Modification of sunflower oil quality by seed-specific expression of a heterologous Δ9-stearoyl-(acyl carrier protein) desaturase gene

P. Rousselin[1], J. Molinier[1], C. Himber[1], D. Schontz[1], P. Prieto-Dapena[2], J. Jordano[2], N. Martini[3], S. Weber[4], R. Horn[4,7], M. Ganssmann[5], R. Grison[6], M. Pagniez[6], A. Toppan[6], W. Friedt[4] and G. Hahne[1]

[1] Institut de Biologie Moléculaire des Plantes, CNRS-ULP, 12, rue du Général Zimmer, F–67084 Strasbourg, France; [2] Instituto de Recursos Naturales y Agrobiologia, CSIC Apartado de Correos 1052, E–41080 Sevilla, Spain; [3] Max-Planck-Institut für Züchtungsforschung, Abteilung Genetische Grundlagen der Pflanzenzüchtung, Carl-von-Linné-Weg 10, D–50829 Cologne, Germany; [4] Institut für Pflanzenbau und Pflanzenzüchtung I, Justus-Liebig-Universität, Heinrich-Buff-Ring 26–32, D–35392 Giessen, Germany; [5] Asgrow/KWS, Domaine de Mérue, F–82170 Monbéqui, France; [6] Biogemma, Domaine de Sandreau, F–31700 Mondonville, France; [7] Corresponding author. E-mail: renate.horn@agrar-uni-giessen.de

Abstract

The coding sequence of Δ9-stearoyl-(acyl carrier protein) desaturase from *Ricinus communis* was introduced into sunflower, under the control of seed-specific promoter and terminator sequences of the late embryogenesis abundant gene from sunflower, *Hads10*. Two independent primary transformants contained three and six copies of the T-DNA, as demonstrated by hybridization using *nptII* as a probe. The transgene proved genetically stable and was transmitted as a Mendelian trait. Transcript analysis of the heterologous Δ9-stearoyl-(acyl carrier protein) desaturase under control of the *Hads10* promoter verified tissue-specific expression in the developing embryos and not in the leaves. Fatty acid composition of the seed oil was followed over five generations under greenhouse and open field conditions. Some of the transgenic lines produced oil with a significantly reduced stearic acid content compared with non-transformed plants under greenhouse and field conditions. However, additional studies need to be performed to assess whether or not physiologically stable lines can be developed from these transgenic lines.

Key words: *Helianthus annuus* — transgenic sunflower —fatty acid biosynthesis — stearic acid — Δ9-stearoyl-(ACP) desaturase gene

Sunflower is one of the four major sources of edible oil worldwide. The fatty acid composition, one of the key factors determining the physical and chemical properties of vegetable oils, is subject to physiological regulation and highly variable owing to environmental factors (Harris et al. 1978, Trémolières et al. 1982, Sarmiento et al. 1998) and the genetic background (Knowles 1988). The main use of sunflower oil is for human nutrition. However, the range of applications for sunflower oil may be extended to industrial applications if 'designer oil' with specific fatty acid composition could be produced. Although cultivars producing oil with particular fatty acid composition have been obtained by conventional breeding techniques (Ivanov et al. 1988, Osorio et al. 1995, Fernandez-Martinez et al. 1997, Miller and Vick 1999), the use of genetic engineering technology can be expected to add precision and novel possibilities to the existing approaches.

Currently available high oleic sunflower lines contain significant amounts of stearic acid (5–6%). This undesirable compound causes problems for certain industrial uses because of the elevated clouding point of the oil. A significant reduction of the stearic acid content would permit more efficient processing of the oil. One possible way to achieve this goal is the seed-specific over-expression of Δ9-stearoyl-(acyl carrier protein) (ACP) desaturase, an enzyme using stearic acid as substrate for the production of oleic acid. The technical feasibility of transgenic approaches has already been demonstrated in several species such as *Arabidopsis thaliana*, *Brassica napus* and *Brassica campestris* (Voelker et al. 1992, Weber et al. 2001, White and Benning 2001). These experiments have shown that modification of the natural fatty acid spectrum of storage lipids is indeed possible by the expression of a heterologous enzyme in the developing seed, or the modification of the expression level of a resident protein. Antisense experiments using a Δ9-stearoyl-(ACP) desaturase cDNA from *B. campestris* in *B. campestris* or *B. napus* resulted in seeds containing high levels of stearic acid (Knutzon et al. 1992).

Information is scarce concerning the expression of foreign genes in sunflower and their stability in subsequent generations because of the absence, until recently, of a reliable transformation protocol (Knittel et al. 1994, Grayburn and Vick 1995, Burrus et al. 1996, Alibert et al. 1999). In this paper, it is shown that transformation of sunflower with a gene useful for practical applications is possible, and that expression of a heterologous Δ9-stearoyl-(ACP) desaturase in sunflower embryos may indeed modify the stearic acid content of the oil produced by these plants. Transgenic sunflowers were investigated for their genetic stability and the phenotypic effect of the introduced gene, i.e. the reduction of stearic acid content, over five generations and under different growth conditions, i.e. greenhouse and open field.

Materials and Methods

Plant materials and culture conditions: The interspecific hybrid line STR 1/95 (sf 2803/91) derived from a cross between *Helianthus annuus* and *Helianthus strumosus* (HA89cms × STR-1974) was used for plant transformation experiments. This line was selected because it demonstrated a significantly higher regeneration potential than other lines or

hybrids in sunflower (Weber et al. 2000). Seeds were dehusked, sterilized in commercial bleach (4%) with one drop of detergent (Johnson brillo, TGB, Harrow, UK) for 50 seeds for 20 min, and rinsed five times with sterile water. After 2 days of germination on medium M0 containing MS mineral salts (Murashige and Skoog 1962), 10 g/l sucrose and 7 g/l agar, cotyledons, root tips and leaf primordia were removed from the seeds as described by Bidney et al. (1992). Intact embryonic axes with a complete meristematic dome and two cotyledonary axillary buds were used as explants for transformation experiments.

