Metabolic Engineering 13 (2011) 414–425

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Metabolic engineering of geranic acid in maize to achieve fungal resistance is compromised by novel glycosylation patterns

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ARTICLE INFO

Article history:
Received 2 November 2010
Received in revised form
13 January 2011
Accepted 31 January 2011
Available online 4 February 2011

Keywords:
Zea mays
Pathway engineering
Terpenoid metabolism
Metabolic profiling
Geraniol synthase
Fungal resistance

ABSTRACT

Many terpenoids are known to have antifungal properties and overexpression of these compounds in crops is a potential tool in disease control. In this study, 15 different mono- and sesquiterpenoids were tested *in vitro* against two major pathogenic fungi of maize (*Zea mays*), *Colletotrichum graminicola* and *Fusarium graminearum*. Among all tested terpenoids, geranic acid showed very strong inhibitory activity against both fungi (MIC < 46 μM). To evaluate the possibility of enhancing fungal resistance in maize by overexpressing geranic acid, we generated transgenic plants with the geraniol synthase gene cloned from *Lippia dulcis* under the control of a ubiquitin promoter. The volatile and non-volatile metabolite profiles of leaves from transgenic and control lines were compared. The headspaces collected from intact seedlings of transgenic and control plants were not significantly different, although detached leaves of transgenic plants emitted 5-fold more geranyl acetate compared to control plants. Non-targeted LC–MS profiling and LC–MS–MS identification of extracts from maize leaves revealed that the major significantly different non-volatile compounds were 2 geranic acid derivatives, a geraniol dihexose and 4 different types of hydroxyl-geranic acid-hexoses. A geranic acid glycoside was the most abundant, and identified by NMR as geranoyl-6-*O*-malonyl-β-D-glucopyranoside with an average concentration of 45 μM. Fungal bioassays with *C. graminicola* and *F. graminearum* did not reveal an effect of these changes in secondary metabolite composition on plant resistance to either fungus. The results demonstrate that metabolic engineering of geraniol into geranic acid can rely on the existing default pathway, but branching glycosylation pathways must be controlled to achieve accumulation of the aglycones.

1. Introduction

Mono- and sesquiterpenoids are the main constituents of essential oils of aromatic plants (Rohloff, 2004). They play major ecological and physiological roles in flower pollination and in responses to biotic or abiotic stress (Yu and Utsumi, 2009). In the search for natural fungicides many antifungal terpenoids were identified, but their role in plant defense remained unclear (Inouye et al., 2001; Kalemba and Kunicka, 2003). Recently, however, the potential role of the antifungal monoterpenes neomenthol and menthol to enhance plant resistance to fungal and bacterial infections has been demonstrated by overexpression and silencing experiments in pepper and Arabidopsis (Choi et al., 2008).

Fusarium graminearum and *Colletotrichum graminicola* are fungal pathogens of maize (*Zea mays*) seriously reducing grain yield and quality (Bergstrom and Nicholson, 1999; Vigier et al., 2001). *F. graminearum* causes maize ear and stalk rot, and seedling blight (Presello et al., 2006; Vigier et al., 2001), and *C. graminicola* induces stalk rot and anthracnose in most maize tissues (Bergstrom and Nicholson, 1999; Sukno et al., 2008). Several studies have examined the *in vitro* effects of essential oils against these two pathogens. For *F. graminearum*, 5 out of 37 examined essential oils inhibited fungal development, and the effects were attributed to the main components of these oils including the monoterpenoids geraniol, citral (oxidation product of geraniol) and carvacrol (Velluti et al., 2004b). For *C. graminicola*, 4 essential

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doi:10.1016/j.ymben.2011.01.011

oils from the *Cymbopogon* genus effectively inhibited its mycelial growth (Somda et al., 2007; Zida et al., 2008), and among the monoterpenes tested citral was found to be the most effective (Dev et al., 2004).

In the past years a large variety of mono- and sesquiterpenoid synthase genes from different plant species have been characterized, and many of them were used for metabolic engineering of plants (Cheng et al., 2007a; Mahmoud and Croteau, 2002; Yu and Utsumi, 2009). Production and increased emissions of target mono- and sesquiterpenoids through heterologous expression of the corresponding terpenoid synthase genes, has been achieved in various plants: geraniol in tomato fruits (Davidovich-Rikanati et al., 2007); linalool in tomato fruits, carnation flowers, Arabidopsis leaves and potato leaves (Aharoni et al., 2003, 2006; Lavy et al., 2002; Lewinsohn et al., 2001); β-pinene and γ-terpinene in tobacco (Lücker et al., 2004); limonene in mint, tobacco and lavender (Diemer et al., 2001; Lücker et al., 2004; Muñoz-Bertomeu et al., 2008; Ohara et al., 2003); nerolidol in Arabidopsis (Kappers et al., 2005); (E)-β-caryophyllene in Arabidopsis, rice and maize (Cheng et al., 2007b; Degenhardt et al., 2009); α-zingiberene in tomato fruits (Davidovich-Rikanati et al., 2008); amorphadiene in tobacco and *Artemisia annua* (Ma et al., 2009; Wu et al., 2006).

Apart from direct products of expressed terpene synthases, volatile or non-volatile derivatives are often produced as well in transgenic plants by the action of endogenous enzymes. Among the many types of different modifications, oxidation, hydroxylation and acetylation of primary terpenoid skeletons are the most common endogenous modifications. For example, when geraniol synthase or linalool synthase were overexpressed, the oxidation products of geraniol (geranial and geranic acid) or linalool (linalool oxide), the hydroxylation product of linalool (hydroxyl-linalool) and the acetylation product of geraniol (geranyl acetate) were found (Davidovich-Rikanati et al., 2007; Lavy et al., 2002; Lewinsohn et al., 2001). Modifications can also take place sequentially resulting in complex transformations. In plants overexpressing amorphadiene synthase for example, amorphadiene is hydroxylated and then oxidized into artemisinic acid and dihydroartemisinic acid (Ma et al., 2009). The volatile terpenoids may be further modified by conjugation to larger moieties such as sugar residues, which usually renders them non-volatile. Among glycosidic conjugations, until now, only glucosylation has been reported. In petunia, Arabidopsis and potato expressing recombinant linalool synthase, glycosides of linalool and hydroxyl-linalool were detected (Aharoni et al., 2003, 2006; Lücker et al., 2001). Analysis of the non-volatile metabolites is, therefore, important for a more comprehensive understanding of the metabolic fate of terpenes in plants engineered for the production of novel metabolites.

In this study, among 15 mono- and sesquiterpenoids which were tested *in vitro* against *F. graminearum* and *C. graminicola*, geranic acid was identified as the most effective one against the two maize pathogens. To produce geranic acid in maize, a geraniol synthase was cloned and overexpressed in maize. Both volatile and non-volatile metabolic profiles of transgenic plants were analyzed, and potential effects of the modified terpenoid content against these fungi were assayed.

2. Materials and methods

2.1. Antifungal compound assays

The pathogenic fungi, *F. graminearum* and *C. graminicola*, were Pioneer Hi-Bred isolates from diseased corn. Cultures of each fungus were maintained on potato dextrose agar (PDA) and stored on silica at −20 °C. Fifteen terpenoids which were pre-selected from previous experiments (data not shown) were selected in this study to determine their specific antifungal effect (Table 1).

Table 1
Effect of mono- and sesquiterpenoids on the hyphal growth of *F. graminearum* and *C. graminicola*.

