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Breeding high-stearic oilseed rape (*Brassica napus*) with high- and low-erucic background using optimised promoter-gene constructs

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Abstract Seed lipids of oilseed rape (*Brassica napus*) usually contain small proportions (<3%) of stearic acid. The objective of this study was to increase the content of stearic fatty acid in rapeseed oil. An antisense down-regulation of the endogenous stearoyl-ACP desaturase (SAD) catalysing the reaction step from stearic to oleic acid in two different genetic backgrounds was studied. The result of down-regulation of the SAD yielded an about 10-fold increase of stearic acid from 3.7% up to 32% in single seeds of transgenic low-erucic acid rapeseed (LEAR), while high-erucic acid rapeseed (HEAR) showed a 4-fold increase of C18:0 from 1% up to 4%. It could be shown in pooled T2 seed material of LEAR rapeseed, that the stearic acid content is highly correlated with the down-regulation of SAD as indicated by the stearate desaturation proportion (SDP). The importance of the promoter strength for the alteration of a trait was confirmed in this study as no change in the fatty acid composition of transgenic plants was achieved with gene constructs controlled by the weak FatB4 seed-specific promoter from *Cuphea lanceolata*.

Keywords *Brassica napus* · Seed oil · Stearic acid · Genetic engineering · Antisense · Promoter

Karim Zarhloul and Christof Stoll have contributed in equal parts to the present work

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Introduction

Stearic acid is used in cosmetic products as an emulsifying agent or as an agent to achieve good consistency. Currently, the main source of stearic acid is animal tallow. Considering the potential transmission risk of bovine spongiforme encephalopathie (BSE) and scrapie, which can occur when raw material from cattle or sheep is used in cosmetics, the use of vegetable oil as a source of stearic acid has become an attractive alternative. Furthermore, stearic acid has little or no influence on the plasma cholesterol concentration in comparison to other saturated fatty acids, especially with chain lengths of 12–16 carbons (Grundy 1994; Yu et al. 1995). Genetic engineering has become a powerful tool in rapeseed (*Brassica napus*) breeding, enabling the introduction of

novel genetic variation to adapted cultivars (Töpfer et al. 1995). This is of great significance since genetic variation, which is an essential prerequisite for successful breeding, is generally narrowed in the course of developing new cultivars. In particular rapeseed breeders do not have the option of resorting to wild species, as they are not available. Therefore, breeders are restricted to re-synthesised rapeseed material, which in turn causes problems in quality breeding as erucic acid is the predominant fatty acid in these seed oils (Weier et al. 1997). Genetic engineering strategies are employed to increase the content of particular fatty acids either using over-expression or down-regulation of certain enzymes (Voelker et al. 1997; Thelen et al. 2002; Drexler et al. 2003a; Singh et al. 2005). With the long-term goal of improving the industrial usefulness of oilseed rape, our work aims at the modification of the saturated fatty acid content by developing transgenic *B. napus* that accumulates stearic acid in seed lipids. We have compared low-erucic acid rapeseed (LEAR) and high-erucic acid rapeseed (HEAR) genotypes differing in a mutation of the fatty acid elongase complex (Roscoe et al. 2001; Puyaubert et al. 2001) for their suitability to down-regulate the stearoyl-ACP desaturase (SAD) gene. The high erucic acid rapeseed, represented by a resynthesized rapeseed line, was selected for its high content of erucic acid and its superior transformation ability. To confirm the significance of the promoter on the gene expression, we have tested two different seed-specific promoters, one from a fatty acid thioesterase gene of *Cuphea lanceolata* (FatB4), and another from a napin gene of rapeseed with respect to their effect on the antisense expression of the SAD gene. Results of molecular breeding aiming at an increase of stearic acid content in rapeseed oil are reported here.

Materials and methods

Plant material

The spring rapeseed cv. 'Drakkar', a cultivar showing good transformation efficiency, was used for genetic modification (e.g. Zarlhoul et al. 1999). The variety 'Drakkar' is a canola type with low erucic-acid rapeseed ($<$ 2% erucic acid of total fatty acids) and a reduced level of glucosinolates ($< 25 \mu mol/g*dM^{-1}$). Seeds of the cultivar were obtained from the breeding company Norddeutsche Pflanzenzucht Hans Georg Lembke KG (Hohenlieth, Germany). Additionally, the line 'RS306' developed from an interspecific cross between *B. rapa* cv. 'Yellow Sarson' and *B. oleracea* (cauliflower) cv. 'Super Regama' (Lühs and Friedt 1994) has been used for transformation. It is designated as a high-erucic acid rapeseed due to an amount of 55–60% erucic acid of total fatty acids. Furthermore, 'RS306' contains more than $25 \mu mol/g*dM^{-1}$ glucosinolates.