Construction of the expression cassette pSKds10EC1: Starting from a *pBluescript II SK*(+) (Stratagene, GenBank # 52324) derivative which contains sequences of the late embryogenesis abundant gene *Hads10* (pSKds10EC1) of sunflower (*H. annuus*, GenBank #AJ224116; Prieto-Dapena et al. 1999) between *Sal*I (−1576 bp from the translation start codon ATG) and *Eco*RI (+1083 bp from the ATG), the translation start codon of the *ds10* gene was replaced by an artificial *Eco*RI recognition site. To this end, *ds10* sequences between *Sal*I (−1576) and +98 relative to the ATG were amplified by polymerase chain reaction (PCR) using *Pfu* DNA polymerase and the oligonucleotide primers, 5′-ATTAACCCTCACTAAAG-3′ (T3 promoter) and 5′-GAGTGAA-CAgAATtc CATCACAACAGGG-3′. The second primer replaced the ATG of the *Hads10* gene by an *Eco*RI site, created by the substitution of the three nucleotides indicated in lower case. The amplification product digested with *Eco*RI and *Sph*I was inserted between the *Sph*I (−126) and the *Eco*RI (+1086) sites in the starting plasmid. The nucleotide sequence of the PCR product was verified by DNA sequencing. Finally, another *ds10* genomic fragment, between the *Eco*RI (+1086 bp from the ATG) and the *Xba*I (+3000 bp relative to the ATG) sites, was inserted in the previously produced plasmid, resulting in the *ds10* expression cassette pSKds10EC1. This cassette is composed of *ds10* sequences including the 5′-flanking region, the promoter and the complete untranslated leader, followed by the engineered unique *Eco*RI cloning site, and additional *ds10* sequences (from +1086 to +3000), including a small part of the intron (84 bp), the second exon (131 bp, not counting the stop codon) and 3′-flanking sequences.

Construction of the transformation vector: The cDNA coding for Δ9-stearoyl-[ACP] desaturase from *Ricinus communis* (RcDes; Accession No: X56508; Shanklin and Somerville 1991), residing on an *Eco*RI fragment in plasmid pRCD1, was isolated and inserted into the unique *Eco*RI site in pSKds10EC1, resulting in two orientations of the *RcDes* cDNA relative to the *ds10* promoter sequence. The sense orientation of the *RcDes* cDNA was identified by restriction analysis of the plasmid

clones, resulting in plasmid pSKds10Des. Subsequent cloning of the *ds10* promoter desaturase cassette as a *Sal*I–*Not*I fragment of pSKds10Des into pBIN19, digested with *Sal*I and *Sma*I after modification of the *Not*I site with T4-DNA polymerase, gave rise to the binary transformation vector pBds10Des (Fig. 1a). The vector pBds10Des was introduced into *Agrobacterium tumefaciens* strain LBA 4404 (Hoekema et al. 1983) by electroporation.

Transformation of plants: Transgenic sunflowers were produced using the protocol established by Burrus et al. (1996), based on the transformation of the explants described above with a method employing *Agrobacterium* co-culture without additional particle bombardment. Putative transgenic shoots, which had not developed their own root system, were grafted on to 3-week-old HA300B plants serving as root stocks (Fischer et al. 1992). Individual inflorescences of flowering putative transgenic T0 plants were selfed and mature seeds harvested for the production of subsequent generations or immediate analysis.

Molecular analysis of transgenic plants: Total DNA was extracted from the leaves of T0 plants (putative transformants) and subsequent generations according to Dellaporta et al. (1983), digested with *Eco*RI and subjected to a Southern analysis (Sambrook and Russell 2001). The blots were hybridized with the 0.5 kb *Nco*I fragment of the *nptII* gene (Fig. 1a) isolated from the vector pRT99 (Töpfer et al. 1988), or with a 1.6-kb *Eco*RI fragment of the plasmid pSKds10Des, harbouring *RcDes* (Fig. 1a). Probes were labelled with ^{32}P-dCTP (Amersham, Freiburg, Germany) using the rediprime DNA labelling system (Amersham).

Total RNA was extracted from mature leaves or immature embryos (28 days after pollination) and subjected to Northern analysis (Verwoerd et al. 1989). The blots were hybridized with the pSKds10Des fragment described above and a ribosomal probe 18S as internal standard.

Greenhouse and field trials: The greenhouse experiment was conducted in 1997 in Strasbourg, France. The first field trial was conducted in 1998 in Mondonville (near Toulouse, France; authorization no. 97.11.29) and the second one in the same location in 1999 (authorization no. 99/059). For the field trial in 1998, plants containing a single copy of the transformation events [1], [2], [3] and [6] were selected, as well as plants containing events [2 and 3] and [2 and 6], simultaneously (see Table 3, later). Plants containing event [1] were hemizygous for the transgene. For events [2], [3] and [6], respectively, two types of plant were used: (1) confirmed homozygous, and (2) hemizygous plants from backcrosses between these homozygous
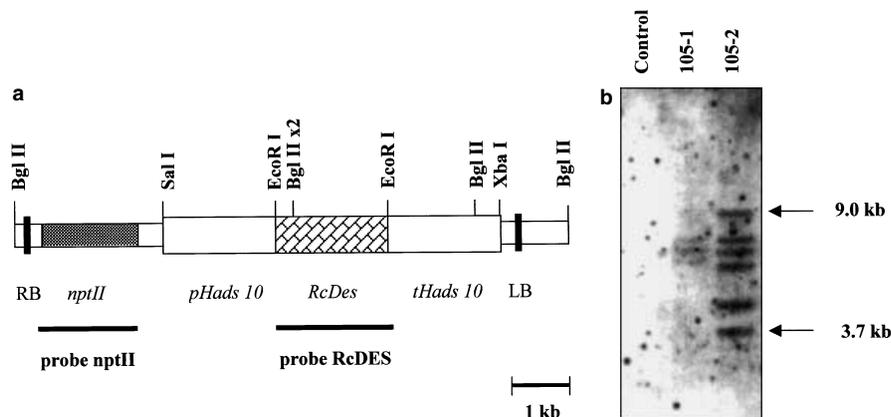