	MCIC (μg mL^{-1})		MIC (μg mL^{-1})	
	CGR	FGR	CGR	FGR
Monoterpenoids				
Geranic acid	7.8	31.3	< 7.8	< 7.8
Carvacrol	125	125	7.8	15.6
Thymol	125	125	15.6	15.6
Perilla alcohol	250	250	125	62.5
Perilla aldehyde	250	500	125	31.3
Citronellal	250	> 500	62.5	62.5
Citronellol	500	500	125	62.5
Geraniol	500	500	125	125
Iso-piperitenone	500	> 500	62.5	62.5
Myrtenol	> 500	> 500	125	125
Carveol	> 500	> 500	250	62.5
Carvone	> 500	> 500	500	250
Sesquiterpenoids				
Farnesol	15.6	> 500	7.8	31.3
Nootkatone	62.5	250	15.6	31.3
Farnesal	62.5	> 500	15.6	125

MCIC (μg mL^{-1}), minimum complete inhibitory concentration, in μg mL^{-1}; MIC (μg mL^{-1}), minimum inhibitory concentration, in μg mL^{-1}; CGR, *C. graminicola*; FGR, *F. graminearum*.

Antifungal effects were studied using a dilution in agar technique (Santos et al., 2005). Each terpenoid was tested at 7.8, 15.6, 31.3, 62.5, 125, 250 and 500 μg mL^{-1}. The terpenoids were dissolved in 95% ethanol and assayed in 24-well plates. Into each well, 5 μl of terpenoid was added and quickly mixed with 500 μL of warm media (1/4 PDB+0.8% SeaPlaque-GTG agarose for *F. graminearum* and 1/4 Czapek-Dox V8+0.8% SeaPlaque-GTG agarose for *C. graminicola*). Wells containing ethanol, water or only medium, instead of terpenoids, were used as negative control. When the medium cooled down, 3 μL of spore suspension (8000 mL^{-1}) was centrally placed onto it. Wells were then sealed with double-layer of parafilm and double-layer of blotter paper. Then the plates were covered and incubated in the dark at 27 °C. The effects of terpenoids were scored after 48 h by measuring spore germination and hyphal elongation for each colony (Duvick et al., 1992). The minimum inhibitory concentration (MIC) is the concentration inhibiting at least 25% hyphal elongation, and the minimum complete inhibitory concentration (MCIC) is the lowest concentration completely inhibiting spore germination and hyphal growth.

2.2. Cloning of full-length LdGES cDNA and overexpressing it in maize

The geraniol synthase gene, *LdGES*, was isolated from *Lippia dulcis*, and the LdGES protein was expressed in *Escherichia coli* to characterize its function (for details, see Supplementary Methods). The *LdGES* was placed under the control of a maize ubiquitin promoter, and *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector (Fig. S3) was used to transform immature embryos of maize (*Z. mays*) genotype PHWWE (Pioneer Hi-Bred) using protocols described previously (Zhao et al., 2001). Maize plants transformed with a vector lacking *LdGES* were used as control.

Three T_0 transgenic plants producing the highest level of new compounds (see below) and one empty vector control plant were

crossed with untransformed PHWWE to get the T_1 generation. Plants were grown in a greenhouse at 20 °C under 18/6 h light/dark photoperiod. After headspace trapping of intact plants, T_1 Plantlets from 3 independent transgenic lines (10 plants per line) were screened by real-time PCR for the expression of *LdGES* (for primers, see below). For LC–MS, GC–MS analysis and measurements of phenotypic traits, 5 positive plantlets from each transgenic line and 5 progenies from the control line were used.

2.3. Transcript analysis

The expression level of *LdGES* was determined by real-time quantitative RT-PCR analysis as described (Schijlen et al., 2007). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (gi22302) was used as reference gene and the primers were designed as reported (Hahnen et al., 2003). For the *LdGES*, the following primers were designed by software Beacon Designer (Palo Alto, CA, USA): forward primer 5′-AATACCACCAACGAGATATGCTAC-3′ and reverse primer 5′-TCCACCATTGAACCACTTTGC-3′ with an expected product size of 129 bp.

2.4. Volatile GC–MS analysis

Volatiles from the headspace of intact plants were collected from 2-week old T_1 and control seedlings in a dynamic headspace trapping system. Hereto, each seedling was placed in a 2-L glass cuvet in a climate chamber at 20 °C. The cuvets were closed with a Teflon-lined lid with a Tenax cartridge (140 mm × 4 mm; 150 mg Tenax [20/35 mesh; Alltech, Breda, The Netherlands]) to purify incoming air and a second Tenax cartridge (140 mm × 4 mm) on the outlet to trap the volatiles. Air was pumped by a vacuum pump through the glass cuvet at about 100 mL min^{-1}. The volatiles were sampled for 2 h.

Volatiles from cut leaves were collected from 8-week old plants. Leaf tips (about 10 cm long) from the second top leaf were harvested and immediately transferred into a 10-mL glass vial filled with tap water. The vial was placed in a 1-L glass cuvet in the climate chamber and sampled as above.

The volatiles trapped in the outlet cartridges were analyzed by Thermodesorption GC–MS using a thermal desorber (Unity, Markes International Limited) and a Trace GC Ultra (Thermo Electron Corporation) coupled with a DSQ mass spectrometer (Thermo Electron Corporation). The cartridges were first purged to waste for 5 min at room temperature using helium at a flow-rate of 30 mL min^{-1} to remove free water and oxygen. Trapped volatiles were then released from the Tenax material in the thermal desorber at 250 °C for 4 min. Volatiles were then collected on a cold trap at 10 °C and desorbed by increasing the temperatures from 10 to 250 °C at 12 °C s^{-1}, and a hold at 250 °C for 2 min. The column used for chromatography was an Rtx-5 ms column (Restek, 30 m × 0.25 mm i.d., 1 μm d.f.). The temperature program of the gas chromatograph was 40 °C for 3.5 min, rising to 280 °C at 10 °C min^{-1} and final time for 2.5 min. The mass spectrometer was set to scan from 35 to 450 *m/z*. The helium flow was constant at 1.0 mL min^{-1}. Ionization potential was set at 70 eV.

For identification, the authentic standards of geraniol, geranyl acetate and geranic acid (present in a mixture of geranic acid and its isomer—neric acid) were run under identical conditions.

2.5. Non-volatile LC–MS analysis

Non-volatile compounds were analyzed using a protocol for untargeted metabolomics of plant tissues (De Vos et al., 2007). In brief, 200 mg young leaf tip from each plant was ground in liquid nitrogen and extracted with 0.6 mL methanol:formic acid (1000:1,v/v). The extracts were prepared by brief vortexing and sonication for 15 min. Then the extracts were centrifuged and filtered through 0.2 μm inorganic membrane filters (RC4, Sartorius, Germany). LC–PDA–MS analysis was performed using a Waters Alliance 2795 HPLC connected to a Waters 2996 PDA detector and subsequently a QTOF Ultima V4.00.00 mass spectrometer (Waters, MS technologies, UK) operating in negative ionization mode. The column used was an analytical column (2.0 mm × 150 mm; Phenomenex, USA) attached with a C18 pre-column (2.0 mm × 4 mm; Phenomenex, USA). Degassed eluent A (ultra pure water:formic acid [1000:1,v/v]) and eluent B (acetonitril:formic acid [1000:1,v/v]) were pumped at 0.19 mL min^{-1} into the HPLC system. The gradient started at 5% B and increased linearly to 35% B in 45 min. Then the column was washed and equilibrated for 15 min before the next injection. The injection volume was 5 μL. The MS–MS measurements were done with following collision energies of 10, 15, 25, 35 and 50 eV. Leucine enkaphalin ($[M-H]^- = 554.2620$) was used as a lock mass for on-line accurate mass correction.

2.6. GC–MS and LC–MS data processing

GC–MS data were acquired using Xcalibur 1.4 (Thermo Electron Corporation) and LC–MS data using MassLynx 4.0 (Waters). The data were then processed using MetAlign version 1.0 (www.metAlign.nl) for baseline correction, noise elimination and subsequent spectral data alignment (De Vos et al., 2007). The processing parameters of MetAlign for GC–MS data were set to analyze from scan numbers 1340–16,000 (corresponding to retention time 2.32–28.05 min) with a maximum amplitude of 1.4×10^8. The parameters for LC–MS data were set to analyze from scan numbers 70–2620 (corresponding to retention time 1.4–49.73 min) with a maximum amplitude of 35,000.

