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The sesquiterpene hydrocarbons of maize (*Zea mays*) form five groups with distinct developmental and organ-specific distributions

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Abstract

The sesquiterpene hydrocarbon chemistry of maize (*Zea mays*) inbred line B73 was analyzed by both direct solvent extraction and headspace sampling. In seedlings, 15 olefinic compounds were identified, and 21 olefins were detected in mature plants after anthesis. Both solvent extracts and collections of headspace terpenes were found to contain the same compounds in the same relative proportions suggesting that there is no selective barrier to release from plants. Approximately 25% of the stored pool was found to be released from young seedlings per hour. The individual sesquiterpenes varied extensively in their abundance among different organs and developmental stages. Compounds could be divided into five different groups such that the members of each group always occur together in the same constant ratios to one another. Each group has a distinct distribution pattern. Group A includes the two dominant compounds, (*E*)-β-farnesene and α-bergamotene, and appears only after herbivore damage in seedlings, but is constitutively present in the leaves and husks after anthesis. The major compounds of group B, including α-copaene, germacrene D and δ-cadinene, were present throughout the seedling but found only in husks of mature plants. The group C compounds, β-bisabolene and an unknown sesquiterpene olefin, are restricted to the roots. The presence of group D and E compounds was confined to the leaves and husk of mature plants. The complex sesquiterpene mixture of group D is identical to the products formed by the previously identified terpene synthase TPS4, suggesting that each of the four other sesquiterpene hydrocarbon mixtures may also represent the products of a single terpene synthase.

Keywords: *Zea mays* L.; Poaceae; Maize; Sesquiterpenes; Volatiles; Terpene biosynthesis; Terpene synthase; Volatile emission; Herbivore-induced terpene formation

1. Introduction

Plants form large numbers of structurally diverse terpene natural products (Connolly and Hill, 1991). Although terpenes play many important roles in primary metabolism, most are secondary products that occur in complex mixtures and exhibit enormous variation among and within species (Harborne and Turner, 1984). The toxicity of many terpenes to herbivorous insects and microbes implicates them in the direct defense of plants (Gershenzon and Croteau, 1991). However, terpenes are also involved in another plant defense strategy termed 'indirect defense', in which volatile terpenes released from herbivore-damaged plants attract enemies of the attacking herbivore (Dicke, 1999). As yet, little is known about the relative importance of terpenes as direct versus indirect defenses in different plant parts. A genetically-tractable model species in which terpene content could be readily modified would represent an ideal experimental system for such studies.

We have begun to investigate the formation and function of terpene natural products in *Zea mays* L. (Degenhardt and Gershenzon, 2000; Köllner et al., 2004; Schnee et al., 2002). In this species, indirect defense against herbivores has been studied in detail at the seedling stage where attack by lepidopteran larvae, such

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as *Spodoptera littoralis*, leads to the emission of terpene volatiles (Turlings et al., 1990, 1991; Turlings and Tumlinson, 1992). These volatiles attract parasitic wasps that use the larvae as hosts, thus reducing damage to the plant (Hoballah and Turlings, 2001). The volatile emission rate after herbivore attack is highest in two week-old seedlings (Turlings et al., 1991) and varies among cultivars and species of the genus *Zea* (Gouinguene et al., 2001). The role of terpenes in direct defense against maize herbivores has also been studied (Binder and Robbins, 1997; Lee et al., 1999). However, the operation of these two defense strategies in different organs and at different growth stages, especially in mature plants, is unknown. Moreover, there is little information about the terpene composition of mature maize except for single analyses of leaves, husk and kernel oil in commercial hybrid cultivars that were not precisely defined (Buttery et al., 1978; Buttery and Ling, 1984). Analyses of silk tissue from several maize cultivars did not detect terpenes (Flath et al., 1978; Zeringue, 2000).