Fig. 1: a. Schematic representation of the T-DNA of the vector pBds10Des carrying the sunflower *ds10* expression cassette with the Δ9-stearoyl-(acyl carrier protein) desaturase of *R. communis* (*RcDes*). RB and LB designate right and left border, respectively. *nptII* is the kanamycin selection cassette including the nopalin synthase promoter and terminator. *pHads10* and *tHads10* are promoter and 3′-flanking region including the last exon of the late embryogenesis abundant gene *Hads10* of sunflower (*Helianthus annuus*). b. Southern analysis of two primary transformants (T0) and a negative control plant (wild-type STR 1/95). Genomic DNA was digested by *Eco*RI and subjected to hybridization with *nptII* as a probe

plants and wild-type plants (null segregants). In addition, the offspring of plant 105-2-21-19-05 was included, which contained the transgene at two loci (events [2] and [3]). F_1 hybrids containing events [2] and [6] were produced by crossing parents (105-2-17-02-09 × 105-2-14-93-05), which were homozygous for the single events [2] or [6], respectively (see Table 3). Plants of each genotype were grown under agronomic conditions (except that authorization was based on the condition of covering the whole plot and each inflorescence by an insect-proof screen to avoid any accidental gene flux). For each plant, a bulk of 20 seeds was analysed for the fatty acid composition and leaf samples were taken. Table 1 summarizes the families and their respective generations for analysis at plant and seed level in the different trials.

The second field trial included plants homozygous for the insertion event [1], as well as plants containing, and segregating for, insertion events [2] and [6] (F_2 generation of F_1 hybrids tested in 1998). Null segregants of the same generation were used as control plants. Standard morphometric data (height, leaf size, time to flowering, etc.) were collected for each family (data not shown); no significant difference could be detected either between transgenic and control plants or between transgenic families.

Fatty acid analysis: Fatty acid analysis of the harvested seeds followed standard protocols (Browse et al. 1986). Extracts from half seeds or bulks of 20 seeds were subjected to acidic methanolysis and the resulting fatty acid methyl esters were analysed by capillary gas chromatography. The fatty acid content was determined by comparison with an internal standard. Statistical analysis of fatty acid data by ANOVA and t-test was performed using MS-Excel 2000 (Microsoft, Seattle, WA, USA).

Results
Production of transgenic plants

Transgenic plants of the interspecific hybrid-derived line STR 1/95 were produced by *Agrobacterium*-mediated transformation of 400 embryonic axes with plasmid pBds10Des. Most of the shoots recovered after selection on kanamycin-containing medium failed to form roots. Thus, 33 shoots were grafted onto non-transgenic root stocks before transfer to the greenhouse. Twenty-one putative transgenic shoots survived this transfer. The majority of the plants recovered were fertile and some plants even produced multiple inflorescences. Individual inflorescences of flowering putative transgenic T0 plants were selfed and harvested separately.

Each individual (T0) inflorescence was characterized by testing leaves that originated from the inflorescence for the presence of the *nptII* gene by PCR. Two of the PCR-positive plants of the T0 generation (105-1 and 105-2) were then confirmed as stable transformants by Southern analysis using *nptII* as a probe. This Southern analysis indicated the presence of three and six insertion events of T-DNA, respectively (Fig. 1). These two primary transformants represent independent transformation events.

Southern analysis of the offspring (T1) of these two plants provided further information about the number of loci at which the transgene had been integrated (Fig. 2). Plant 105-2 and its offspring represented stable transformants. According to the segregation data of 24 T1 plants of this line, the six insertion events of the T-DNA were located at five independent loci (Fig. 2). Insertion events [4] and [5] appear to be genetically linked. This analysis also provided evidence for the presence of both *nptII* and *RcDes* genes in the transgenic plants (Fig. 2a).

In contrast, analysis of the entire offspring derived from the confirmed primary transformant 105-1 indicated that the T-DNA had not been transmitted to any of the 26 T1 plants (data not shown). The most probable explanation for this is that plant 105-1 had been chimeric for the transformation event, which had not reached the germline sectors.

Expression of the Δ9-stearoyl-(ACP) desaturase gene from *R. communis*

The expression of the introduced gene was monitored by Northern analysis (Fig. 3) of immature zygotic embryos and leaves derived from T1 plants (105-2-17 and 105-2-21). Transcripts were detected in immature embryos (28 days after pollination) of both transgenic plants. The corresponding band had the size expected for the transcription product of the introduced *Ricinus* Δ9-stearoyl-(ACP) desaturase gene (1,600 nt) and was absent from leaves of the same plants as

Table 1: Summary of the families and their respective generations for analysis at the plant and seed level in the different trials

