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Short communication

The influence of monoterpene synthase transformation on the odour of tobacco

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Abstract

Monoterpenes are an important class of terpenoids that are commonly present in plant essential oils. These can be extracted from plants and are used in the flavouring and perfumery industry. Monoterpene synthases are the key enzymes in monoterpene biosynthesis, as they catalyse the cyclisation of the ubiquitous geranyl diphosphate (GDP) to the specific monoterpene skeletons. Tobacco is one of the most studied model plants, it can easily and efficiently be transformed, and is a suitable model to study the release of plant volatiles. Thus, we have isolated monoterpene synthases from lemon, transformed tobacco with these cDNAs and have used human panelists to study the change in fragrance of the transgenic in comparison to the wild type plants. In a triangle test, we found that subjects were capable of smelling significant differences between leaf samples. However, as a result of variability in panel ratings, no significant difference between two sets of transgenic flowers and the wild type tobacco flowers was found for the generated attributes in a descriptive test.

Keywords: Lemon monoterpene synthases; Transgenic tobacco; Fragrance

1. Introduction

The largest class of plant secondary metabolites is that of the terpenoids. Over 36,000 individual structures of this class have been reported (Hill, 2002). Terpenoids determine the fragrance of a considerable number of plants and they are commonly present in essential oils (McGarvey and Croteau, 1995). Terpenoids are produced by a range of plant organs and have many biological roles. They are important for the interaction between plants and their environment such as for defence against herbivores and pathogens (Bouwmeester et al., 1999), and as attractants for pollinators and seed dispersers (McGarvey and Croteau, 1995). In addition, terpenes protect leaves against

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high-temperature damage by enhancing membrane stability under heat stress (Kreuzwieser et al., 1999). Moreover, terpenoid containing essential oils have been shown to have an influence on human behaviour (Ilmberger, 2001). One of the subclasses of the terpenoids, the monoterpenes are also of high economic value as they are widely used in products of the flavouring, perfumery, food and drink, detergent and cosmetics industry (Verlet, 1993). Monoterpenes have a typical smell and some, for example limonene, display cancer chemoprevention properties (Bardon et al., 1998; Crowell and Gould, 1994).

Terpenes have a unique structure: they consist of an integral number of five-carbon (isoprene) units. Two such units form the basis for the monoterpenes (C_{10}), and subsequent additions of more isoprene units form the basis for the sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenoids ($>C_{40}$) (Gershenzon and Croteau, 1993). Primary monoterpenes are formed from the general precursor geranyl diphosphate (GDP) through the action of monoterpene synthases (Croteau, 1987). Different initial cyclisation processes catalysed by monoterpene synthases lead to the vast variety in monoterpene skeletons (Fig. 1). Several monoterpene synthases are able to produce more than one product (McGarvey and Croteau, 1995).

By introducing genes encoding monoterpene synthases, some research groups have reported on the formation of new monoterpenes or on the increase of already existing terpenes in transgenic plants (Lavy et al., 2002; Lewinsohn et al., 2001; Lücker et al., 2001). To date, none of these studies have reported on the successful alteration of fragrance in plants.

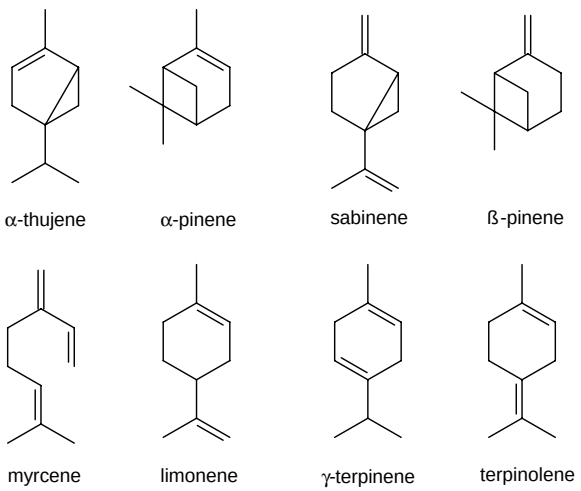

Fig. 1. Representative members of various monoterpene structures.