Bacterial strains and vectors

Agrobacterium tumefaciens strain GV3101(pMP90RK) (Koncz and Schell 1986) was transformed with the constructs pAS*Bn*DES1, pAS*Bn*DES2 and pAS*Bn*DES3 using a conventional electroporation protocol (Dower et al. 1988).

The gene of interest used for three antisense constructs is a seed-specific Δ9-desaturase (SAD) cDNA, which was cloned via PCR according to the sequence information provided by Slocombe et al. (1994) from a rapeseed cDNA library made of mRNA from developing seeds [B. Klein, unpublished]. This SAD cDNA was cloned in antisense orientation into a promoter-terminator cassette of the seed-specific fatty acid thioesterase gene of *Cuphea lanceolata* (FatB4) and in a promoter-terminator cassette of a seed-specific napin gene of *B. napus* (Syring-Ehemann 2001). From these two intermediate plasmids the complete gene cassettes were transferred to the binary vector pLH9000 (AF458478) resulting in the constructs pAS*Bn*DES1 controlled by the FatB4 promoter and pAS*Bn*DES2 driven by the napin promoter. The gene cassette controlled by the napin promoter was also cloned in pLH9500 (AF458479) to obtain the construct pAS*Bn*DES3. The last two constructs differ only in the orientation of the antisense SAD gene in relation to the selectable marker, which in all three cases

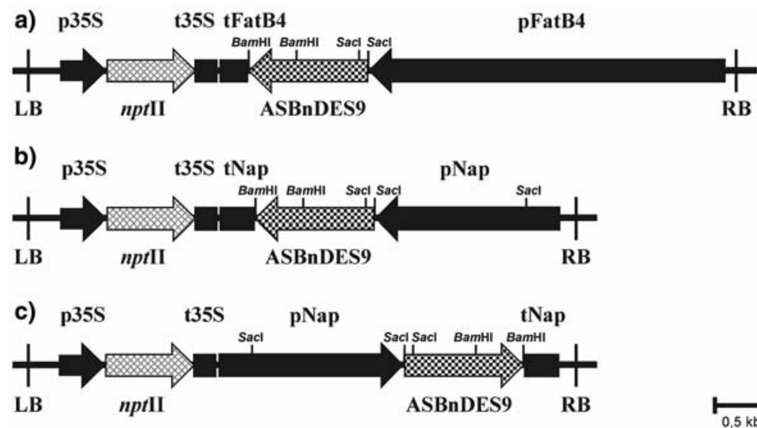

Fig. 1 Schematic representation of the T-DNA region of the binary vectors. A = pAS*Bn*DES1, B = pAS*Bn*DES2, C = pAS*Bn*DES3. LB = left border, RB = right border, p35s/t35s = Cauliflower mosaic virus promoter/terminator, pFatB4/tFatB4 = *C. lanceolata* promoter/terminator, pNap/tNap = napin promoter/terminator,*npt*II = neomycine phospho-transferase II, AS*Bn*DES9 = antisense Δ9-desaturase

represented the *npt*II gene controlled by the constitutive 35S promoter (Fig. 1).

Preparation of agrobacteria for co-culture

Agrobacteria were grown on YEB medium (5 g beef extract, 1 g yeast extract, 5 g peptone, 5 g sucrose, 1 M $MgSO_4$, pH 7.2) solidified with 18 g l^{-1} bacto agar. The medium was supplemented with the appropriate antibiotics in order to select single colonies. A volume of 3 ml of liquid YEB medium, i.e. YEB medium without agar, was inoculated with a single colony and grown overnight on a rotary shaker at 28°C and 200 rpm. This overnight culture was used directly for the co-cultivation step of rapeseed transformation.

Transformation procedure

For genetic transformation and subsequent selection steps the method of De Block et al. (1989) was applied with several modifications. These include the use of (1) etiolated rapeseed hypocotyls instead of hypocotyls grown in the light, (2) ticarcillin/potassium clavulanate, i.e. Betabactyl™ (SmithKline Beecham Pharma, Germany), for elimination of the agrobacteria after co-cultivation, and (3) Gelrite™ (Serva, Heidelberg/Germany) as a gelling agent in the tissue culture media. First, the seeds were surface sterilised in 70% ethanol for 5 min, then for a further 10 min in a 3% NaOCl solution. The oilseed rape seedlings were germinated on A1 medium in the dark. After 7 days (d) the etiolated hypocotyls were cut in 1-cm-segments and co-cultivated with *A. tumefaciens* for 3 d in a liquid A3 medium using 9 cm petri dishes. Following co-cultivation, the hypocotyl explants were placed on petri dishes (2 cm high, 14.5 cm in diameter), 25 explants per dish containing A5 medium with kanamycin (50 mg l^{-1}) as a selective agent. Further sub-culturing of the explants was conducted at intervals of 2 weeks. After 6–8 weeks of selection the first shoots regenerated and were then removed from the hypocotyl explants and transferred to A6 medium (with 15 mg l^{-1} kanamycin), on which they continued growing until phenotypically normal shoots appeared. These were transferred to A8 rooting medium and after formation of roots were placed on soil in the greenhouse. The respective control plants passed the same tissue culture steps without any infection of *A. tumefaciens*.