Generation Plant	Seeds	Material/family analysed	Experimental level
T0		105-1; 105-2	Confirmation of transgenic status; copy number PCR; Southern analysis
T1		105-2-1–105-2-24	Segregation Southern analysis
T1		105-2-5; 105-2-11; 105-2-14; 105-2-17; 105-2-21	Greenhouse trial 1997 Southern analysis
	T2		Fatty acid analysis (half seeds)
T4		105-2-14-93-11; 105-2-17-22-14; 105-2-17-24-03; 105-2-21-05-15; 105-2-21-19-05	Field trial 1998 PCR; Southern analysis
	T5		Fatty acid analysis (bulks)
T4		F_1 hybrids: 105-2-17-18-19 × 105-2-17-22-01; 105-2-17-18-21 × 105-2-21-02-03; 105-2-17-18-06 × 105-2-14-93-01; 105-2-17-02-09 × 105-2-14-93-05	PCR; Southern analysis
	T5		Fatty acid analysis (bulks)
T5		105-2-17-24-03-02; 105-2-17-24-03-09	Field trial 1999 PCR; Southern analysis
	T6		Fatty acid analysis (bulks)
T5		F_2 population: cross 105-2-17-02-09 × 105-2-14-93-05	PCR; Southern analysis
	T6		Fatty acid analysis (bulks)

a

| | 105-2 | 105-2-1 | 105-2-2 | 105-2-3 | 105-2-4 | 105-2-5 | 105-2-6 | 105-2-7 | 105-2-8 |

nptII

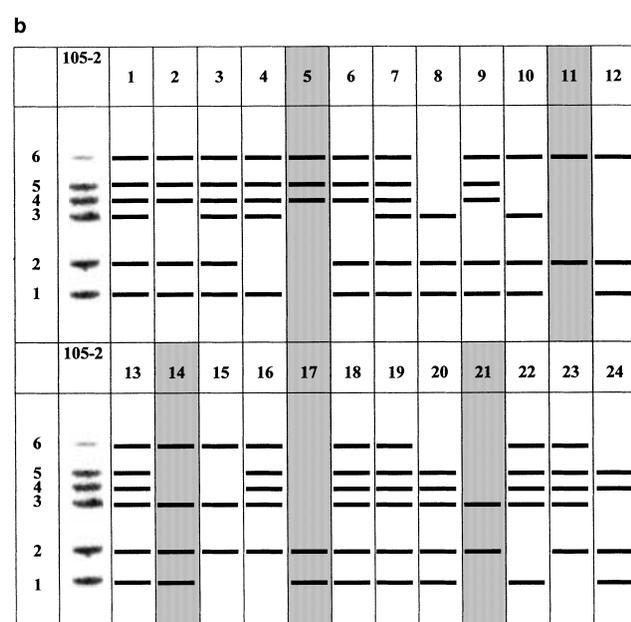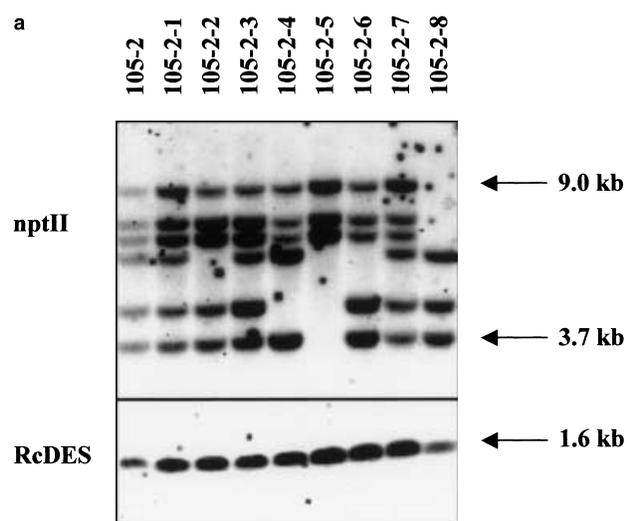← 9.0 kb

← 3.7 kb

RcDES

← 1.6 kb

b

Fig. 2: a. Southern analysis of primary transformant 105-2 and eight T1 plants of its progeny. Genomic DNA was digested by *Eco*RI and subjected to hybridization with *nptII* or *Ricinus communis* Δ9-stearoyl-(acyl carrier protein) desaturase (RcDES) as a probe. Localization of the probes in the vector pBds10Des is shown in Fig. 1a. b. Schematic representation of Southern analysis of 24 T1 plants derived from primary transformant 105-2 (T0). The different insertion events (shown on the left as Southern blot of plant 105-2; probe, *nptII*) are numbered (1–6). Shaded sectors indicate the plants selected for further analysis (Table 2)

well as from wild-type control embryos. Therefore, the *Hads10* regulatory sequences of the construct indeed seemed to direct the expression of the *Ricinus* Δ9-stearoyl-(ACP) desaturase towards the expected tissues and the anticipated developmental stage, i.e. the developing embryo.

Fatty acid composition of seeds obtained under greenhouse conditions

The transgenic plants are expected to produce oil with reduced stearic acid content. T2 seeds of the T1 plants 105-2-5, 105-2-11, 105-2-14, 105-2-17 and 105-2-21 were analysed

| 105-2-17 | | 105-2-21 | | WT | |
| L | E | L | E | L | E |

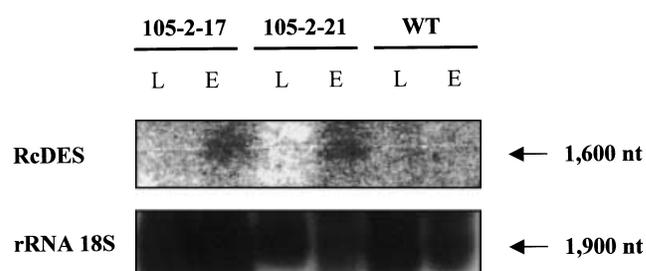**RcDES** ← 1,600 nt

rRNA 18S ← 1,900 nt

Fig. 3: Northern analysis of leaf material (L) and immature embryos (E) (28 days after pollination) of two T1 plants (105-2-17 and 105-2-21) and a wild-type control plant (WT) grown under greenhouse conditions; 20 μg of total RNA were loaded per lane. The band of the size expected for the *Ricinus communis* Δ9-stearoyl-(acyl carrier protein) desaturase (1,600 nt), visible only in developing embryos of transgenic plants, is marked. Control hybridization was performed with a ribosomal probe 18S