A prerequisite for investigating the ecological role of terpenes in maize is a thorough examination of terpene composition in a genetically well-defined maize cultivar. We chose the inbred line B73 as the subject for this study since it does not exhibit allelic polymorphisms and has been established as the model variety for molecular and genetic studies of maize (Gai et al., 2000). A terpene analysis of B73 will also provide a valuable tool for study of the biochemistry and molecular biology of terpene biosynthesis in this species. Here, we report on the spatial and temporal variation of sesquiterpene hydrocarbon formation in maize line B73. The 21 identified compounds form five distinct groups in which substances co-occur in a constant ratio to one another. Each group has a distinct pattern of occurrence in different organs and developmental stages.

2. Results and discussion

2.1. Comparison of sesquiterpene content as measured by solvent extraction and headspace collection

The isolation of sesquiterpenes from maize has previously been carried out by dynamic headspace collection (Buttery and Ling, 1984; Turlings et al., 1991) and steam distillation (Thompson et al., 1974; Flath et al., 1978; Buttery et al., 1978). However, headspace collection does not sample compounds stored in plant tissue, and steam distillation is likely to produce artifacts as a result of degradation and rearrangements (Chaintreau, 2001). To overcome these limitations, we employed a simple pentane extraction to isolate stored sesquiterpenes along with dynamic headspace collection. Both treatments were carried out on two-week old seedlings of the maize inbred line B73 that had been subjected to 18 h of feeding by *Spodoptera littoralis* Boisd. (the Egyptian cotton leafworm), a treatment that increases the formation of sesquiterpenes (Hoballah and Turlings, 2001). Compounds were identified by GC–MS and quantified by GC–FID using an internal standard. Both sampling methods resulted in the isolation of the same sesquiterpene hydrocarbons in similar proportions (Fig. 1). The total sesquiterpene hydrocarbon content in the herbivore-damaged leaves was 733 ng g^{-1}, as determined by pentane extraction, while sesquiterpene emission was 170 ng h^{-1} g^{-1}, indicating that ≈25% of the stored pool was released per hour. The very similar sesquiterpene composition of the plant extract and the headspace suggests that there is no selective barrier to release. Given that the vapor pressures of the individual sesquiterpene hydrocarbons are very similar to one another (Connolly and Hill, 1991), emission may be expected to occur by passive diffusion. These substances

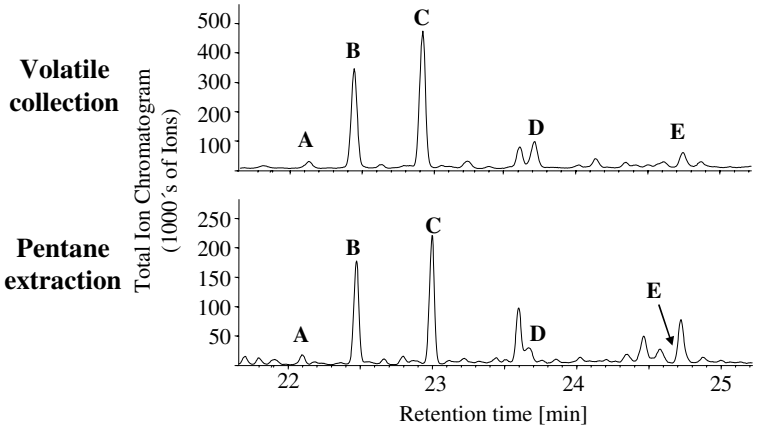
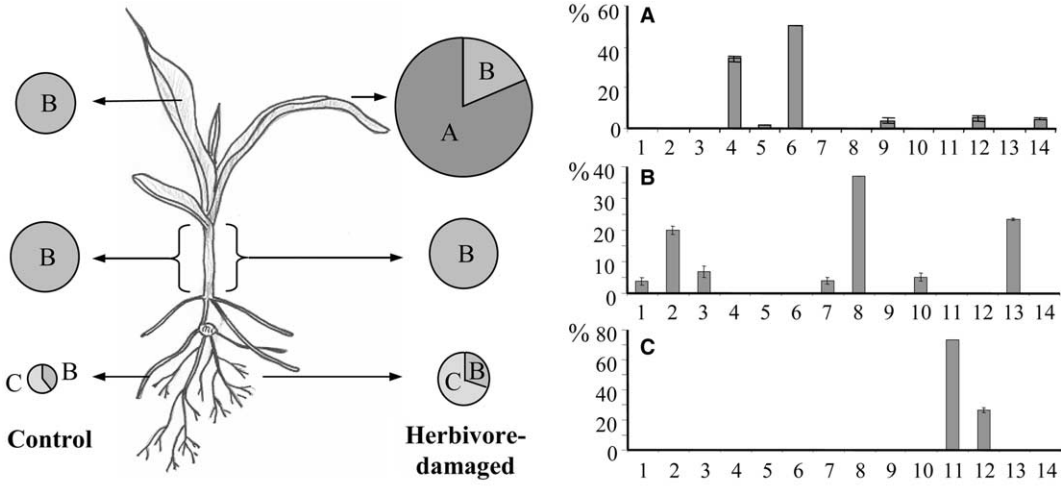