Characterisation of the transgenic plants by ELISA assay and Southern analysis

The activity of the neomycine phosphotransferase II (NPTII) as a selectable marker was detected

using an ELISA according to manufacturer's instructions (NPTII ELISA KIT, 96-well kit, Eppendorf 5 Prime Inc., 5603 Arapahoe Ave, Boulder, CO 80303, USA). DNA was isolated from fresh leaf tissue as described by Doyle and Doyle (1990). For Southern analysis, 10 µg genomic DNA of the transgenic plants was digested either by using *Bam*HI for plants harboring the pAS*Bn*DES1 and pAS*Bn*DES2 construct or *Sac*I for plants harboring the pAS*Bn*DES3 construct (Promega, Madison, WI, USA). The digested DNA was fractionated by gel electrophoresis, denatured and transferred to a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech, Freiburg, Germany) using 0.4 M NaOH and hybridised according to Reed and Mann (1985). A hybridization probe was prepared by PCR amplification of a 324 bp nptII fragment using the primer pair nptII-s (5′-TGC TCC TGC CGA GAA AGT ATC C-3′) and nptII-as (5′-CGG GTA GCC AAC GCT ATG TCC T-3′) and labelling with ^{32}P dCTP using Klenow-Polymerase (Boehringer, Mannheim, Germany) (Fig. 2). Primary individuals regenerated from the co-cultured explants were named T1 plants (primary transformants), and their progeny were correspondingly called T2 seeds or plants, respectively.

Lipid analyses

For the fatty acid determination in the plant seeds, the extraction and analyses procedures described by Weier et al. (1997) were used. The evaluation was performed after petroleum ether extraction using pooled samples (0.3 g) of T2 seeds harvested from T1 plants (Weier et al. 1997). The sum of very long-chain saturated fatty acids (VLCSFA) included the contents of eicosanoic (arachidic, C20:0), docosanoic (behenic, C22:0) and tetracosanoic (lignocerinic, C24:0) acid. The stearate desaturation proportion (SDP) was calculated as follows:

$$\mathrm{SDP} = \frac{C18:1 + C18:2 + C18:3 + C20:1 + C22:1 + C24:1}{C18:0 + C20:0 + C22:0 + C24:0 + C18:1 + C18:2 + C18:3 + C20:1 + C22:1 + C24:1}[\%]$$

Statistical analyses

Statistical analyses were performed with the program SPSS (SPSS for Windows, Rel. 12.0.1 2003. SPSS Inc., Chicago, IL, USA). Multiple comparisons between mean values of the different variants (untransformed control, pAS*Bn*DES1, pAS*Bn*DES2, pAS*Bn*DES3) were carried out by LSD test in cases of homogeneous variances and by Tamhane test for non-homogeneous variances. Furthermore, box and whisker plots have been used, which enable the identification of maximum, minimum, median values, upper quartile, lower quartile and outliers, respectively.

Results

Effect of different gene constructs on stearic acid content

Primary transformants, represented by T2 seeds, harboring the pAS*Bn*DES1 construct did not show an effect on the fatty acid composition in comparison to the control plants (Table 1). Thus, the expression of the transgene controlled by the FatB4 promoter from *Cuphea lanceolata* did not lead to the desired phenotype. Furthermore, a slight augmentation in the content of palmitic, stearic and linoleic acid in the seeds was determined (Table 1).

In contrast, transgenic LEAR plants harboring the constructs pAS*Bn*DES2 or pAS*Bn*DES3 regulated by a napin promoter showed a shift in the fatty acid composition of pooled T2 seeds (Fig. 3a), particularly characterised by

Table 1 Fatty acid composition of T2 seeds of transgenic 'Drakkar' with integrated pAS*Bn*DES1 gene construct. Minimum, mean and maximum fatty acid contents in % of the total fatty acids

Variant	*n*	C16:0	C18:0	C18:1	C18:2	C18:3
Control	2	3.2–**3.5**–3.7	2.2–**2.7**–3.0	69.8–**72.3**–74.8	11.2–**11.5**–11.8	4.1–**5.1**–6.0
pAS*Bn*DES1	17	3.3–**5.2**–8.2	2.0–**3.4**–5.2	46.3–**62.9**–72.1	11.9–**16.1**–25.3	4.6–**7.4**–9.9

n = Number of plants/lines

Table 2 Fatty acid composition of *Brassica napus* T2 seeds (0.3 g pooled samples) of control, low- (LEAR) and high-erucic acid rapeseed (HEAR) lines harboring gene constructs pAS*Bn*DES2 or pAS*Bn*DES3. Mean values ± standard errors of selected fatty acids in relation to the total fatty acid content (%)

