two to three times with similar results. The K_m values were determined using seven substrate concentrations with four repetitions each. The enzyme activity was stable for at least 1 month when stored at −80°C. The concentration of the purified protein was determined by the method of Bradford (1976) using the Bio-Rad reagent with BSA as a standard.

Gas Chromatography–Mass Spectrometry

A Hewlett-Packard model 6890 gas chromatograph was employed with the carrier gas He at 1 mL/min, splitless injection (injector temperature of 220°C), a Chrompack CP-SIL-5 CB-MS column [5% (phenyl)-methyl-polysiloxane, 25 m × 0.25 mm i.d. × 0.25 μm film thickness; Varian], and a temperature program from 40°C (3-min hold) at 5°C/min 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 40 to 350 atomic mass units. Products were identified by comparison of retention times and mass spectra with authentic reference compounds.

RNA Hybridization

Plant RNA was prepared with the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. A 400-bp fragment containing the first two exons of *tps23* was used as a probe, generated by linear PCR with the primer 5′-GAACTTCAAAAATACATCAGA-3′ and the complete ORF as a template. The probe was labeled with [^{32}P]ATP using the Strip-EZ PCR procedure (Ambion). Blotting on a Nytran-Plus nylon membrane (Schleicher and Schuell), hybridization, and washing were performed (Sambrook, 1989). The blots were scanned with a Storm 840 PhosphorImager (Molecular Dynamics). All RNA hybridization experiments were performed in two biological replicates.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database. The cDNA sequences for *tps23* alleles and its promoters from the different maize lines were deposited in GenBank (http://www.ncbi.

nlm.nih.gov) with the accession numbers EU259634 (genomic sequence from inbred line B73), EU259632 (coding sequence *tps23-Graf1*), EU259633 (coding sequence *tps23-Del1*), EU259635 (coding sequence *tps23-Del2*), EU259636 (1.8-kb promoter fragment *tps23-Del1*), and EU259637 (1.8-kb promoter fragment *tps23-Del2*). The apparent orthologs of *tps23* in teosinte species have the accession numbers EU259638 (*Z. diploperennis*), EU259639 (*Z. m. huehuetenangensis*), EU259640 (*Z. luxurians*), EU259641 (*Z. m. mexicana*), EU259642 (*Z. m. parviglumis*), and EU259643 (*Z. perennis*). The accession numbers for the maize terpene synthases TPS10 and TPS4 are AAS88571 and AAX99146, respectively. The accession numbers of (*E*)-β-caryophyllene synthases from other plants are AAO85539 (*Arabidopsis* At TPS27), AAL79181 (*Artemisia annua* QHS1), AAU05952 (*Cucumis sativus*), and ABJ16553 (*Oryza sativa* Os TPS3), and the accession number of the *Arabidopsis* diterpene synthase At DTPS is NP_178064.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Amino Acid Alignment of the Putative TPS23 Orthologs from Maize and the Teosinte Species *Z. parviglumis*, *Z. luxurians*, *Z. mays mexicana*, *Z. diploperennis*, *Z. perennis*, and *Z. huehuetenangensis*.

Supplemental Figure 2. Sesquiterpene Products of the Putative TPS23 Orthologs.

Supplemental Data Set 1. Amino Acid Alignment Used to Produce the Dendrogram Presented in Figure 7.

Supplemental Data Set 2. Amino Acid Alignment Used to Produce the Dendrogram Presented in Figure 11.

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