Fig. 1. Comparison of sesquiterpenes isolated from herbivore-damaged leaves of 14 day-old maize seedlings by headspace collection (a) or direct extraction (b). Compounds were separated by GC on a DB5 column and identified by MS. A: (*E*)-β-caryophyllene, B: (*E*)-α-bergamotene, C: (*E*)-β-farnesene, D: germacrene D, E: β-sesquiphellandrene.

could diffuse through the cuticle as a result of their lipophilicity or be emitted through stomata after release into internal air spaces. The high rate of sesquiterpene emission suggests that, rather than serving as a direct defense against herbivores, these compounds act as signals in indirect defense or perhaps possess other roles in protecting maize against pathogens or abiotic stresses.

2.2. Sesquiterpene content and composition of young plants

To study the sesquiterpene hydrocarbon pattern in organs of young maize plants, we analyzed two-week old seedlings of the maize inbred line B73 which were undamaged or had been fed on by *Spodoptera littoralis* for 18 h. The pentane extraction of leaf, sheath and root tissue revealed a complex pattern of 14 olefinic compounds (Fig. 2). Most of these had been previously observed in a study of terpenes in other maize varieties (Hoballah et al., 2004). The isolated sesquiterpenes could be classified into groups of substances that consistently co-occurred with each other in the same proportions in the different organs. For example, α-copaene, germacrene D and δ-cadinene were present in all assayed tissues of the plant in the same relative amounts and were designated group B (Fig. 2). The roots of the seedlings contained two additional compounds, β-bisabolene and an unknown sesquiterpene hydrocarbon whose mass spectrum could not be found in the databases for terpenes (Adams, 1995; Joulain and König, 1998). These two terpenes made up 60% of the sesquiterpene hydrocarbons in roots and were designated group C. In herbivore damaged leaves, the terpene concentration increased 5.4-fold, mostly through the accumulation of a third group of terpenes that was dominated by (*E*)-α-bergamotene and (*E*)-β-farnesene. These compounds, designated group A, form a major part of the volatile blend (Fig. 1) which has also been reported from a large number of maize cultivars and related grasses by Gouinguene et al. (2001, 2003). All varieties analyzed were found to release significant amounts of (*E*)-β-farnesene and (*E*)-α-bergamotene despite a large variation in the absolute quantity of the volatiles.