Gene construct	*n*	C18:0	C18:1	C18:2	C18:3	VLCSFA
LEAR T2 seeds						
Control (cv. 'Drakkar')	10	2.9 ± 0.1	74.1 ± 0.8	11.3 ± 0.6	5.6 ± 0.4	0.6 ± 0.2
pAS*Bn*DES2	81	4.0* ± 0.1	69.9* ± 0.4	13.5* ± 0.2	5.4 ± 0.1	1.3* ± 0.1
pAS*Bn*DES3	37	7.3* ± 0.7	62.8* ± 1.2	14.3* ± 0.4	6.6 ± 0.2	2.9* ± 0.4
HEAR T2 seeds						
Control (cv. 'RS306')	14	0.8 ± 0.2	12.7 ± 0.4	10.6 ± 0.2	7.3 ± 0.3	2.2 ± 0.5
pAS*Bn*DES2	22	1.2* ± 0.1	11.5* ± 0.3	11.6* ± 0.2	8.0 ± 0.2	3.7* ± 0.4
pAS*Bn*DES3	31	1.2* ± 0.1	10.7* ± 0.3	11.6* ± 0.1	8.2* ± 0.2	4.0* ± 0.4

n = Number of plants/lines; * significantly different from the control ($P < 0.05$)

significantly increased means of 4% and 7.3% of stearic acid in the seed oil (Table 2). Increased amounts up to 11% and 22% C18:0 were found in the seed oil of plants transformed with pAS*Bn*DES2 and pAS*Bn*DES3 respectively (Fig. 3a); for comparison, the controls had a maximum content of 3.7% stearic acid in the seed oil (Fig. 3a). The analysis also revealed significantly decreased oleic acid contents (69.9% and 62.8%) and significantly increased levels of linoleic acid (up to 13.5% and 14.3%) for the constructs pAS*Bn*DES2 and pAS*Bn*DES3, respectively. In addition to these modifications, we have also observed an increased sum of very long-chain saturated fatty acids (VLCSFA) in the transformants with increased stearic acid content in the seed oil (Table 2, Fig. 3). Mean values of LEAR T2 seeds with the pAS*Bn*DES2 and pAS*Bn*DES3 constructs were 1.3% and 2.9% showing a significant difference to the control seeds (0.6% VLCSFA). The maximum amounts of VLCSFA were 2%, 4.9% and 9.8%, respectively (Fig. 3a). In order to determine the relationship of SAD down-regulation and the augmentation of stearate in the transgenic plants the correlation between stearic acid content and the percentage of stearic acid desaturation (SDP) in T2 seeds of plants harboring the gene constructs pAS*Bn*DES2 and pAS*Bn*DES3 was calculated. A high correlation between the stearic acid content and the SDP in plants of both gene constructs was found. For pAS*Bn*DES3 the variation of stearic acid can almost exclusively be explained by the activity of the SAD ($r = -0.99$, $r^2 = 98\%$) while the corresponding values for the construct pAS*Bn*DES2 are $r = -0.94$ ($r^2 = 88\%$). HEAR plants with an entirely different genetic background (resynthetic line 'RS306') have also been transformed with the most promising gene constructs pAS*Bn*DES2 and pAS*Bn*DES3. This resulted in a significantly higher average content of stearic acid (Table 2) and maximum contents of up to 2.5% and 4% compared to the controls (Fig. 3b). Oleic and linoleic acid contents were modified in a similar manner (Table 2) in the LEAR plants, i.e. a significant decrease of oleic acid to 11.5% and 10.7% for pAS*Bn*DES2 and pAS*Bn*DES3, and a significant increase of linoleic acid up to 11.6% for both gene constructs.

Increased mean values of VLCSFA contents for the pAS*Bn*DES2 or pAS*Bn*DES3 constructs