Recently, three *Citrus limon* cDNAs encoding the enzymes γ-terpinene synthase (GenBank accession number: AF514286), (+)-limonene synthase (AF514287) and β-pinene synthase (AF514288) (Lücker et al., 2002) were transformed either separately or together to *Nicotiana tabacum* 'Petite Havana' SR1 (Lücker et al., 2003). GC–MS analyses showed that the terpenoid products of these enzymes and their side products were produced and emitted by leaves and flowers of the transgenic plants.

The present study reports on the effect of these transgenes on fragrance perception by humans, based on a discrimination test using plant leaves and a descriptive analysis using flowers. The difference between the two methods is that the discrimination test is used to determine whether a sensory difference exists between two products (in this case plant leaves) based on an overall difference in impression, whereas the descriptive analysis is an analytical approach in which trained panelists provide quantitative descriptions of a product (in this case plant flowers) based on its attributes as determined by the panel (Stone and Sidel, 1993).

2. Materials and methods

2.1. Plant material

In order to obtain a plant producing several new monoterpenes, three full length *Citrus limon* monoterpene synthase cDNAs, γ-terpinene synthase (B93), (+)-limonene synthase (C62) and β-pinene synthase (D85) (GenBank accession numbers AF514286, AF514287 and AF514288, respectively) were transformed to wild type *N. tabacum* 'Petite Havana' SR1 plants as described by Lücker et al., (2003). Crossings were made between primary transformants containing one insert of the three representative monoterpene synthase cDNAs and one plant was found to emit all three main products of the introduced monoterpene synthases (Lücker et al., 2003). Control plants (SR1), plants transformed with an empty vector (CAM1), plants transformed solely with the β-pinene synthase

(D85) and plants transformed with the β-pinene synthase, (+)-limonene synthase and γ-terpinene synthase (BCD) were grown under greenhouse conditions under 16-h photoperiod.

2.2. Headspace trapping of leaf volatiles and GC–MS

Leaf samples of about 4 g (fresh weight) from SR1, CAM1 and BCD tobacco lines were cut and kept turgescent by placing the leaf petiole in floral foam blocks (Smithers-Oasis Belgium N.V., Houthalen, Belgium), that were saturated with water and wrapped with aluminium foil. Subsequently, the headspace volatiles were collected on a glass cartridge (140 mm × 4 mm) containing 150 mg Tenax TA (20/35 mesh, Alltech, Breda, The Netherlands) and analysed using GC–MS as described by Bouwmeester et al. (1999). GC oven temperature was programmed from 45 °C (1 min hold) to 280 °C, at a rate of 10 °C min^{-1}, with a final hold of 10 min.

2.3. Sensory discrimination procedure

A triangle test was used for discrimination testing. Twenty participants (20–40 years old) were tested in a triangle test, in which it was their task to determine which one of the three samples smelled most different from the other two (Stone and Sidel, 1993). The stimuli used were tobacco leaves placed in 600 ml glass jars with a 7.5 cm screw-cap. Each participant completed two trials. In one trial, they were presented with two jars containing leaves from the BCD plant line, and one containing leaves from the SR1 control plant line. Conversely, in the other trial they were presented with two jars containing leaves from the SR1 control plant line and one jar containing leaves from the BCD plant line.

2.4. Flower fragrance, screening and selection of panel for descriptive analysis

Around 1 g of BCD, D85 and SR1 flower samples from four different flower developmental stages (8, 10, 11 and 12) (Koltunow et al., 1990) were used as fragrance stimuli. For each sample, four flowers were placed into a block of floral foam saturated with water and wrapped with aluminium foil. The flowers were presented in 600 ml glass jars with ∅ 7.5 cm screw-caps. For training purposes, duplicates of flowers of each plant line were used.