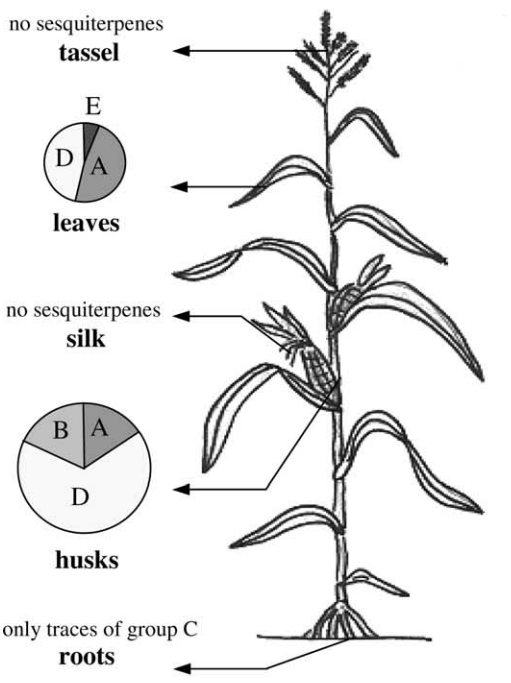


Group	Leaves		Sheath		Roots	
	Control (ng/g)	Herbivory (ng/g)	Control (ng/g)	Herbivory (ng/g)	Control (ng/g)	Herbivory (ng/g)
A	0	591 ± 144	0	0	0	0
B	135 ± 19	142 ± 50	186 ± 20	176 ± 39	10 ± 4	24 ± 10
C	0	0	0	9	15 ± 4	57 ± 22
Σ	**135 ± 19**	**733 ± 216**	**186 ± 20**	**176 ± 39**	**25 ± 7**	**81 ± 30**

Fig. 2. Sesquiterpene hydrocarbons in seedlings of the inbred line B73. Compounds were extracted from leaves, sheaths and roots of 14 day-old seedlings after treatment with *Spodoptera littoralis* for 12 h. Compounds were identified by GC–MS by comparison of mass spectra and retention times with those of authentic standards, and were quantified by comparison of FID responses to those of an internal standard. Compounds were placed in groups A through C based on co-occurrence in the same relative proportions. Graphs A through C show the relative amounts of terpenes within each respective group. The circular diagrams display the relative proportion of each group in each organ, and the table lists the total amounts of extracted terpenes. Means and standard errors result from three to six independent measurements. The compounds are listed in the order of increasing retention time. Compound 1: unknown, 2: α-copaene, 3: (*E*)-β-caryophyllene, 4: (*E*)-α-bergamotene, 5: sesquisabinene A, 6: (*E*)-β-farnesene, 7: unknown, 8: germacrene D, 9: zingiberene, 10: α-muurolene, 11: unknown, 12: β-bisabolene, 13: δ-cadinene, 14: β-sesquiphellandrene.

2.3. Sesquiterpene content and composition of mature plants

For the analysis of sesquiterpene hydrocarbons in mature maize plants, both pentane extraction and headspace collection were used. Total sesquiterpene content of mature leaves was ≈2–5 ng g^{-1}, while an average of 3.5 ng h^{-1} g^{-1} was emitted, suggesting that there was no significant storage in this tissue. To determine the sesquiterpene composition of mature plants, headspace sampling gave higher sensitivity and better resolution than pentane extraction due to a lower background of other constituents (Buttery and Ling, 1984) and was therefore chosen for presentation in Fig. 3. The 21 sesquiterpene hydrocarbons found in the mature plant include all of those previously detected in the seedling, except α-muurolene, with the addition of eight new compounds. Their distribution patterns differ from those in young plants. The terpenes forming group B, found throughout young seedlings, were confined to the husk tissue of mature plants. The characteristic blend of group A, found only in herbivore-damaged leaves of seedlings, appeared in leaves and husk tissue of the mature plant. These two tissues also contained a second, complex mixture of bisabolane-, sesquithujane- and bergamotane-type sesquiterpenes that was designated group D. Two major sesquiterpenes of group D, sesquisabinene A and β-bisabolene, are also minor components of group A, making it difficult to resolve the exact contribution of groups A and D to the total



group	leaves (ng/h/g)	husks (ng/h/g)
A	1.7 ± 0.5	1.8 ± 0.1
B	0	2.1 ± 0.7
D	1.6 ± 0.4	7.7 ± 1.7
E	0.2 ± 0.1	0
Σ	**3.5 ± 0.9**	**11.6 ± 1.9**