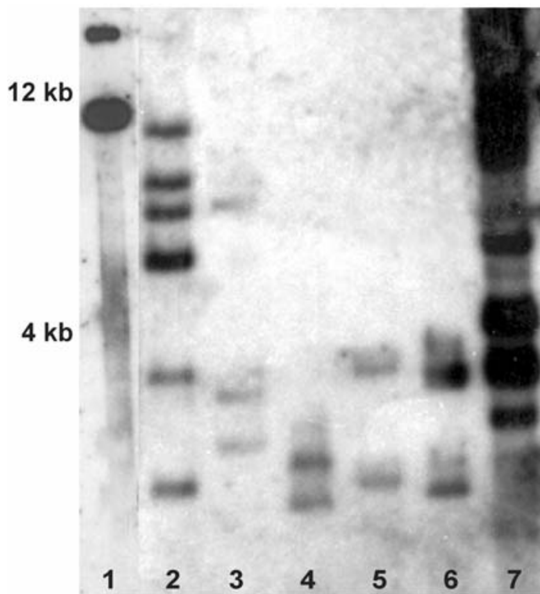

Fig. 2 Southern hybridisation analysis of six LEAR T1 plants from the construct pAS*Bn*DES3: 1 = Plasmid DNA, 2 = DES3 1.A1, 3 = DES3.2.A3, 4 = DES3.1A3, 5 = DES3.1.A2, 6 = DES3.4.A3, 7 = DES3.1.A3. DNA was digested using SacI and hyridized by 324bp fragment of the *npt*II gene (see materials and methods)

(3.7% and 4%) as compared to the control (2.2%) were determined in T2 seeds (Table 2). In this case, transgenic plants carrying both constructs were significantly different from the control. The highest estimated VLCSFA contents were 6.6% and 13.9% for pAS*Bn*DES2 and pAS*Bn*DES3, respectively (control 2.7%) (Fig. 3b). Correlations were also calculated for the HEAR plants to estimate the relationship between the down-regulation of the endogenous SAD and stearic acid content. It was determined that for the plants harboring pAS*Bn*DES2 94% ($r = -0.97$) and pAS*Bn*DES3 98% ($r = -0.99$) of the variation of stearic acid can be explained by the activity of the SAD.

Independent from LEAR and HEAR genotypes and phenotypes, we have observed an orientation effect of the antisense cassette in the gene construct used with regard to the selectable marker on the achieved level of stearic acid. In the construct pAS*Bn*DES2 the gene of interest and selectable marker gene are oriented tail-to-tail, while in pAS*Bn*DES3 it is a head-to-tail orientation (Fig. 1). The latter orientation causes a more efficient gene expression as shown by the significant differences between the means (Fig. 3a).

We have also determined the transgene copy number of 15 LEAR T1 plants to study their influence on the stearic acid amount. Only those lines have been selected which represent the highest stearic acid contents in the respective T2 seeds. The number of integrated genes varied from one to 10. The selected genotypes showing the highest stearic acid amounts (22% and 18.7%) contained six and four gene copies, respectively. In general we found a moderate correlation between copy number and C18:0 content for all the 15 plants tested ($r = 0.54$).

Discussion

Three different gene constructs have been tested for their potential to alter the fatty acid composition in rapeseed. For the seeds derived form plants harboring the pAS*Bn*DES1 construct a slight increase of the palmitic and stearic acid contents was found. However, this minor change was simultaneously observed in corresponding control plants, which showed a comparable poor seed development like the transgenic seeds (data not shown). Therefore, we assigned these modifications to the poor seed development due to adverse environmental conditions.

Two of the three gene constructs, pASBnDES2 and pASBnDES3, have been tested in low- and high-erucic acid genotypes revealing an interesting difference between the genotypes in their potential to accumulate stearic and VLCSFA acids. For LEAR plants harboring pASBnDES2 and pASBnDES3 a significant increase of stearic and linoleic acid contents and a concomitant decrease of oleic acid has been observed in transgenic plants. This significant modification is a likely result of the down-regulation of the endogenous SAD, which catalyses the desaturation step from stearic to oleic acid (Table 2). This assumption is supported by the high correlation for the relationship between the down-regulation of the endogenous SAD and the stearic acid content in the transgenic plants. On the one hand, the oleoyl-CoA, as a precurser of linoleic acid is