using half-seed preparations. Fatty acid composition of those T2 seeds that showed the lowest level of stearic acid in each family are given in Table 2 as well as the corresponding insertion events in the plants derived, which were detected by Southern analysis. The stearic acid content of these selected single seeds ranged from 2.0% to 3.9%, which is significantly lower than the mean stearic acid content of the control at 5.7% ± 1.0%. In addition, some of the half-seeds showed a saturated fatty acid content below 10%, compared with 12.6% ± 1.4% of the control. Figure 4 shows the global distribution of stearic acid and unsaturated fatty acids in seeds of T1 plant 105-2-17 (hemizygous for insertion events [1] and [2]; 32 seeds) and of T1 plant 105-2-11 (hemizygous for insertion events [2] and [6]; 30 seeds) compared with 30 seeds derived from the wild-type line STR 1/95 (control grown simultaneously under the same conditions in the greenhouse). The expected effect of the transgene, i.e. a reduction of the stearic acid content and/or reduction in saturated fatty acids, is apparent in the global distribution of individual values since some seeds derived from plant 105-2-11 (insertion events [2] and [6]) showed a particularly reduced level of stearic acid (less than 3.8%) and some seeds of plant 105-2-17 as well as of plant 105-2-11 had a reduced content of saturated fatty acids (less than 10%).

Fatty acid composition of seeds obtained under field conditions

Field trial 1998

The results of the field trial in 1998 for selected plant families are shown in Table 3. Wild-type control plants (null segregants of the same generation as the transgenic plants used) had a mean stearic content of 4.4 ± 0.4%. Analysis of variance revealed significant differences of stearic acid content between families (F = 14.11 at P = 0.05), however, none of the transgenic families had a significantly reduced mean stearic acid content compared with the control population. No major changes were detected in the ratio between saturated and unsaturated fatty acids in the T5 seeds.

The individual results of the fatty acid analysis of F_1 hybrid plants containing events [2] and [6] (cross 105-2-17-02-09 × 105-2-14-93-05) and of nine progenies derived from plant 105-2-17-24-03 (containing insertion event [1] in a hemizygous state) are shown in Fig. 5 (data represent a bulk of 20 seeds for each plant). Stearic acid contents are plotted against unsatur-

Table 2: Fatty acid composition of selected single seeds harvested from T1 plants 105-2-5, 105-2-11, 105-2-14, 105-2-17 and 105-2-21, grown in the greenhouse 1997. The table presents data for those half-seeds that contained the lowest values in each family. The corresponding half-seeds were grown into plants and analysed for the presence of the insertion events, which are numbered according to Fig. 2b

| T2 seed	Insertion event	Palmitic acid (%)	Stearic acid (%)	Oleic acid (%)	Linoleic acid (%)
105-2-5-4	4, 5 and 6	5.9	3.0	32.3	58.8
105-2-11-10	2 and 6	5.8	3.6	36.9	53.7
105-2-11-14	2 and 6	8.5	2.0	19.1	70.4
105-2-11-20	2	8.0	3.5	19.0	69.5
105-2-11-22	2 and 6	7.8	3.5	17.5	71.2
105-2-14-75	nd[1]	6.8	3.1	25.2	64.9
105-2-14-99	nd	6.6	3.1	53.8	36.5
105-2-14-110	nd	8.2	3.1	21.2	67.5
105-2-17-4	1	5.8	3.9	34.4	55.9
105-2-17-13	1 and 2	5.5	3.9	39.4	51.2
105-2-17-28	1 and 2	6.5	3.9	28.4	61.2
105-2-21-14	2 and 3	6.9	3.5	21.4	68.2

[1] nd, not determined.
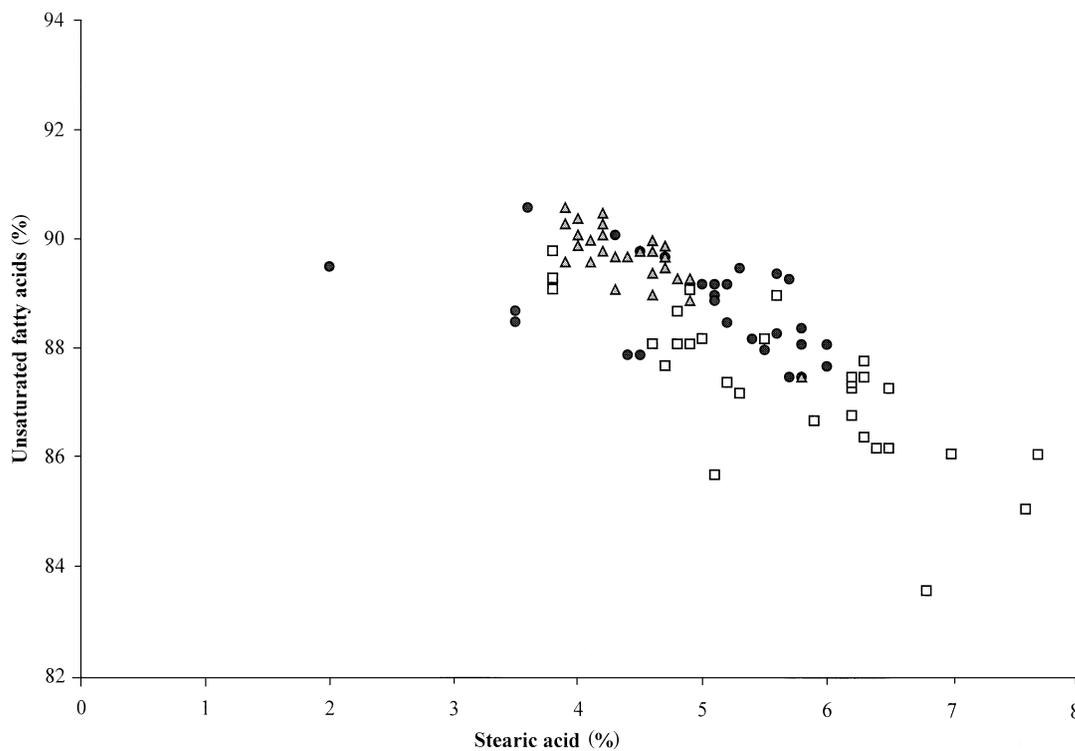
Fig. 4: Greenhouse trial (1997). Stearic acid and unsaturated fatty acids in the seeds (half-seed analysis) obtained from plant 105-2-11 (●; hemizygous for events [2] and [6]) and plant 105-2-17 (▲; hemizygous for events [1 and 2]), as well as seeds of the wild-type line STR 1/95 that had served as donor material for the initial transformation experiments (□, control)