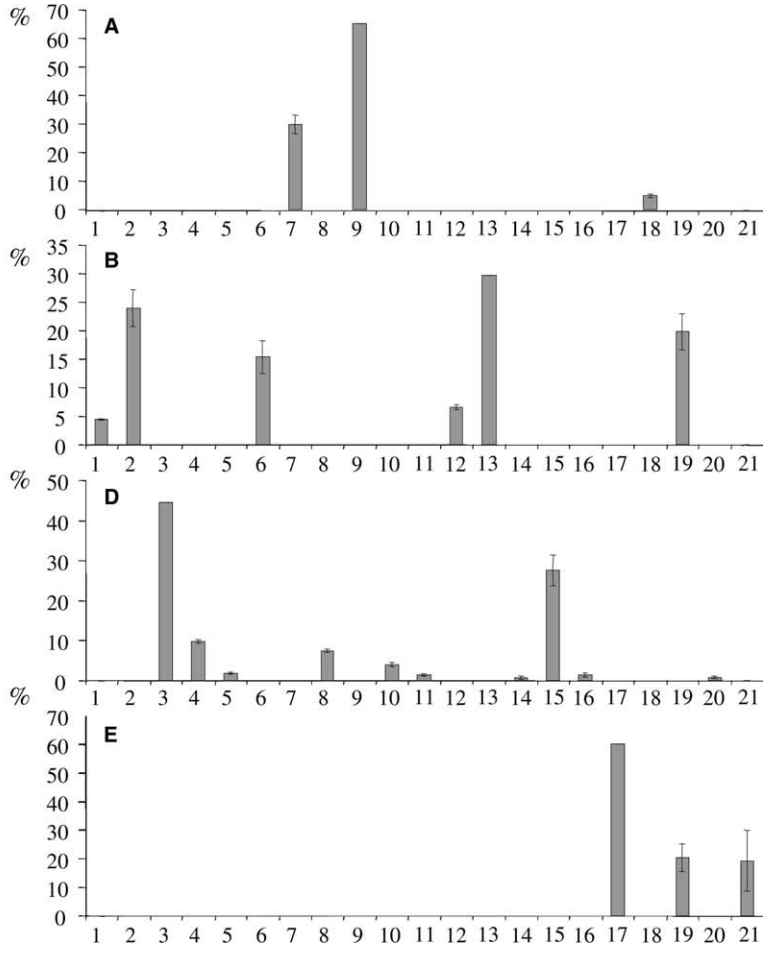


Fig. 3. Sesquiterpene hydrocarbons in mature plants of the inbred line B73. Compounds were collected from the headspace of various organs and identified and quantified as described in Section 3. Solvent extraction gave a similar composition, but the results of headspace collection analyses are presented here because these had a much lower background of other constituents, and so were easier to interpret. Compounds were placed in groups A, B, D or E based on co-occurrence in the same relative proportions. Graphs A, B, D and E show the relative amounts of terpenes within each respective group. The circular diagrams display the relative proportion of each group in each organ, and the table lists the total amounts of terpenes. Means and standard errors result from three to six independent measurements. The compounds are listed in the order of increasing retention time. Compound 1: unknown, 2: α-copaene, 3: 7-*epi*-sesquithujene, 4: sesquithujene, 5: (*Z*)-α-bergamotene, 6: (*E*)-β-caryophyllene, 7: (*E*)-α-bergamotene, 8: sesquisabinene A, 9: (*E*)-β-farnesene, 10: sesquisabinene B, 11: α-humulene, 12: unknown, 13: germacrene D, 14: zingiberene, 15: β-bisabolene, 16: (*Z*)-γ-bisabolene, 17: γ-cadinene, 18: β-sesquiphellandrene, 19: δ-cadinene, 20: (*E*)-γ-bisabolene, 21: α-cadinene.

composition of leaves and husks. In Fig. 3, sesquisabinene A and β-bisabolene are only shown as group D products. The third group of leaf sesquiterpenes in mature plants, designated group E, consists of γ-cadinene, δ-cadinene and α-cadinene but contributes less to the total terpene blend than do groups A or D. The roots of mature plants only contained low amounts of group C terpenes. No sesquiterpene hydrocarbons were detected in the male inflorescence and silk of the plant, and there were no significant changes in sesquiterpene levels caused by simulated *S. littoralis* herbivory. Since larvae of this species will not feed on mature maize plants, we mimicked herbivore damage by artificially wounding the leaves and applying oral secretions of the larvae. However, this treatment did not alter sesquiterpene emission as it did in young plants (data not shown).