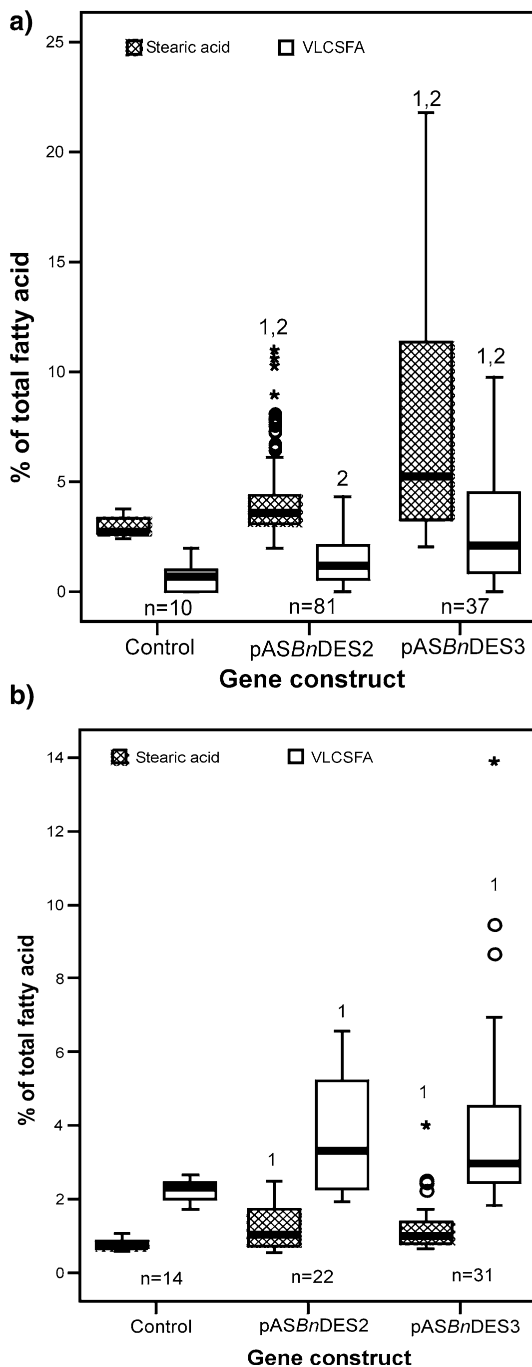

Fig. 3 (**a**, **b**) Box and whisker plot of stearic and very long chain saturated fatty acid (VLCSFA) contents of transgenic T2 seeds in comparison to the controls in LEAR (**a**) and HEAR (**b**) lines. n = number of analysed plants. 1 = significantly different from the control at $P < 0.05$, 2 = significant difference between pAS*Bn*DES2 and pAS*Bn*DES3 at $P < 0.05$. O = outliers with a 1.5 distance from the nearest quartile, * = extreme values with more than 3 box lengths from the upper quartile

reduced by the down-regulation of the SAD, while on the other hand, the enzyme activity of the oleoyl-desaturase is not inhibited. This was expected to lead to an additional decrease in oleic and increased levels of linoleic acid as shown in Table 2.

Just like in LEAR plants a significant increase of stearic acid was also achieved in HEAR plants, although the effect of SAD down-regulation was smaller than in the former material. However, this had to be expected due to the generally lower amounts of stearic acid in HEAR plants. Oleic and linoleic acid contents of HEAR individuals were modified in the same manner (Table 2) as in LEAR plants, which is also explained by the down-regulation of the SAD and the residual enzyme activity of the oleoyl-desaturase resulting in a reduced oleoyl-CoA content. Furthermore, an increased total content of very long-chain saturated fatty acids (VLCSFA) was observed in the LEAR and HEAR transformants with increased stearic acid content (cf. Table 2, Fig. 3). In a similar investigation on rapeseed increased levels of VLCSFA were also observed along with a raised stearate content in the seed oil (Knutzon et al. 1992). In this context two hypotheses were mentioned: (1) an acyl-ACP-specific plastid fatty acyl synthase allows the stearoyl chain further rounds of elongation (Knutzon et al. 1992); (2) the remaining activity of the cytosolic oleoyl-CoA elongase system may be responsible for the increased amount of eicosanoic (C20:0), behenic (C22:0) and lignocerinic acid (C24:0) found in high stearate transgenic plants (Knutzon et al. 1992; Hawkins et al. 1998). Here, the elongation system goes through four successive steps which consist of the following reactions: (i) condensation (3-ketoacyl-CoA synthase), (ii) keto-acyl-CoA reduction (3-ketoacyl-CoA reduc tase), (iii) dehydration (3-OH-acyl-CoA dehydratase) and (iv) enoyl-CoA reduction (trans-2,3-enoyl-CoA reductase).

In general, LEAR and HEAR rapeseed plants are characterized by different amounts of VLCFA caused by two recessive alleles at two gene loci, E1 and E2 (Barret et al. 1998; Fourmann et al. 1998). These genes control the first enzyme of the elongase multienzyme complex, the ß-ketoacyl-CoA synthase, which is

responsible for the higher amount of VLCFA in HEAR seeds and for the shift towards oleic acid in the LEAR seeds (Roscoe et al. 2001; Puyaubert et al. 2001, 2005). There is evidence for the co-existence of two different elongation systems in rapeseed, an acyl-CoA dependant elongase and an ATP-dependant elongase using an endogenous primer for the condensation with malonyl-CoA (Domergue et al. 1999; Hlousek-Radojcic et al. 1995). Due to the co-existence of different elongation systems it may be assumed that a broad range of substrates can be used for further elongation. In addition to the initial condensing step (condensation of C18 fatty acid with malonyl-CoA) (Lassner et al.1996; Millar and Kunst 1997; Han et al. 2001), an altered specificity of the KCS enzyme in favour of stearate is considered to be causal for elevated VLCSFA contents. This assumption is supported by the fact that in our transgenic LEAR and HEAR plants, in spite of a higher ratio of oleic to stearic acid in transgenic high-stearate plants, preferentially C18:0 and not C18:1 was used as substrate for the elongation leading to an increased amount of