Eleven participants were screened for smoking history, respiratory disease, occupational exposure to volatile chemicals, ability to detect the odour of menthone (a terpene) and ability to generate odour descriptors for terpenes. This involved taking careful sniffs from two 0.25% solutions in propylene glycol of γ-terpinene and β-pinene presented in small glass vials with ∅ 2.5 cm screw-caps and writing down their immediate sensory impressions. Panelists, who were capable of detecting the lowest presented concentration of menthone (0.0078% v/v in propylene glycol) without error, and of generating odorant attributes for the above stimuli, were selected for participation in the panel.

2.5. Panel training for descriptive test

Panelists participated in three training sessions, which occupied 30–45 min each. During *Session 1*, participants sniffed from each of the BCD, D85 and SR1 flower samples and wrote down their sensory impressions. The descriptors used by the panelists were discussed among the panel. During *Session 2*, the combined fragrance descriptors were presented to the panel. Panelists then smelled each sample again and tried to identify the appropriate descriptors for each of the samples. If necessary, new descriptors were generated and definitions for each of the descriptors were agreed upon. During *Session 3*, the participants rated each of the descriptors generated on 100 mm Visual Analogue Scales varying from ‘very little’ to ‘very much’ for each of the samples in individual booths using computer software (Fizz, 2002a). Subsequently, they discussed the results and rank-ordered the samples from lowest to highest based on each of the attributes, in an attempt to reach agreement among the panel on the qualitative and quantitative aspects of the descriptors.

2.6. Final session and statistical analyses for descriptive test

During the final session, the individual panelists rated each of the tobacco flower samples twice, in random order on the previously selected descrip-

tors. Computerised testing and data collection was performed using Fizz software (Fizz, 2002a). To check whether there were significant differences for each of the generated attributes, panel ratings were analysed using ANOVA (Fizz, 2002b; Hair et al., 1987).

3. Results and discussion

3.1. GC–MS analysis of SR1, CAM1 and BCD leaf headspace

According to GC–MS analysis, there were no significant differences between the headspaces of SR1 and CAM1 plants (Fig. 2). SR1 was then considered a suitable negative control to proceed with further experimentation. However, there were significant differences in the volatile profiles between BCD and SR1 leaves. Major products such as sabinene, β-pinene, (+)-limonene and γ-terpinene, and minor products such as α-thujene, α-pinene and terpinolene were detected in the headspace of BCD but not SR1 leaves (Figs. 1 and 2). These results agree with the volatile products of the introduced genes when these genes were expressed in *E. coli* (Lücker et al., 2002) confirming that transformation had been successful.

3.2. Results from sensory discrimination test using BCD and SR1 leaves

In a triangle test, the chance probability associated with the test is 1/3. The triangle test trials performed with 20 individuals yielded 22 correct scores. The following formula was used to test whether participants were able to discriminate between the fragrance of leaves from transgenic and control plants: $Z = (k - (1/3)n)/\sqrt{(2/9)n}$ (Meilgaard et al., 1991), where k is the number of correct answers; n the total number of presentations, with $k = 22$ and $n = 40$. $Z = 2.91$ exceeds the critical value for Z at $\alpha = 0.05$, which is 1.645. Hence, the null hypothesis that subjects were unable to smell the difference between the leaves from transgenic plants and control plants was rejected with $P < 0.05$. During the test, it was noticed that there were differences not only between but also within treatments (plant lines). For example, differences in intensity were reported between the samples of the BCD leaves. Nevertheless, subjects were capable of smelling the differences between treatments with statistical significance.