Previous studies on the terpene content of mature maize plants examined isolated organs from a variety of commercially available hybrids. From leaves of the variety 'Jubilee', a blend of eight sesquiterpene hydrocarbons was identified (Udayagiri and Jones, 1992) and eleven sesquiterpene hydrocarbons were described in the commercial varieties 'Bonanza' and 'Stylepak' (Buttery and Ling, 1984). Whereas the major leaf sesquiterpenes of B73 were 7-*epi*-sesquithujene, (*E*)-β-farnesene, (*E*)-α-bergamotene and β-bisabolene, 'Jubiliee', 'Bonanza' and 'Stylepak' produced mostly cyclosativene and α-ylangene, neither of which could be detected in B73. The commercial varieties also contained much higher concentrations of β-caryophyllene than B73. The high variation in terpene composition in maize indicates that this trait is very polymorphic. In accordance with earlier studies, we found a four to fivefold higher sesquiterpene hydrocarbon concentration in husks than in leaves (Buttery and Ling, 1984) and no terpenes in the silk (Flath et al., 1978; Zeringue, 2000).

The results presented here document significant changes in sesquiterpene hydrocarbon content during maize development. Total sesquiterpene content declined dramatically, while the simple pattern of groups A, B and C in young seedlings is replaced by a more complex pattern with five groups in mature plants. In addition, groups A and B changed their distribution patterns. In the case of group A, which was formed in young seedlings only after herbivore damage, compounds were present constitutively in mature plants, although we cannot exclude that they may have been induced by abiotic stress or mechanical damage during their growth in a pest-free green house. Changes in sesquiterpene content and composition during development have been noted in many other plant species (e.g., Chou and Mullin, 1993; Flesch et al., 1992) with mature plants frequently exhibiting much lower sesquiterpene content than younger plants (Alonso-Amelot et al., 1992; Haraguchi et al., 1993).

2.4. Sesquiterpene co-occurrence and biosynthetic relationships

The relatively constant proportions of sesquiterpene hydrocarbons within each of the five groups suggest that the members of each group share a similar mode of biosynthesis. Sesquiterpenes are derived from the C_{15} acyclic intermediate, farnesyl diphosphate, by catalysis of sesquiterpene synthases (Cane, 1999; Gershenzon and Kreis, 1999). A striking feature of these enzymes is their tendency to form multiple products from the common substrate farnesyl diphosphate, often with a wide variation of basic carbon skeletons. Three members of the sesquiterpene synthase gene family in maize have been characterized recently, of which two are reported to encode enzymes forming multiple products (Köllner et al., 2004; Schnee et al., 2002). The products of one enzyme, terpene synthase 4 (TPS4) are identical and in the same relative proportions as the constituents of group D in mature plants (Köllner et al., 2004). In fact, transcripts of the corresponding gene *tps4* accumulate to high levels in husks of mature plants, a pattern very similar to that observed for the occurrence of group D products. TPS4 thus seems responsible for the formation of group D sesquiterpene olefins in mature plants as no further active terpene synthases with high sequence similarity to TPS4 were found in line B73. Each of the other four groups of sesquiterpenes in the maize plant could also represent the products of a single terpene synthase.

2.5. Sesquiterpene variation and plant defense

If the sesquiterpene olefins of maize have a role in plant defense, one might expect the complex organ-specific and developmental patterns observed to have been selected for by herbivore pressure. Optimal defense theory predicts that plants evolve to allocate defenses in a way that maximises reproductive fitness. Organs and developmental stages should have higher levels of defenses if they are more susceptible to herbivores or pathogens, or have higher value to the plant (Rhoades, 1979). In agreement with this prediction, we observed high levels of terpenes in young seedlings, a stage reported to be most susceptible to herbivore attack (Hoballah and Turlings, 2001), with the highest levels being attained right after herbivore damage had been initiated. In older plants, the highest sesquiterpene hydrocarbon levels were found in husks, which protect the developing seeds, probably the most valuable organs of this annual plant. In contrast, the leaves of mature plants, which are less valuable for reproductive fitness than the seeds, had very low levels of terpenes. This study showed that the proportion of sesquiterpenes volatilized per unit time represents a very substantial fraction of total sesquiterpene content, regardless of plant age, suggesting that

terpene-mediated indirect defense may be consistently more important than direct defense in line B73.