In order to elucidate which mass signals originate from the same metabolite, all the detected masses were clustered by an in-house developed software package based on a Multivariate Mass Spectra Reconstruction (MMSR) approach (Tikunov et al., 2005). The mass signal intensities (expressed as peak height using metAlign) obtained from transgenic plants and empty vector control plants were compared using the Student's *t*-test. Masses with a significant ($p < 0.05$) intensity change of at least 2-fold were verified manually in the original chromatograms.

To annotate significantly different compounds, accurate masses were manually calculated, taking into account only those scans with the proper intensity ratios of analyte and lock mass (between 0.25- and 2 (Moco et al., 2006)), and elemental formulae generated within 5 ppm deviation from the observed mass. In addition, mass-directed LC–MS/MS experiments were performed on differential compounds. To obtain proper MS/MS spectra only molecular ions with signal intensities higher than 500 ion counts per scan were selected.

2.7. Purification and identification of geranoyl-6-O-malonyl-β-D-glucopyranoside

Plant extracts were prepared as described above, and the most dominant new compound in the transgenic plants, later identified as geranoyl-6-*O*-malonyl-β-D-glucopyranoside, was initially purified with an analytical HPLC system coupled to a PDA detector. This purification method, however, was not suitable for NMR analysis, as the fractions collected were not sufficiently pure, mainly due to column bleeding and the multiple injections needed to purify sufficient amounts for NMR analysis.

Therefore, the compound was purified with a preparative LC–MS system, consisting of an Agilent 1200 HPLC with a flow splitter and connected to a Bruker MicroTOF MS. The split ratio was set to 1:100. The column used was an Alltima C18 (5 μm) LC column (22 mm × 150 mm). Degassed eluent A (ultra pure water:formic acid [1000:1,v/v]) and eluent B (methanol:formic acid [1000:1,v/v]) were pumped at 2 mL min^{-1} into the LC system. The gradient started at 50% B and increased linearly to 70% B in 40 min. This was the optimal gradient for separation of the mass of interest from the rest of the matrix. Then the column was washed and equilibrated for 30 min before next injection. For each measurement, 500 μL of extract was manually injected. The mass spectrum was monitored with software Hystar (version 3.2). The fraction containing the molecular ion of the desired compound ($[M-H]^-=831.33$) was manually collected in a glass tube. The plant extract and the collected fractions were kept on ice or at 4 °C, as the compound in extraction solvent was unstable at room temperature. The fraction collected from one preparative LC–MS run was freeze-dried, re-dissolved in methanol and then re-analyzed by analytical LC–MS as described above. The compound collected in this fraction eluted at the expected retention time (49.1 min) and showed the expected mass spectrum. Then, this fraction was collected from 7 sequential preparative LC–MS runs in the same way. The fractions were pooled, freeze-dried and re-dissolved in deuterated dimethyl sulfoxide (DMSO) to be analyzed by NMR. The amount of the compound purified from these 7 runs (3.5 mL extract injected) was estimated to be 20 μg.

NMR measurements were carried out with a 600 MHz Bruker Avance III NMR spectrometer equipped with a 5 mm cryoprobe. All measurements were performed at 298 K. Gradient enhanced versions were used when applicable. One and two-dimensional NMR spectra were obtained (COZY, HSQC, HMBC and TOCSY). Calibration was done relative to the solvent resonance of DMSO at 2.54 ppm. The amount was checked by integration of the intensity of the signals of the compound. The Avance III NMR instrument was calibrated by measuring the integral of reference molecules at different receiver gain values of the instrument.

2.8. Phenotypic trait measurements and fungal infection assays

Plant height and leaf numbers of T_1 maize plants were measured at the tasseling stage. Chlorophyll content of leaves was measured as previously described (Mocquot et al., 1996). The fresh kernel weights were measured directly after harvesting.

The leaf sheaths of leaf 4 or 5 of T_1 plants at the V5 stage (plants with 5 fully developed leaves) were inoculated with 50 μL of spore suspension (5×10^6 mL^{-1}) after wounding the leaf sheath on both sides about half way between edge and midrib with a small screwdriver. Plants were grown in the greenhouse. The leaf sheath was covered with plastic wrap for 5 days. Nine days after inoculation the area of lesions was measured. Fifty positive T_1 plants from 10 independent transgenic lines (5 plants per line) and 50 control plants were assayed.

2.9. Distribution of materials

Novel materials described in this publication may be available for noncommercial research purposes upon acceptance and signing of a material transfer agreement. In some cases such materials may contain or be derived from materials obtained from a third party. In such cases, distribution of material will be subject to the requisite permission from any third-party owners, licensors, or controllers of all or parts of the material. Obtaining any permission will be the sole responsibility of the requestor. Plant germplasm will not be made available except at the discretion of the owner and then only in accordance with all applicable governmental regulations.

3. Results

3.1. In vitro antifungal effect of mono- and sesquiterpenoids

We examined 12 monoterpenoids and 3 sesquiterpenoids for their antifungal activity against 2 major maize pathogenic fungi, *F. graminearum* and *C. graminicola* (Table 1). In this *in vitro* antifungal assay, every terpenoid was tested at a series of concentrations ranging from 7.8 to 500 μg mL^{-1} for 48 h to determine the minimum complete inhibitory concentration (MCIC) and minimum inhibitory concentration (MIC). The most effective growth inhibitor of both fungi was geranic acid with a MIC of less than 7.8 μg mL^{-1} and MCIC of 31.3 μg mL^{-1} for *F. graminearum* and 7.8 μg mL^{-1} for *C. graminicola* (Table 1).

Previously, concentrations ranging from 7.8 to 31.3 μg mL^{-1} (i.e. 46–186 μM) were easily achieved for monoterpene alcohols in several transgenic plants (Aharoni et al., 2003; Lücker et al., 2001). To investigate this possibility for maize, we cloned a geraniol synthase and overexpressed it in maize.

3.2. Cloning of the LdGES cDNA and introduction into maize

LdGES, a full length cDNA of a geraniol synthase gene, was cloned from *Lippia dulcis*, which is a strongly aromatic herb of the *Lamiales* family, also known as *Phyla dulcis* (Trevir.) Moldenke. The *LdGES* gene (GU136162) contains an open reading frame of 1755 nucleotides encoding a protein of 584 amino acids. Nucleotide blast against the Nr database revealed that it shared the highest degree of homology with geraniol synthase from *Ocimum basilicum* (*ObGES*; Iijima et al., 2004) (For alignment of the deduced amino acid sequences of *LdGES* and other geraniol synthases, see Fig. S1.).

The entire reading frame of *LdGES* was cloned into a vector for expression in *E. coli*. In the presence of geranyl diphosphate (GDP), the only product produced by the recombinant LdGES protein was geraniol (Fig. S2). No product was detected when the enzyme was supplied with farnesyl diphosphate (data not shown).

The coding region of the cDNA encoding LdGES was placed under the control of the strong 35S enhancer–ubiquitin–promoter

Table 2
LdGES expression levels relative to the expression level of *GAPDH* in maize T_1 transgenic plants and control plants.

Plants	Mean	SD	Plants	Mean	SD
T_1 plants			*T_1 plants*		
ger 18-1	1.31	0.09	ger 20-2	1.54	0.06
ger 18-3	1.45	0.08	ger 20-4	1.35	0.07
ger 18-4	1.57	0.07	ger 20-6	1.25	0.03
ger 18-6	1.45	0.06	ger 20-8	1.18	0.05
ger 18-7	1.48	0.12			
ger 19-2	1.26	0.07	*Control plants*		
ger 19-4	1.35	0.07	c 31-1	n.d.	n.d.
ger 19-6	1.26	0.07	c 31-4	n.d.	n.d.
ger 19-8	1.27	0.04	c 31-2	n.d.	n.d.
ger 19-9	1.83	0.05	c 31-5	n.d.	n.d.
ger 20-1	1.13	0.06	c 31-3	n.d.	n.d.