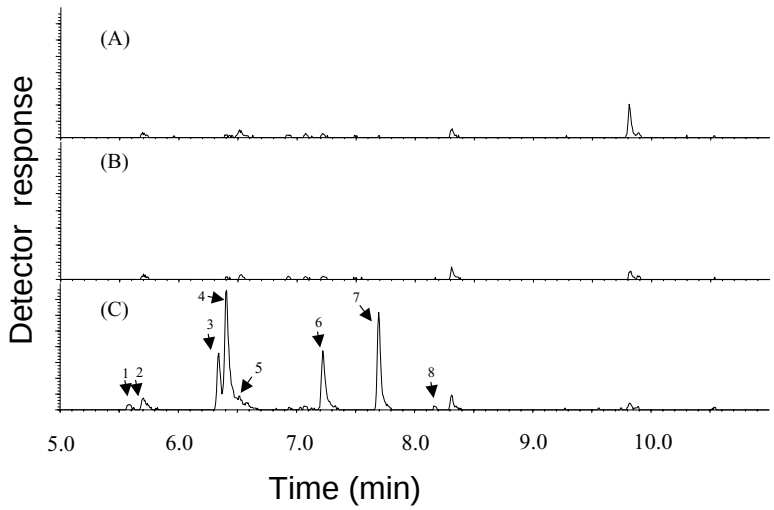

Fig. 2. Headspace gas chromatography/mass spectrometry analysis of intact leaves of tobacco lines. Lines were control SR1 plants (A), empty vector-transformed plants (CAM1) (B) and plants transformed with the lemon limonene synthase, β-pinene synthase and γ-terpinene synthase (BCD) (C). (1) α-thujene, (2) α-pinene, (3) sabinene, (4) β-pinene, (5) β-myrcene, (6) limonene, (7) γ-terpinene, and (8) terpinolene.

3.3. Results from the sensory descriptive test of BCD, D85 and SR1 flowers

A descriptive test was conducted to generate the sensory profiles corresponding with each of the transgenic flowers, in order to investigate whether the floral profiles of the transgenic BCD and D85 flowers differed from that of the control SR1 flowers.

After screening 11 prospective panelists, 7 panelists were selected for participation in the study. The selected panel consisted of four males and three females with ages varying between 26 and 50 years. Six of the panelists did not currently smoke and one smoked cigarettes occasionally. The panel participated in three training sessions in order to generate sensory attributes and become familiar with the procedures. In a process of elimination, eight descriptors were agreed upon. These descriptors, which covered the odorant characteristics of all three samples, were: "Green odour", "sour", "sweet", "stale/moldy", "flowery", "fresh", "prickling" and "overall intensity". In a final test session, the flowers of BCD, D85 and SR1 were evaluated, in duplicate, for each of the attributes. Fig. 3 displays the sensory odorant profiles corresponding to each of the plants. As can be seen, the odorant profiles of the flowers are very comparable. The most notable difference occurred on "overall intensity", with the flowers of D85 being perceived as the most intense (mean $= 59.8 \pm 13.8$ versus 49.1 ± 18.5 for BCD and 47.4 ± 24.9 for SR1, respectively). In addition, the trend in the "sweet" attribute may perhaps be associated with the presence of (+)-limonene in the BCD-line. (+)-Limonene is commonly described as 'mild/light', 'sweet', 'citrus', 'fruity', 'fragrant' (Aldrich, 1997; Dravnieks, 1985). The "sweet" impression can not be due to the presence of γ-terpinene and β-pinene because they were described as 'fresh', 'oil', 'petrol', 'turpentine' and 'herbs', for the first, and 'pine', 'medicine', 'camphor' and 'alcohol' for the latter, by the panel. However, none of the differences in ratings between the tobacco lines reached statistical significance (Table 1). This was probably caused by the large standard deviations in attribute ratings across panelists, which indicates a lack of agreement on perceived attributes. All this demonstrates that the evaluation of the individual components that together make up the overall floral fragrance profile is a difficult, highly analytical task.