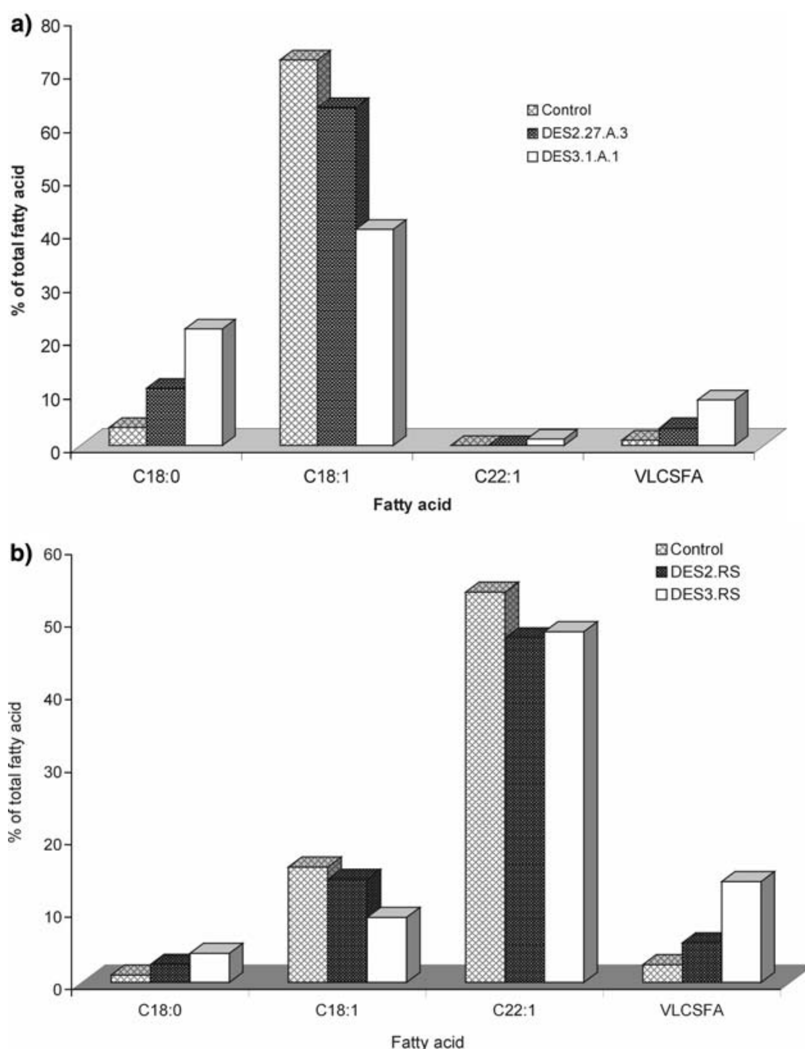

Fig. 4 (**a**, **b**) Effect of stearic acid augmentation on the amount of accumulated very long chain saturated fatty acids (VLCSFA) in LEAR (**a**) and HEAR (**b**) lines. Analysis was conducted on T2 pooled seed material of the best plant concerning the highest amount of stearic acid in control, pAS*Bn*DES2 and pAS*Bn*DES3 plants

VLCSFA as shown in Fig. 4a, b. We further assume that this altered activity is relatively weak, so that it is only manifested with sufficient substrate concentration, i.e. stearic acid. However, this assumption does not expel that a residual activity of the oleoyl-CoA elongase system leads to the determined decrease in oleic acid and an increase in linoleic acid in LEAR and HEAR rapeseed plants. For these two genotypes two major controlling steps seem to be involved in the formation of stearic acid. On the one hand, down-regulation of the SAD was leading to a high stearic acid accumulation of up to 40% (Knutzon et al. 1992) and 32% (present work) stearic content in single seeds of the best transgenic plants. On the other hand, the expression of a thioesterase (Gram FatA1) from *Garcinia mangostana* and its site-directed mutant led to the accumulation of stearate of up to 22% (Hawkins and Kridl 1998) and 40% (Facciotti et al. 1999) in the oil of *B. napus* pooled seed material.

The comparison of LEAR and HEAR genotypes revealed differences in their amount of stearic acid and VLCSFA in the seeds. We address this to the elongase multienzyme complex, the ß-ketoacyl-CoA synthase, and the resulting different metabolic pathways in both genotypes. In addition to that, it might also be possible that the LEAR in contrast to HEAR plants can tolerate higher levels of stearic acid due to physiological aspects. The fluidity of fatty acids tends to be lower the longer and the more saturated they are so that the main fatty acid components might also influence the different accumulation potentials of LEAR (> 60% C18:1) and HEAR (> 55% C22:1). For example, Downey and Taylor (1996) interpreted the concomitant increased levels of linolenic acid in high-stearic acid canola lines (Knutzon et al. 1992) as a counteraction on the higher temperature and oxidative stability resulting from elevated levels of stearate. In the actual work, significantly increased levels of linoleic acid in both genetic backgrounds might be interpreted as a response of the plant on the increase of non-fluid acyl groups. The higher levels of oleic acid in LEAR plants could also enable the plant to cope with the higher amounts of stearic acid in the transgenic LEAR seeds in comparison to the HEAR plants, which show a lower but still significant increase of stearic acid.