Transgene expression levels were determined by quantitative RT-PCR. n.d., not detectable. Expression levels of the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, were set to 1. Values for each sample are based on 3 technical replicates.

combination (Fig. S3) and introduced into maize plants. Plants transformed with the vector lacking *LdGES* were used as controls. Expression levels of the *LdGES* gene in T_1 transgenic plants were determined by quantitative real-time RT-PCR. Out of 30 analyzed plants (10 plants per line), 15 of them (5 plants per line) were positive with an *LdGES* transcript level from 1.13 (ger 20-1) to 1.83 (ger 19-9), relative to the level of the household gene glyceraldehyde-3-phosphate dehydrogenase (Table 2).

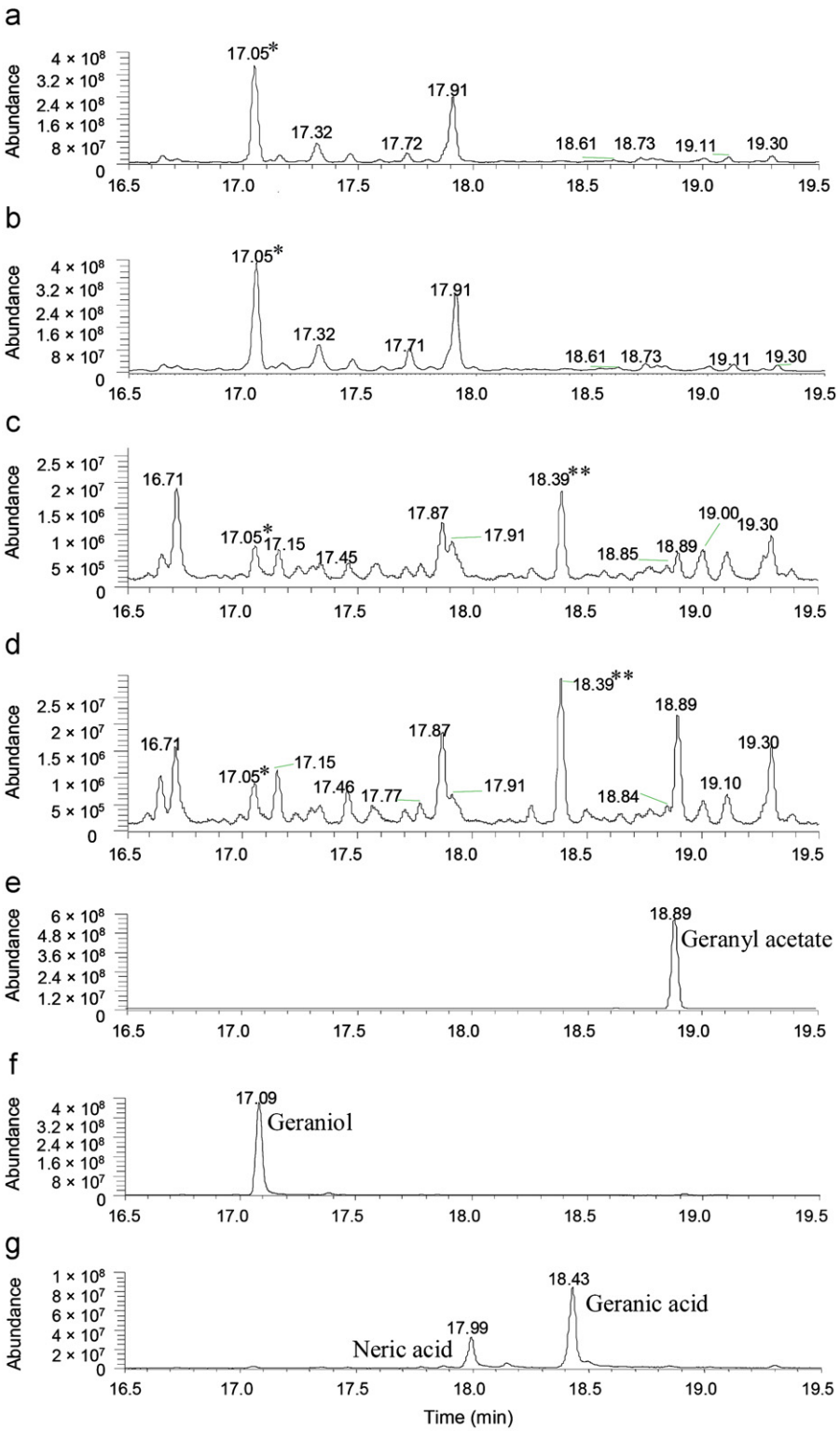

Fig. 1. GC–MS chromatograms obtained by dynamic headspace trapping of intact plants and cut leaves of control and *LdGES* expressing plants. (a) GC–MS chromatograms of headspaces trapped from intact control (b) and *LdGES* expressing plant 19-2. There was no significant difference between them. (c) GC–MS chromatograms of headspaces trapped from cut leaves of control (d) and *LdGES* expressing plant 19-2. Compared to control, cut leaves of *LdGES* expressing plant emitted 5-fold more geranyl acetate as the only significant difference. GC–MS chromatograms of authentic standards of geranyl acetate (e), geraniol (f) and geranic acid (g) (neric acid is the isomer of geranic acid). * The peak eluting at 17.05 min is not geraniol, as the mass spectrum of this peak is different from that of geraniol. Geraniol was not detected in any sample. ** The peak eluting at 18.39 min is not geranic acid, as the mass spectrum of this peak is different from that of geranic acid. Geranic acid was not detected in any sample.

3.3. Effect of LdGES on headspace emissions from intact plants and detached leaves

Volatiles were collected from the headspace of intact plants and detached leaves from transgenic and control plants at the same developmental stage and analyzed by GC–MS (Fig. 1a–d). The primary product of the LdGES protein, geraniol, was not detected in any plant analyzed (Fig. 1a–d, f). The desired anti-fungal compound, geranic acid, was not detected either (Fig. 1a–d, g). Subsequently, an untargeted metabolomics approach and comparison of all mass signals was carried out on intact and detached leaves. Only when comparing the headspace collected from detached leaves of transgenic and control plants were significant differences found. MetAlign software detected 2241 mass signals with a signal-to-noise ratio higher than 3, and that represented 191 metabolites (mass clusters) according to the MMSR clustering script (Tikunov et al., 2005). For each extracted mass, the average mass intensity was used to calculate the intensity ratio of transgenic ($n=15$) to control plants ($n=5$), and significant differences between plants were determined by the Student's t test. Among all 2241 detected masses, 120 masses (i.e. 5.4%) showed at least 2-fold intensity difference ($P<0.05$) between transgenic and control plants. More masses were found to be significantly increased (97) than decreased (23) in the transgenic plants. Differential masses were checked manually for differential peak areas in the original chromatograms, and compounds were identified by comparing to authentic standards. Geranyl acetate was the only compound found to be significantly different, showing 5.05-fold increase. Detailed analysis of the mass clusters revealed that 76 significantly increased masses corresponded to geranyl acetate. For the other 21 significantly increased and 23 decreased masses, at most 1 or 2 masses per compound were different in the t-test, while manual inspection of their chromatographic peak areas showed no significant differences. Therefore, these differential masses were regarded as noise and not significantly different between transgenic and control plants. Within transgenic plants, the emission of geranyl acetate displayed a positive correlation with the expression level of *LdGES* ($R^2=0.88$).