Leaves were not the only organs showing increased sesquiterpene content after herbivore attack in this study. Herbivore damage to the aerial parts of the plant resulted in a substantial increase in sesquiterpene accumulation in the roots, indicating the existence of long-distance signalling between above- and below-ground organs of maize. Above-ground herbivory has been previously shown to alter the quality of roots for below-ground herbivores (Bezemer et al., 2003). However, the role of maize terpenes against subterranean herbivores and pathogens remains to be studied. Now that the sesquiterpene chemistry of the model maize line B73 has been documented, researchers interested in studying the function of these compounds in maize defense can begin to try to obtain plants with altered terpene profiles that can be tested with herbivores. By exploiting the expanding genetic resources of this line (physical and genetic maps, expressed sequence tags, transposon insertion mutants) and the recent availability of cloned terpene biosynthetic genes from B73, plants can be sought that have modified terpene composition in a uniform genetic background.

3. Experimental

3.1. Plant and insect material

Seeds of the maize (*Zea mays* L.) inbred line B73 were provided by KWS seeds (Einbeck, Germany) and 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 22/18 °C (day/night) and 65% relative humidity. Twelve to fifteen day old-plants (15–25 cm high, 4–5 expanded leaves) were used in all experiments. Eggs of *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae) were obtained from Aventis (Frankfurt, Germany) and were reared on an artificial wheat germ diet (Heliothis mix, Stonefly Industries, Bryan, TX, USA) for about 10–15 d at 22 °C under an illumination of 750 μmol $(m^2)^{-1}$ s^{-1}. For the herbivory treatments, three third 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.

3.2. Headspace collection

An automated collection system (Analytical Research Systems, Gainesville, FL, USA) 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 cm high × 20 cm diameter) 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 l 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 Super Q (80/100 mesh, Alltech, Deerfield, IL, USA), at the base of the chamber. Air was drawn through the trap at a rate of 1 l 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. All collections were performed between 10:00 AM and 2:00 PM to avoid differences due to diurnal rhythms. After the 4 h collection period, the trap was rinsed with 0.2 ml dichloromethane containing 400 ng nonyl acetate as an internal standard and the sample was analyzed by GC–MS.

3.3. Solvent extraction

The plant material was harvested, frozen in liquid nitrogen and ground to a fine powder in a mortar. Three grams of tissue were extracted with 10 ml pentane for 1 h at room temperature with constant rotation. After centrifugation at 1800 *g* for 5 min to sediment the tissue, 800 ng nonyl acetate were added to the pentane supernatant as an internal standard, and the solution cleared with 25 mg activated charcoal. The pentane was concentrated under a stream of nitrogen to a volume of 200 μl and stored at −20 °C overnight to remove waxes and other high molecular weight lipids by precipitation. The pentane phase containing the terpenes was analyzed by GC.

3.4. Gas chromatography

A Hewlett-Packard model 6890 gas chromatograph 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&W Scientific, Folsom, CA, USA) and a temperature program from 40 °C (3 min hold) at 5 °C min^{-1} to 240 °C (3 min hold). Quantification was performed with the trace of a flame

ionization detector (FID) operated at 250 °C. Peaks were compared with that of the internal standard assuming equal response factors. The coupled mass spectrometer was a Hewlett-Packard model 5973 with a quadrupole mass selective detector, transfer line temperature – 230 °C, source temperature – 230 °C, quadrupole temperature – 150 °C, ionization potential – 70 eV and a scan range of 50–400 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, USA) or Bedoukian (Danbury, CT, USA), 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 elsewhere (Köllner et al., 2004) or kindly supplied by Wilfried A. König, Hamburg (essential oils of *Oreodaphne porosa* and *Aloysia sellowii*). All analyses were performed at least six times. Means and standard errors of the FID measurements are shown.

Acknowledgements

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