We have observed a weak correlation between transgene copy number and stearic acid content, which however may be due to the small number of genotypes tested. For LEAR and HEAR plants not just the amount of transcribed antisense RNA is important, but also the time frame of gene expression, i.e. at different stages of seed development, in order to obtain an efficient inhibition. The rational of this hypothesis was documented by Knutzon et al. (1992). These authors combined two SAD genes in antisense orientation controlled by two different promoters, napin and ACP, in order to achieve a high gene expression prior to and throughout the time period of synthesis of stearoyl-ACP desaturase mRNA in developing *Brassica* seeds resulting in contents of up to 40% C18:0 in the best single seeds.

In the present work, an effect of the orientation of antisense cassettes has been observed. This can be explained by an impact of the inherent enhancer of p35S used in the selectable marker cassette on the napin promoter in pAS*Bn*DES3. An enhancer is assumed to act at a distance of about 2 kb. Therefore, the stronger antisense effect detected in plants transformed with the pAS*Bn*DES3 construct might be due to the fact that the distance between p35S and the napin promoter is shorter in pAS*Bn*DES3 than in pAS*Bn*DES2.

In the present work just like in other publications, the outstanding importance of the promoter for an appropriate expression of the transgene has been demonstrated. Although the same antisense gene was present in all the three constructs we assume that the FatB4 promoter driving the antisense SAD was too weak to achieve the desired phenotype. Promoters from dicot donor plants are usually considered to function in the same manner in other dicot recipient plants. However, in a study by Drexler et al. (2003b) the expression strength and the specificity of four seed-specific promoters, KCS, napin, LeB4 and USP, were compared using the *gus*A reporter gene. This study showed that the promoters from dicot plants do not always work as well in the recipient as they do in the donor plant. The

authors found that the two promoters from rapeseed (KCS and napin) exhibited only low levels of glucuronidase (GUS) activity in linseed (*Linum usitatissimum*) whereas the other two promoters from *Vicia faba* resulted in a high level of GUS activity. Furthermore, transient expression experiments using immature seeds from linseed and rapeseed embryos bombarded with seven different promoter-GUS constructs (USP, LeB4, KCS, napin, DC3, FatB4 or CaMV 35S) showed the napin promoter to have the highest activity. The FatB4 promoter was too weak to result in detectable GUS activity, hence it was not effective in developing embryos of rapeseed. Our study with stable integrated transgenes in rapeseed confirmed the results of Drexler et al. (2003b) since only the napin, but not the FatB4 promoter constructs, gave the expected modification of seed fatty acid composition. Thus, to ensure the expression of a gene of interest and subsequently get the desired altered phenotype a strong tissue-specific promoter is needed.

In our study we were able to considerably raise the stearic acid content in both LEAR and HEAR genotypes in pooled T2 seeds. In addition, increased levels of VLCSFA were found in pooled T2 seeds of LEAR and HEAR as a consequence of the suppression of a specific enzyme using an antisense approach.

One documented superior alternative to the antisense approach for silencing of plant genes is RNA interference effected by sequence-specific degradation of dsRNA (Smith et al. 2000). The better efficiency and efficacy of the hairpin RNA-mediated gene-silencing compared to the antisense or co-suppression approach has been shown by the down-regulation of a Δ12-desaturase in Arabidopsis (Stoutjesdijk et al. 2002). Another work aimed at the gene-silencing of the Δ9- and Δ12-desaturase in cotton, resulting in high stearic and high oleic cotton seed oils (Liu et al. 2002). In comparison to the stearic acid levels in the controls of cotton and rapeseed (2–3% each) the stearic acid contents of the best transgenic genotypes of cotton (40% stearic, Liu et al. 2002) and rapeseed (40% stearic, Knutzon et al.1992) did not differ although different strategies, i.e. RNAi silencing and antisense, were used by these authors. Nevertheless, it would be interesting to have a direct comparison between the antisense approach and the hairpin RNA-mediated gene-silencing in rapeseed. However, it can be concluded that genetic engineering is not only a powerful tool to elucidate genetically encoded biosynthetic pathways, but also for creating new phenotypes as has been shown with the genetic modification of SAD. A next step will be the inclusion of these prototypes in oilseed rape breeding programmes in order to create environmentally stable high-stearic genotypes combined with high agronomic value and market potential.

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