3.4. Effect of LdGES on the profile of non-volatile compounds from leaves

As monoterpene alcohols such as geraniol may (partly) be conjugated to other compounds rendering them non-volatile, we also analyzed the non-volatile metabolites in transgenic ($n=15$) and control plants ($n=5$). Aqueous methanol extracts from young leaves were prepared and analyzed by accurate mass LC–MS in negative mode (Fig. 2a and b). In order to reveal differential compounds, the LC–MS profiles of transgenic plants and control plants were compared in an untargeted manner using Metalign followed by MMSR clustering of extracted signals, as described above for the GC–MS profiles.

In total, 5869 mass signals were extracted, which grouped into 257 clusters representing 257 metabolites. Among all 5869 masses, 641 masses (i.e. 10.9%) showed at least 2-fold intensity difference ($P<0.05$) between transgenic and control plants. More masses were found to be significantly increased (466) than decreased (175) in the transgenic plants. Differential masses with a signal intensity higher than 500 (i.e. about 50-fold higher than the noise) were subsequently analyzed by LC–MS/MS (Table 3). According to their MS/MS spectra, these compounds were putatively identified as derivatives of geraniol: 2 different glycosides of geranic acid, 1 geraniol dihexose and 4 different isobaric forms (i.e. different retention times, but identical accurate mass) of hydroxyl geranic acid (or neric acid)-hexose. The amounts of these compounds did not correlate with the expression levels of *LdGES* (data not shown). Without authentic standards mass spectrometry has fundamental limitations in resolving the different possible chemical structures, so that it is not obvious to which carbon the hydroxyl group is attached and to which position the hexose is conjugated.

The newly produced compound that was most abundant, as determined by UV absorption (220 nm) resulting from the double bond of geranyl-compounds, showed up as m/z of 831.3287 (Fig. 2c) and eluted at a retention time of 49.12 min (Fig. 2b). In control plants, the amount of this compound is at least 2000-fold lower as it is lower than the detection limit (Fig. 2f–g). This compound was selected for further identification by LC–MS/MS and NMR.

3.5. Identification of the most distinct new compound in leaves of transgenic plants by LC–MS/MS and NMR

In order to identify the most abundant new compound in the extracts of transgenic maize plants, the apparent parent mass was fragmented by LC–MS/MS in negative mode. The selected mass 831.3287 appeared to be a $[2M-H]^-$ adduct of 415.1608. Within the MS/MS fragments of 415.1608, we detected an ion with mass 167.1076, i.e. a -0.9 ppm deviation from the elemental formula of geranic acid ($C_{10}H_{15}O_2$,). This MS/MS experiment suggested that the selected compound is a derivative of geranic acid. In order to unambiguously identify the structure of the compound, we subsequently purified the molecule for NMR analysis using preparative LC–MS. To estimate its average concentration, the molecule was purified from the transgenic plant with average mass intensity of mass 831 (the intensity in this particular plant was 2063, i.e. $<10\%$ difference from the accurate average intensity 2255.2; $n=15$). During purification, it turned out that the molecule was unstable at room temperature, posing some challenges to the purification. This was solved by keeping the plant extracts and collected fractions at 0–4 °C. Based on one and two-dimensional NMR data, we could assign the structure of the molecule to geranoyl-6-*O*-malonyl-β-D-glucopyranoside (Fig. 3 for detailed ^{13}C NMR and ^{1}H NMR data, see Table S1). This compound has not been reported in the plant kingdom before. The NMR analysis allowed us to estimate the average compound concentration in transgenic maize leaves at about 17 $\mu g\, g^{-1}$ FW. Assuming a leaf water content of 90%, this concentration corresponds to 45 μM. We propose that the most likely biosynthetic pathway of geranoyl-6-*O*-malonyl-β-D-glucopyranoside is the conversion of the product of LdGES, geraniol, into its corresponding acid, geranic acid, by two dehydrogenation/oxidation steps, followed by further stepwise conjugation of the acid moiety with malonyl-glucose (Fig. 4).

3.6. Effect of LdGES on plant phenotype and susceptibility to fungal attack

Overexpression of monoterpene synthases may be directly toxic and/or lead to reduced steady state levels of terpenoids essential in primary metabolism and plant development such as gibberellic acid and carotenoids, and result in bleached leaves and delayed plant growth (Aharoni et al., 2003, 2006; Davidovich-Rikanati et al., 2007). We, therefore, investigated the phenotype and development of the transgenic plants. We observed that the *LdGES* expressing plants were phenotypically indistinguishable from the control plants (Fig. 5). The plant height, leaf number, chlorophyll content and kernel weight of 3 transgenic lines and a control line were measured (5 plants were used per line).

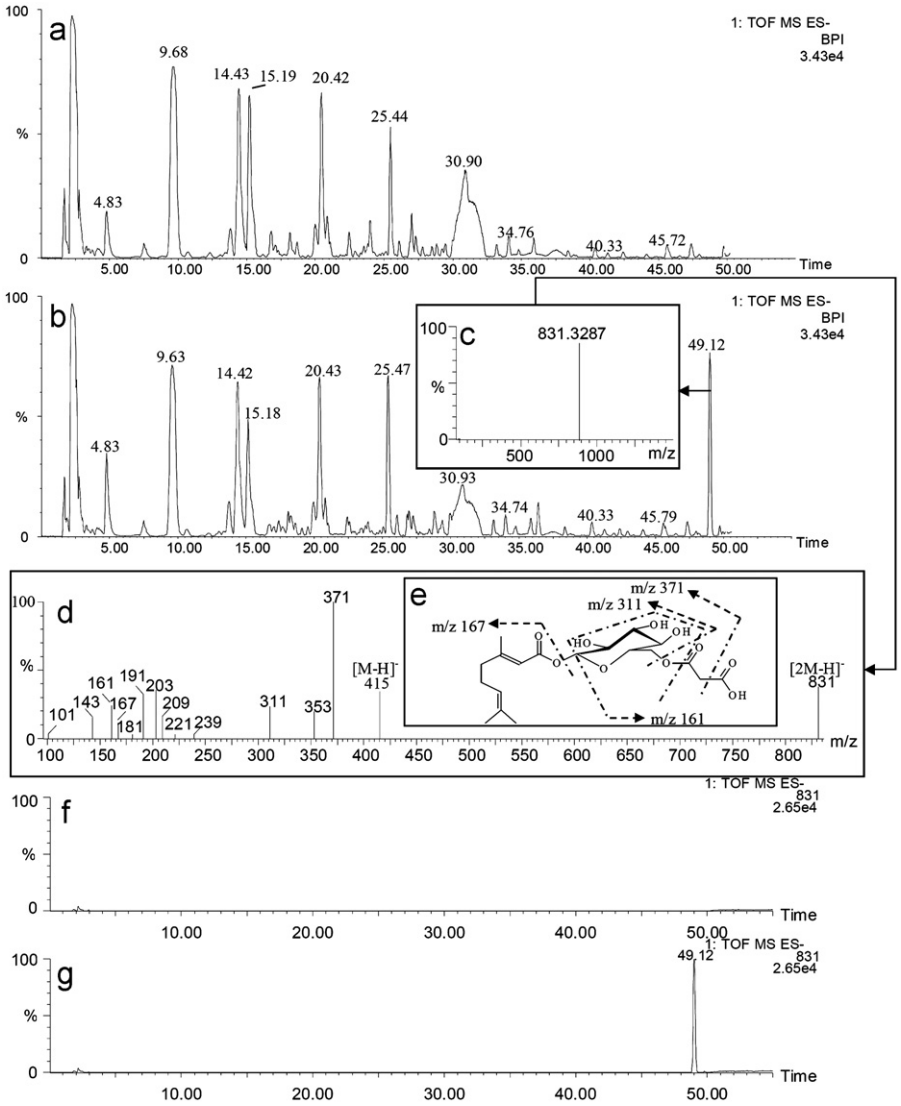

Fig. 2. Negative mode LC–QTOF–MS chromatograms of aqueous-methanol extract of leaves of an empty vector control plant (a) and *LdGES* expressing plant 19-2 (b). (c) The accurate mass of the compound eluting at 49.12 min uniquely found in the transgenic lines. (d) The MS/MS spectrum of the 49.12 min compound. (e) Scheme of collision-induced MS/MS-fragmentation of mass 415 eluting at 49.12 min, which was later identified by NMR as geranoyl-6-*O*-malonyl-β-D-glucopyranoside. The ion trace of *m/z* 831, i.e. $[2M-H]^-$, was extracted from the chromatograms of the empty vector control (f) and *LdGES* expressing plant (g).