ated fatty acids to overcome the fact that the interspecific hybrid-derived line STR1/95 had been shown to segregate for high and low oleic acid content.

Two progenies (105-2-17-24-03-02 and 105-2-17-24-03-09) showed low stearic acid contents of 3.1% and 2.3%, respectively. These values were significantly below the others and the wild-type control (mean 4.4 ± 0.4%). In addition, the total saturated fatty acid content was reduced to 9.7% and 8.8% in these lines. These two plants proved to be the only ones that were homozygous for the insertion event [1], as demonstrated by Southern analysis of their offspring. The individuals with higher stearic acid content were all either hemizygous or null segregants. These results suggest that the allele number may influence the phenotype produced by insertion event [1].

The plants containing both insertion events [2] and [6] constituted hemizygous F_1 hybrids obtained from parents that were homozygous for insertion events [2] or [6]. For each F_1 plant, the presence of both insertion events was confirmed by Southern analysis. The seeds produced by these plants constitute segregating F_2 generations. The fatty acid profile of the seeds of these F_1 plants showed only a slightly reduced content of stearic acid compared with the control (Table 3) which however, was not significant, as indicated by t-test (P = 0.18). This can be explained by the fact that bulks of 20 seeds derived from these plants were used for the fatty acid analysis, which represent mixtures of different segregation events, including null segregants. In contrast, seeds containing simultaneous insertion events [2] and [6] had previously shown

Table 3: Fatty acid composition (mean ± SE) of seeds harvested from field-grown plants (1998). A bulk of 20 seeds from each individual plant (generation T4) was used for fatty acid analysis. The values obtained for the individual plants were used to calculate the mean (± SE) for each family. The two families selected for the 1999 field trials are indicated in bold

Plant family	Plants (n)	Insertion event	Genetic state of the transgene	Fatty acid composition of T5 seeds (%)			
				Palmitic acid	Stearic acid	Oleic acid	Linoleic acid
Wild type	28	—		6.6 ± 0.4	4.4 ± 0.4	24.9 ± 6.2	64.1 ± 6.1
105-2-17-24-03	**9**	**1**	**Segregating**	**6.6 ± 0.3**	**4.8 ± 1.2**	**29.1 ± 3.8**	**59.5 ± 3.0**
105-2-17-22-14	9	2	Homozygous	5.6 ± 0.4	5.2 ± 0.5	42.2 ± 4.7	46.7 ± 4.7
105-2-17-18-19 × 105-2-17-22-01	11	2	Hemizygous	6.2 ± 0.6	4.5 ± 0.4	26.6 ± 5.3	62.7 ± 5.1
105-2-21-02-15	7	3	Homozygous	6.5 ± 0.5	6.2 ± 0.4	24.8 ± 1.5	62.5 ± 1.4
105-2-17-18-21 × 105-2-21-02-03	10	3	Hemizygous	6.2 ± 0.4	4.5 ± 0.3	24.9 ± 6.2	60.2 ± 4.1
105-2-14-93-11	10	6	Homozygous	6.1 ± 0.3	5.3 ± 0.5	29.5 ± 5.5	59.2 ± 5.4
105-2-17-18-06 × 105-2-14-93-01	20	6	Hemizygous	6.1 ± 0.3	4.6 ± 0.1	24.6 ± 4.4	64.8 ± 4.3
105-2-21-19-05	7	2 and 3	Homozygous	6.8 ± 0.3	5.3 ± 0.3	23.8 ± 2.1	64.1 ± 2.1
105-2-17-02-09 × 105-2-14-93-05	**10**	**2 and 6**	**Hemizygous**	**6.2 ± 0.6**	**4.2 ± 0.3**	**28.0 ± 6.4**	**61.6 ± 6.0**

a significant reduction of the stearic acid content in their seeds in greenhouse experiments compared with the control (Table 2). A second field trial was therefore conducted with the corresponding offspring (F_2 plants) in order to examine more closely the effect of the two insertion events on the stearic acid content in the F_3 seeds.