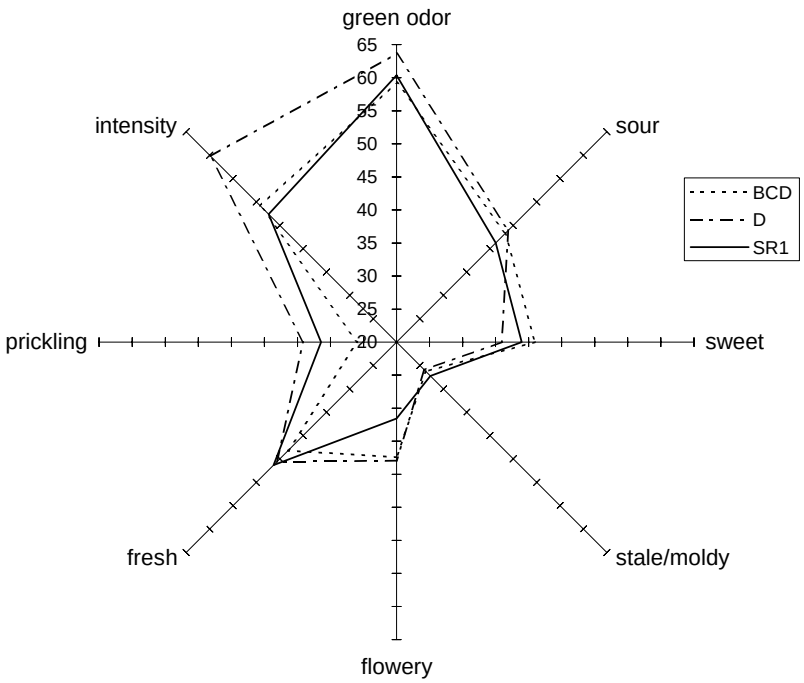

Fig. 3. Odorant profiles of tobacco flowers. The mean overall Coefficient of Variation (%) is 49.8.

Table 1
Results from ANOVA analysis on the ratings of eight attributes of tobacco flower fragrance as a function of flower sample and panelists

Attribute	Product			Panelist		
	d.f.	*F*-value	*P*-value	d.f.	*F*-value	*P*-value
Green odour	2	0.27	0.760	6	4.96	0.003**
Sour	2	0.27	0.77	6	11.37	0.001***
Sweet	2	0.98	0.39	6	20.49	0.0001***
Stale/moldy	2	0.04	0.97	6	3.85	0.01**
Flowery	2	0.96	0.40	6	10.10	0.0001***
Fresh	2	0.19	0.9	6	14.74	0.0001***
Prickling	2	1.37	0.27	6	8.55	0.0001***
Intensity	2	2.20	0.14	6	3.55	0.01*

Note: (*) significant at 5%; (**) significant at 1%; (***) significant at 0.1%.

3.4. Future prospects

The petals of flowers remain the main source of fragrance in most plants (Pichersky et al., 1994). However, many modern flower varieties, such as carnation, lack the characteristic original fragrance (Clery et al., 1999). The use of a transgenic approach could lead to the re-introduction of fragrance compounds or could be used to improve the fragrance of ornamental plants and flowers. Several groups have adopted a transgenic plant approach (Lavy et al., 2002; Lücker et al., 2001, 2003; Mahmoud and Croteau, 2001) and GC–MS analyses have confirmed their success. Although the goal of this metabolic engineering was—without exception—to change the olfactory characteristics, this was not proven by any of the groups and our present work was one of the first serious attempts to do so.

It is clear that flower fragrance has a complex character determined by a mixture of low molecular weight volatile molecules. Due to the inaccessibility of this character, to the limitations of human's sense of smell, and to the variable nature of a scent, no simple and efficient analytical method to screen for genetic variation has been developed yet (Vainstein et al., 2001). Our results show that the discrimination between samples differing in the emission of single fragrant volatiles is feasible. For a better olfactory description of these differences, more research is required and for example a different method of presentation of the samples to the panel may improve the sensitivity. The additional research and advanced analytical tools should then allow to study more exhaustively the effects of transformation of plants with genes encoding biosynthetic enzymes on the olfactory properties of the transgenic plants and to unravel the relationship between plant fragrance and human olfactory impression.

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