Table 3
Non-volatile metabolite significantly increased in transgenic plants putatively identified by LC–MS–MS.

Ret (min)	Av intensity (C)	Av intensity (T)	Ratio (T/C)	Accurate mass found	Mol form	Δmass (ppm)	MS–MS fragments	Putative ID	MM
49.12	n.d.	2255.2	–	831.3298	$C_{19}H_{28}O_{10}$	1.4	415, 371, 353, 311, 239, 221, 209, 203, 191, 189, 181, 167, 161, 143	Geranoyl-6-*O*-malonyl-β-D-glucopyranoside[a] ($[2M-H]^-$)	416.1683
48.25	1.5	583.1	388.73	473.2025	$C_{22}H_{34}O_{11}$	0.4	167	Geranic acid[c]	474.2101
36.66	422.8	2204.3	5.21	523.2405	$C_{23}H_{40}O_{13}$	2.7	477, 316, 161	(Geraniol dihexose) FA	524.2469
24.12	155.0	1489.3	9.61	345.1565	$C_{16}H_{26}O_8$	4.5	207, 183, 179	Hydroxy geranic acid-hexose[b]	346.1628
22.84	5.5	499.5	90.82	391.1621	$C_{16}H_{26}O_8$	4.3	345, 183	(Hydroxy geranic acid-hexose[b]) FA	346.1628
19.75	59.5	707.6	11.89	345.1566	$C_{16}H_{26}O_8$	4.8	121, 119, 101	Hydroxy geranic acid-hexose[b]	346.1628
19.31	160.3	1865.8	11.64	345.1559	$C_{16}H_{26}O_8$	2.8	161, 121, 119, 101	Hydroxy geranic acid-hexose[b]	346.1628

The significantly changed metabolites with mass intensity higher than 500 in either transgenic or control plants were chosen to be analyzed by LC–MS/MS.
Ret (min), retention time, in minutes; Av, average; C, empty vector control plants; T, transgenic plants; ratio (T/C), ratio of mass signal between transgenic plants (T) and empty vector control plants (C); mol form, molecular formula of the metabolite; Δmass (ppm), deviation between the found accurate mass and real accurate mass, in ppm; putative ID, putative identification of metabolite; MM, monoisotopic molecular mass of the metabolite. n.d., not detectable, compounds with intensity less than 1 are not detectable.

[a] The structure of geranic acid-malonyl-glucopyranoside was confirmed by NMR.
[b] The compound could also be hydroxyl neric acid-hexose; ( ) FA, formic acid adduct.
[c] Geranic acid conjugated with agarobiose, carrobiose, difructose anhydride or their isomers.

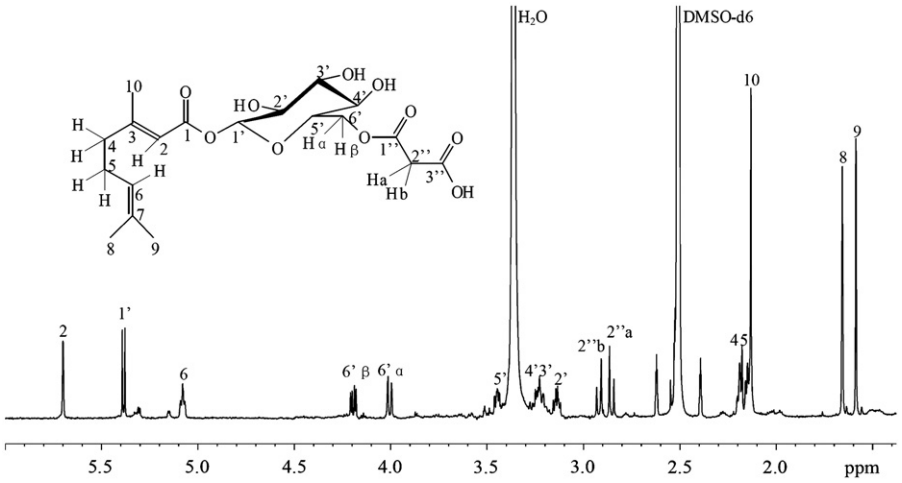

Fig. 3. 600 MHz ^{1}H NMR spectrum of geranoyl-6-*O*-malonyl-β-D-glucopyranoside.

Statistical analysis showed no significant differences between transgenic and control plants for any of the measured traits (Table 4). This indicates that overexpression of *LdGES* at the reported levels and under the conditions tested does not negatively affect maize plant growth and development.

Fifty plants from 10 different T_1 generation transgenic maize lines (5 plants/line) and 50 control plants were tested in the greenhouse for resistance of leaf sheaths to infection by *F. graminearum* and *C. graminicola*. There was no significant difference between transgenic and control plants in lesion area resulting from inoculation by either fungus (Table 5). The increased content of geraniol and geranic acid derivatives, therefore, did not lead to changes in fungal resistance under the applied experimental conditions.

4. Discussion

In this study, we aimed to introduce fungal resistance into maize by metabolic engineering. We took a stepwise approach of first testing a wide range of terpenoids and overexpressing the most promising candidate. Among the 15 terpenoids that were tested against two maize pathogenic fungi *F. graminearum* and *C. graminicola*, geranic acid displayed the strongest antifungal activity (Table 1). The MIC of geranic acid was lower than 7.8 μg mL^{-1} (46 μM) for both fungi, and for these fungi it represents the most potent antifungal activity reported to date. Previously, citral, dimethyloctanol, terpineol and linalyl acetate were tested against *C. graminicola* and citral was the most effective with a MIC of 870 μg mL^{-1} (5723 μM) (Dev et al., 2004). Only complex essential oils have been screened for their inhibitory effect against *F. graminearum*, however, the MICs of the most effective oils were higher than 1000 μg mL^{-1} (Singh et al., 2008; Velluti et al., 2004a). The antifungal effects of the individual components of these essential oils are not known, but if they represent more than 1% of the oil, they must be less effective than geranic acid.

Previously, geranic acid has been produced *de novo* in transgenic tomato fruits by overexpressing geraniol synthase (Davidovich-Rikanati et al., 2007). Thus, we cloned and overexpressed a geraniol synthase gene from *L. dulcis* (*LdGES*) under control of an ubiquitin promoter in maize plants. In leaves of *LdGES* expressing maize plants, we detected 7 geraniol derivatives with significantly higher concentrations than control plants. Remarkably, the most dominant compound geranoyl-6-*O*-malonyl-β-D-glucopyranoside in transgenic maize was not detected at all in the control plants, whereas the other 6 geraniol-derived compounds did occur, although at relatively low levels. In view of the presence of these endogenous geraniol derivatives, we expect a geraniol synthase gene to be expressed in maize leaves. So far the only reported candidate gene is the Terpene Synthase 1 (TPS1) gene. This gene has been reported to be capable of producing geraniol and several other mono- and sesquiterpenes in an *in vitro* assay (Schnee et al., 2002). However, as the authors pointed out, it is likely to function as a sesquiterpene synthase *in vivo*, since it lacks an N-terminal signal peptide for chloroplast targeting and it is more effective to convert farnesyl diphosphate than geranyl diphosphate (Schnee et al., 2002). We presume, therefore, that the operational geraniol synthase gene of maize still needs to be identified (for alignment of all published geraniol synthases, see Fig. S1).