Field trial 1999

The results of the field trials in 1999 are presented in Fig. 6. It is obvious that the stearic acid content measured as a bulk of 20 seeds for each F_2 plant (population size 237 individuals, segregating for events [2] and [6]), was spread over a wide range from 2.5% to 6.5%, while the mean value of the control plants was 5.2 ± 0.5%. A proportion amounting to 40% of the progenies investigated showed stearic acid contents below the threshold. The low stearic acid individuals would be interesting candidates for introduction into further breeding programmes. However, the total content of saturated fatty acids was above 10%, except for one F_2 plant. The low values of stearic acid for the lines 105-2-17-24-03-02 (bulk of 20 seeds each; 11 plants) and 105-2-17-24-03-09 (bulk of 20 seeds each; eight plants) homozygous for event [1] that had been observed in the 1998 field trial could not be verified in 1999 as all plants analysed showed values that were not significantly different from those of the eight control plants.

Discussion

Although the transformation of sunflower has become a routine procedure, it is still a cumbersome enterprise. The production of a large number of independently transformed plants still requires a heavy investment in time and material, and, owing to their chimeric nature, not all of the primary transformants can be expected to produce transgenic offspring. In this respect, the frequent occurrence of multiple transgene insertion events at different loci is a welcome phenomenon that was exploited to produce, by genetic segregation, offspring with single but different insertion events.

The overall objective of this work was to test the feasibility of modifying the fatty acid composition of sunflower oil by means of genetic engineering, specifically by creating genotypes that produce reduced amounts of stearic acid. To this end, transgenic sunflower plants were produced that expressed the Δ9-stearoyl-(ACP) desaturase gene from *Ricinus communis* (*RcDes*) (Shanklin and Somerville 1991) under the control of sunflower embryo-specific *Hads10* regulatory sequences (Prieto-Dapena et al. 1999).

Southern analysis of primary transformant 105-2 showed that six copies of the T-DNA had been integrated at five independent loci. Since a transformation system for sunflower was not available until recently (Knittel et al. 1994, Grayburn et al. 1995, Burrus et al. 1996, Alibert et al. 1999), information relating to the characterization of transgenic sunflowers is scarce (Hahne 2001, Weber et al. 2001). It was thus important to test the transformants for their genetic stability. The five independent transformation events segregated as expected according to the Mendelian laws. Homozygous and hemizygous lines were obtained as predicted and the individual transformation events could be combined at will using conventional breeding schemes. No sign of genetic instability was observed in any of the lines tested in five generations of greenhouse and field trials.
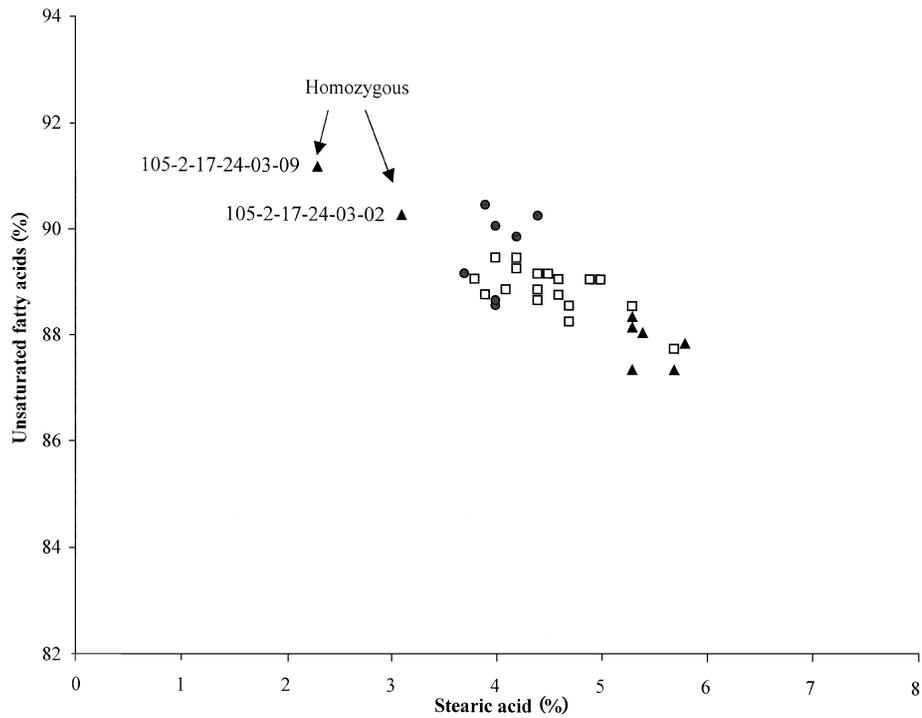
Fig. 5: Field trial (1998). Stearic acid and unsaturated fatty acids in the seeds from nine progenies of plant 105-2-17-24-03 (▲; containing event [1] in homozygous (indicated) or hemizygous or null allele form) and seeds from 10 resynthesized F_1 hybrid plants (105-2-17-02-09 × 105-2-14-93-05) containing events [2] and [6] (●; hemizygous for both events), as well as 18 null segregants of the same generation (□, control). Bulks of 20 seeds were analysed for each plant
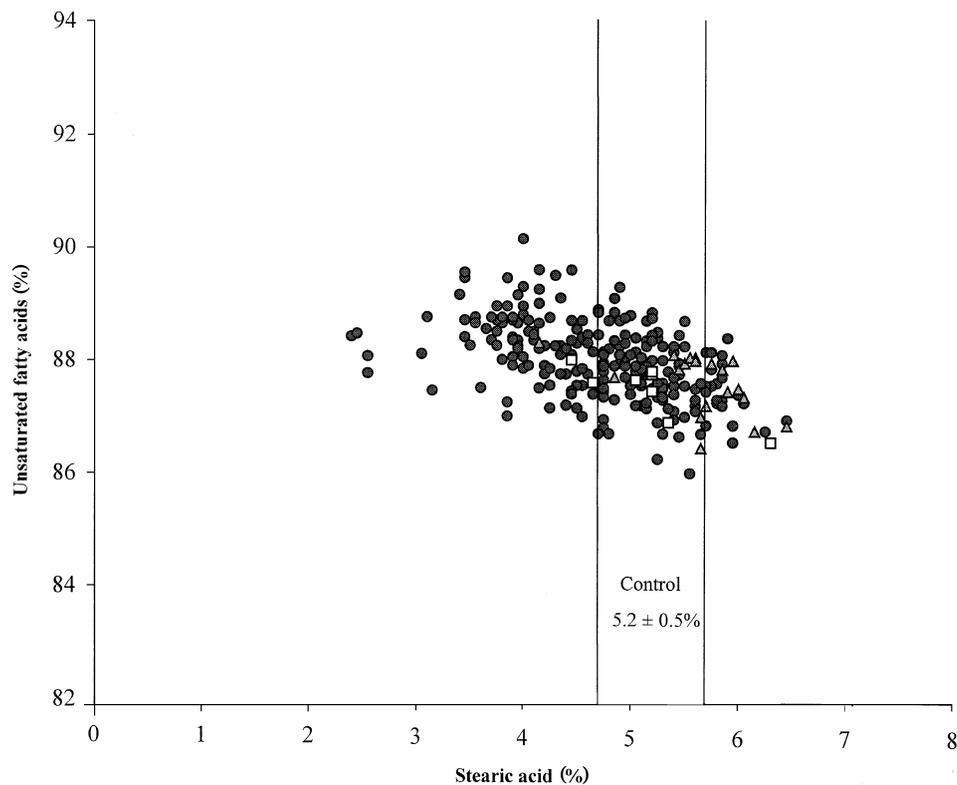