We suggest that the most likely reason for the observed differences in the amounts of accumulating compounds between the transgenic and control plants is the difference between the native promoter and the ubiquitin one used in this study. The maize ubiquitin promoter is known to be active in most cell types of the leaf (Schunmann et al., 2004). The spatial expression pattern of the native geraniol synthase gene remains to be investigated once the gene has been cloned. We would predict that utilizing promoters which are more cell type-specific will reduce the number of different derivatives and conjugates found upon over-expressing terpene synthase genes in plants, and thus that could be one strategy for the metabolic engineering of more specific products.

The identification of the novel compound, geranoyl-6-*O*-malonyl-β-D-glucopyranoside, was made possible by applying a non-targeted LC–MS approach to analyze any changes in the non-volatile metabolite profile resulting from the expression of terpene synthases. In previous studies analyzing the non-volatile conjugates in plants overexpressing monoterpene alcohols, either indirect methods of detection were used, based on the release of aglycons by glycosidases, or targeted methods based on available standards were used. For example, Lücker et al. (2001) described glucosylation of monoterpene alcohols with linalool synthase overexpression in petunia, but this was analyzed using a synthetic standard of (*R*,*S*)-linalyl β-D-glucopyranoside, and did not exclude the presence of additional, possibly more abundant glycosides. Commercial glycosidase treatments linked with GC–MS have been used to identify non-volatile glycosides of linalool in transgenic Arabidopsis and potato plants (Aharoni et al., 2003, 2006), however, both the nature of the original conjugations and the effectiveness of the method to hydrolyze all types of glycosides remained unknown. Thus, the presented non-targeted

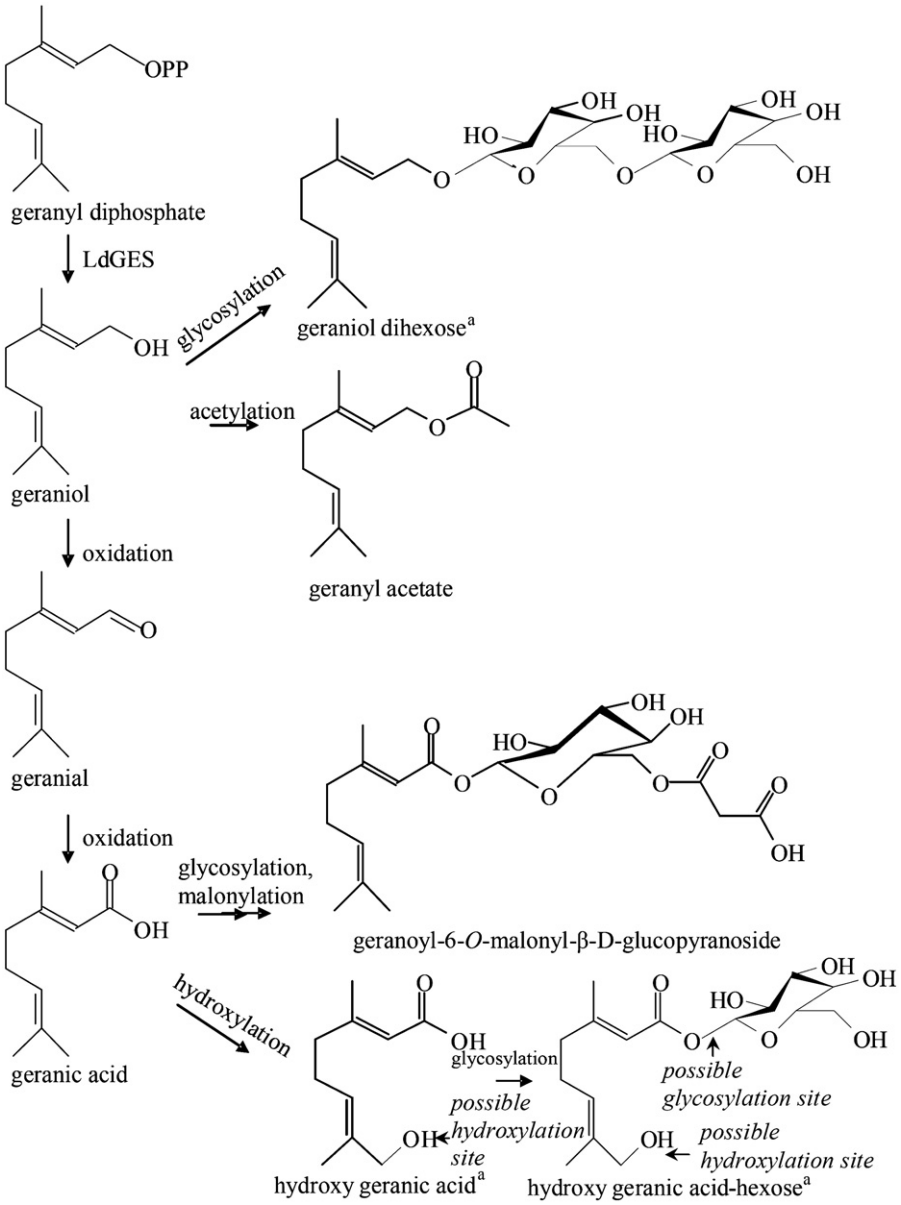

Fig. 4. Metabolism of geraniol in transgenic maize plants overexpressing *LdGES*. [a]The compounds were putatively identified based on LC–MS/MS analysis. It is not clear yet, which type of hexose is conjugated, where the hexose is attached to the aglycones, how the two hexoses are connected and in the case that there are more than one possible hydroxylation and glycosylation sites it is uncertain which site is used. The structures given in this figure are based on the most common structures of naturally occurring terpene glycosides.

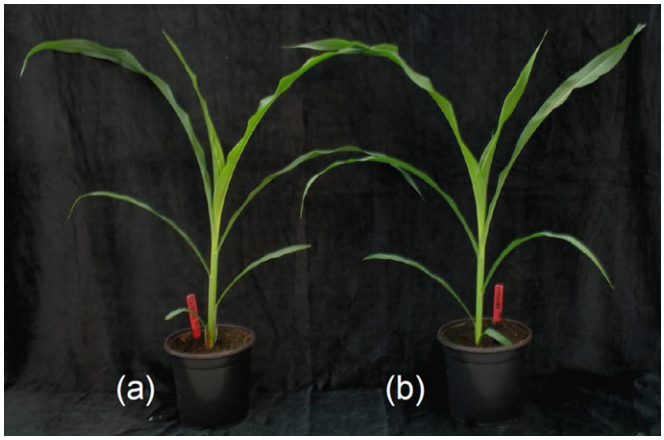

Fig. 5. Phenotype of control plant (a) and *LdGES* expressing plant 19-2 (b).

analysis is basic to know the metabolic fate of heterologously expressed terpenoids, although each non-targeted method is also limited by the extraction method, chromatography behavior and molecular ionization potential. As a result, also here, additional products cannot be excluded.

From a biochemical point of view, glycosyl conjugation reduces chemical reactivity of compounds (Von Rad et al., 2001), while malonyl conjugation can facilitate the transport of glycosylated compounds into the vacuole, a process mediated by ATP-binding-cassette transporters (Liu et al., 2001). Malonylation may also contribute to the water-solubility of the glycosylated compound and prevent glycolysis by glycosidases (Heller and Forkmann, 1994). Reduced susceptibility to glycosidases may have experimental and biological consequences. Firstly, the amount of glycosylated terpenoids reported by others in transgenic plants may have been underestimated, since this amount has been determined by measuring liberated terpenoids after

Table 4
Plant height, leaf number, chlorophyll content and kernel weight of T_1 transgenic plants and control plants.

Lines	Plant height (cm)		Leaf number		Chlorophyll (mg/g FW)		Kernel weight (g)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
c 31	261.21a	30.16	13.8a	0.45	2.96a	0.50	0.46a	0.04
ger 18	266.20a	8.70	14.4a	0.55	3.19a	0.35	0.45a	0.04
ger 19	268.82a	13.10	14.2a	0.84	2.59a	0.40	0.41a	0.05
ger 20	256.64a	15.53	13.6a	0.55	2.67a	0.11	0.47a	0.03

Five plants from each line were measured. Values followed by the same letter within a column are not significantly different (ANOVA test: $P > 0.05$).

Table 5
Fungal resistance of T_1 transgenic and control plants measured by lesion area when infected with *F. graminearum* and *C. graminicola*.

Plants	*C. graminicola*		*F. graminearum*	
	Lesion area (cm^2)		Lesion area (cm^2)	
	Mean	SD	Mean	SD
Control plants	0.708a	0.23	2.589a	0.63
T_1 plants	0.670a	0.19	2.778a	0.82

Fifty control or T_1 transgenic plants were tested. Values followed by the same letter within a column are not significantly different (ANOVA test: $P > 0.05$).

glycolysis with commercial glycosidases (Aharoni et al., 2003, 2006), which much less effectively hydrolyze malonylated glycosides (Ismael and Hayes, 2005; Roscher et al., 1997). Secondly, malonylation may also compromise potential release of the aglycon by endogenous or fungal glycosidases and this could potentially result in reduced biological effects under biotic or abiotic stress.

From a physiological point of view, glycosyl and malonyl conjugations are considered to aid in the accumulation, storage or transport of secondary metabolites which may be phytotoxic (Crouzet and Chassagne, 1999; Hatzios, 1997). High concentrations of geraniol, for example, have been shown to be phytotoxic to maize, causing oxidative stress to membranes (Zunino and Zygadlo, 2004), but the compound potentially plays important biological roles in plant communication to attract beneficial insects (James, 2005) and repel some insect pests (Halbert et al., 2009; Wei et al., 2004). Temporary storage and subsequent, stress-induced enzymatic release of geraniol from its glycosides could, therefore, be used in maize as a defense strategy. Enzymatic release may not even be necessary: glycosides can also possess strong bioactivity themselves, as for example resveratrol glycosides against the alfalfa fungal pathogen *Phoma medicaginis* (Hipskind and Paiva, 2000). Here, we show that geraniol and geranic acid are both stored naturally in maize with additional hydroxylations and/or various glycosylations. This suggests similar potential roles of glycosides of geraniol or its derivatives in plant communication or defense against biotic agents.

The goal of this work was to engineer fungal resistance into transgenic maize by expressing a fungicidal concentration of geranic acid. The average concentration of geranoyl-6-*O*-malonyl-β-D-glucopyranoside in *LdGES* expressing maize leaves was around 17 $\mu g\, g^{-1}$ FW (45 μM). This concentration was in the range of previously reported levels of glycosides of heterologous terpenes in transgenic plants. For example, 5–10 $\mu g\, g^{-1}$ FW (14–28 μM) linalyl-β-D-glucoside has been found in petunia plants overexpressing a linalool synthase from *Clarkia breweri* (Lücker et al., 2001), and up to 110 $\mu g\, g^{-1}$ FW (643 μM) hydroxylinalool has been estimated to be present as glycosides in Arabidopsis plants overexpressing a linalool synthase from strawberry (Aharoni et al., 2003). In our experiments, resistance to fungal infections did not significantly improve, however, despite the fact that the average concentration of geranoyl-6-*O*-malonyl-β-D-glucopyranoside (45 μM) was in the same range as the MIC of geranic acid for both fungi (< 46 μM). The reasons why we did not observe significant antibiotic effects in the fungal bioassays (Table 5) may, therefore, be the lack of bioavailability of the geranic acid and/or inappropriate subcellular localization of the compound in relation to the infection strategy of the fungus. The bioavailability could be improved by the down-regulation of genes involved in glycoside biosynthesis (Lochlainn and Caffrey, 2009) or by the up-regulation of genes producing other more bioactive glycosides of geranic acid (Freitag et al., 2006; Pickens and Tang, 2009). However, the corresponding glycon transferases and malonyl transferase genes have not been identified yet. Alternatively, the bioavailability could be improved by co-expression of an appropriate glycosidase either in the same subcellular compartment under an inducible promoter or constitutively in separate compartments if the fungal infection would destroy subcellular compartmentation. As mentioned above the release of geranic acid from malonyl-glucoside may be compromised by the presence of the malonyl group. Some glucosidases have been isolated, however, which can effectively release the aglycone also from malonylated glycosides, such as a malonylesterase from chickpea (Hinderer et al., 1986) and an isoflavone conjugate-hydrolyzing β-glucosidase from soybean (Suzuki et al., 2006). These could be used in future strategies to induce the release of the aglycone upon fungal infections.

No significant difference was detected between the headspaces of intact *LdGES* expressing and control plants (Fig. 1a and b). This indicates that in the seedling all products of LdGES were stored in the plant tissues. However, detached leaves of *LdGES* expressing plants emitted 5-fold more geranyl acetate than control leaves (Fig. 1c–e), suggesting that the release of geranyl acetate was wound-inducible in both *LdGES* expressing and control maize plants and dependent on the relative availability of geraniol as substrate. Geranyl acetate has also been reported in the headspace of maize seedling after infestation by the caterpillars, *Spodoptera littoralis* (D'Alessandro and Turlings, 2006) and *Helicoverpa armigera* (Yan and Wang, 2006), but not in the headspace of undamaged control seedlings (D'Alessandro and Turlings, 2006; Yan and Wang, 2006). Together with other caterpillar-induced volatiles, geranyl acetate was suggested to be involved in plant direct or indirect defense to the caterpillars (D'Alessandro and Turlings, 2006; Yan and Wang, 2006), and *LdGES* expressing plants could be used, therefore, in further studies to establish the ecological function of geranyl acetate in maize–insect interactions. However, for this purpose also other genes involved in the geranyl acetate biosynthetic pathway or transcriptional factors controlling this pathway could help to control the production of geranyl acetate in maize to establish its role (Peebles et al., 2010, in press).

LdGES expressing maize plants all exhibited a completely normal phenotype and development (Fig. 5, Table 4), despite the fact that in other plant species severe phenotypes have been observed as a result of overexpression of monoterpene synthase genes. Arabidopsis (Aharoni et al., 2003) and potato (Aharoni et al., 2006) expressing a linalool synthase displayed bleached leaves and retarded growth. Tomato fruits expressing geraniol synthase failed to develop the normal deep red color, because of a 50% drop in lycopene content (Davidovich-Rikanati et al., 2007). In our *LdGES* expressing maize plants, several semi-polar compounds were significantly down-regulated, but could not be further identified due to their low signal intensity in the LC–MS analyses. The down-regulation of these compounds could represent the cost of overexpressing LdGES. However, the chlorophyll

content of the transgenic lines examined was not affected (Table 4) and no obvious phenotype was observed (Fig. 5), suggesting that maize plants have sufficient levels of geranyl diphosphate to support the accumulation of the induced geraniol derivatives as well as of the endogenous (essential) isoprenoids. Engineering of isoprenoids in maize is, therefore, a feasible option in future research programs.

Acknowledgments

We thank Jeff Robson for generating the maize transformation vector, Jay Gilliam for performing *in vitro* antifungal assays, Julie Ritland for plant disease resistance assays, Bert Schipper for assistance in LC–MS analysis and Yury Tikunov for his help in MS data analysis. Ric de Vos acknowledges additional funding from the Center for BioSystems Genomics (CBSG), which is part of the Netherlands Genomics Initiative.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2011.01.011.

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