Fig. 6: Field trial (1999). Stearic acid and unsaturated fatty acids in seeds obtained from lines 105-2-17-24-03-02 and 105-2-17-24-03-09 (△; homozygous for event [1]) and seeds from the segregating F_2 population of resynthesized hybrids (105-2-17-02-09 × 105-2-14-93-05) containing events [2] and [6] (●), as well as seeds of null segregants of the same generation (□, control). A bulk of 20 seeds was analysed for each plant

Analysis of fatty acid composition of the seed oil gave evidence for a significant decrease in the amount of stearic acid in the seeds obtained from transgenic plants. Some progenies also showed a content of saturated fatty acids below 10% while other plants with reduced stearic acid content had a higher content of palmitic acid. Certain insertion events appeared to have a stronger influence on the phenotype than others and, for these, homozygous plants had a more pronounced phenotype than hemizygous ones, at least in the greenhouse and in one field trial (1998). The second field trial (1999) did not confirm the superior phenotype of the lines 105-2-17-24-03-02 and 105-2-17-24-03-09 homozygous for event [1], as the progenies showed values for stearic acid that were not significantly different from the controls. The reasons for this remain unknown from the data obtained to date. In contrast, the low-stearic phenotype related to events [2] and [6] when present in the homozygous state, observed in the greenhouse and the first field trial, could be confirmed in the second field trial. However, the plants containing the two events individually or in combination behaved differently between the different growth environments.

The biochemical result of the greenhouse and field trials is complex. In addition, the natural variation within these lines with interspecific backgrounds is still sufficiently high to mask the effects of the transgene in overall comparisons at least partly. Care must be taken to compare the results with appropriate control plants, i.e. null segregants of the same generation. Under this condition, it is clear that several of the integration events have a significant effect on the stearic acid concentration while others had no discernible effect. Events [1], [2] and [6] have shown a statistically significant contribution to the reduction of the stearic acid to levels well below the mean value of the appropriate control population. The contribution of the transgene was strongly modulated by environmental effects: values obtained at different sites (greenhouse/field) and values obtained at the same site but in different years (field trials 1998 and 1999) were not immediately comparable even for control plants. This is not an unexpected observation (Harris et al. 1978, Trémolières et al. 1982, Sarmiento et al. 1998).

The study presented here shows that the experience in genetic engineering of seed oil composition, gathered with the easy-to-transform *B. napus* (Weber et al. 2001), can be transposed, in principle, to the much more difficult to transform sunflower (Hahne 2001). Several conclusions have become apparent from this extensive trial where hundreds of plants have been analysed for their genetic, biochemical and morphometric characteristics. (1) The transgenes introduced are stable and behave as Mendelian traits. (2) The presence and expression of the foreign construct have no effect on the morphology and other agronomic characters of the plants, other than the seed oil composition. These two findings allow one to envisage the incorporation of transgenic sunflowers as starting material in traditional breeding strategies. (3) The genetic construct incorporated results in a subtle but statistically significant alteration of the biochemical phenotype.

However, a definitive conclusion about suitability of this approach for sunflower breeding programmes requires the screening of a larger population of primary transformants. Further experimentation must address problems such as the portability of the transgene and the stability of its expression in different sunflower lines with varying genetic backgrounds.

The exploitation of the natural variability (0.3–9.2% stearic acid and 2.0–15.5% palmitic acid) in the genus *Helianthus*

(Korell et al. 1996) through interspecific hybridization and induced mutagenesis also offer the possibility to obtain cultivated sunflower with reduced levels of stearic acids or reduced total saturated fatty acids. Sunflower mutants with decreased content of palmitic acid (4.7 ± 4%) and stearic acid (2.0 ± 3%) have been produced by *N*-nitroso-*N*-methylurea (NMU) and ethyl methanesulphonate treatment (EMS) by Miller and Vick (1999). However, total saturated fatty acid content was only below 10% in one of the three lines characterized. However, these authors also observed adverse effects derived from combining the alleles providing low stearic acid content and those providing low palmitic acid content. Additional research is necessary to develop sunflower lines with stable reduced levels of stearic acid and/or total saturated fatty acids for an improved industrial application.

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