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Genetic manipulation of γ-linolenic acid (GLA) synthesis in a commercial variety of evening primrose (*Oenothera* sp.)

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Keywords: *Agrobacterium*, Δ^6-desaturase, evening primrose, fatty acid, genetic modification, γ-linolenic acid (GLA).

Summary

A robust *Agrobacterium*-mediated transformation procedure was developed for Rigel, a commercial cultivar of evening primrose, and used to deliver a cDNA encoding a Δ^6-desaturase from borage under the control of a cauliflower mosaic virus (CaMV) 35S promoter. Analysis of the transformed plants demonstrated an altered profile of polyunsaturated fatty acids, with an increase in γ-linolenic acid and octadecatetraenoic acid in leaf tissues when compared with control lines.

Introduction

Evening primrose (*Oenothera* sp.) is grown commercially for its seed oil, which contains a high proportion of polyunsaturated fatty acids (PUFAs), including γ-linolenic acid (GLA, $18{:}3\Delta^{6,9,12}$) (Hudson, 1984; Kies, 1989; Mukherjee and Kiewitt, 1987). GLA is an essential fatty acid of major importance in animal cells, but is relatively uncommon in plants. It is found at significant levels in only a few (taxonomically unrelated) species, including evening primrose, blackcurrant (*Ribes nigrum*) and borage (*Borago officinalis*) at approximately 10%, 15% and 20%, respectively, in their total seed (oil). GLA is increasingly being used as a food supplement and has potential for pharmaceutical applications in the treatment of medical conditions, such as diabetes, eczema, inflammation, stress-related disorders, cardiovascular disease and cancer (Barre, 2001; Horrobin, 1987, 1992; Horrobin and Lapinskas, 1993; Horrobin and Morse, 1995; Lapinskas, 1993). Evening primrose and borage are currently the primary agricultural sources of GLA; however, neither species is ideal for commercial oil production as they have low yields in comparison with conventional (agronomically adapted) oil crops (Fieldsend, 1995).

Transgenic approaches to engineer GLA production in both model and agriculturally adapted seed oil crops have been successful. Tobacco plants over-expressing a fatty acid Δ^6-desaturase from cyanobacteria or from borage led to the accumulation of GLA and octadecatetraenoic acid (OTA, $18{:}4\Delta^{6,9,12,15}$) in leaves (Reddy and Thomas, 1996; Sayanova *et al*., 1997). The expression of Δ^6-desaturase enzymes from the fungus *Pythium irregulare* or *Mortierella alpine*, both under the control of seed-specific (napin) promoters, resulted in high levels of GLA in seed lipids of the high-yielding oil crops *Brassica juncea* (Hong *et al*., 2002) and *Brassica napus* (Liu *et al*., 2001), respectively. The obvious advantages of using food crops, such as canola, to produce pharmacologically active compounds include the predictably high yields and the availability of efficient cultivation and harvesting systems. However, the commercial growing of commodity food crops that have been genetically modified with pharmacologically active compounds has been criticized, and there is increasing pressure from regulatory authorities and food producers to keep pharmaceutical crops entirely separate from the food supply (Fox, 2003).

The approach taken here was to genetically enhance the GLA content of the non-food, medicinal plant, evening

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primrose, which is already specifically cultivated for its high-value fatty acids and for which there is a dedicated cultivation, extraction and packaging pipeline. To pursue this approach, it was first necessary to develop a tissue culture and transformation protocol for a commercial *Oenothera* sp. variety. It has been demonstrated previously that non-commercial *Oenothera* sp. accessions are amenable to *in vitro* micropropagation, including the production of undifferentiated, slow-growing callus (Skrzypczak *et al*., 1994), and protocols for regenerating other evening primrose model cultivars (e.g. *O. hookeri*) from leaf protoplasts have been developed (Kuchuk *et al*., 1998). Transformation by *Agrobacterium tumefaciens* co-cultivation has also been reported in *O. hookeri* (Mehra-Palta *et al*., 1998). We have previously regenerated fertile adult plants from immature leaves and cotyledons of the commercial *Oenothera* sp. cultivars, Rigel, Merlin and Vulcan (Mendoza de Gyves *et al*., 2001), but genetic transformation of a commercial cultivar has not been reported previously.

We describe here the first *Agrobacterium*-mediated genetic transformation of a commercial cultivar of *Oenothera* sp. (Rigel) with a cDNA encoding a fatty acid Δ^6-desaturase (Sayanova *et al*., 1997). Fatty acids extracted from leaves of transformed plants were shown to accumulate GLA and OTA to 28% of total fatty acids, with none found in untransformed controls. Although no change in the fatty acid profile was observed in seed tissues, this report demonstrates the potential for the use of targeted expression of the borage Δ^6-desaturase gene to enhance the proportion of high-value fatty acids in evening primrose seed oil.

Results

Selection

To determine the selection pressure appropriate for transformation experiments, the natural level of resistance of *Oenothera* sp. variety Rigel to kanamycin was tested. Untransformed *in vitro* cultures originating from cotyledon explants revealed a marked inhibition of growth, even at the lowest concentration tested (10 mg/L), with higher doses resulting in death (Figure 1e). Kanamycin at 10 mg/L was originally judged to give good discrimination between transformed and untransformed tissue, and was used in all subsequent experiments; however, the final number of 'escape' plants that survived antibiotic selection, but were polymerase chain reaction (PCR)-negative for the transgene (35/50), was relatively high, and indicates that the selection pressure should be raised in future experiments.

Transformation

The transformation procedure was repeated seven times, each using 200 cotyledon pieces and 200 immature leaf pieces. The 107 plants that survived selection, and were transferred from tissue culture to soil, all originated from cotyledon explants (Figure 1b–d). All plant cultures originating from the immature leaf explants died either before or during the selection process. Fifty of the 107 plants were chosen at random for PCR analysis. Twelve were positive for both the selectable marker gene (*npt*II) and the borage Δ^6-desaturase cDNA, which co-segregated in the progeny of these plants. In a further three plants, only the *npt*II gene could be detected (Table 1). The transformation efficiency (i.e. the percentage of cotyledon explants that gave a transgenic plant) was 2.3%. Southern analysis of selected transgenic plants confirmed the integration of the borage Δ^6-desaturase cDNA and enabled an estimation to be made of the transgene copy number. Genomic DNA was digested with *Xba*I, which released the borage Δ^6-desaturase cDNAs as a 2.2 kb fragment, *Bam*HI, which also released one of the two Δ^6-desaturase cDNAs as a 2.2 kb fragment and left the other as part of a larger genomic fragment, and *Eco*RI, which cut once within the T-DNA (see Supplementary material). T-DNA copy numbers were estimated from the number of hybridized bands released by *Bam*HI (not including the 2.2 kb fragment) and

Table 1 The presence of the transgenes Δ^6-desaturase and *npt*II was tested in all lines by polymerase chain reaction (PCR). Southern analysis was performed on lines selected for fatty acid analysis to confirm the presence of the intact borage Δ^6-desaturase transgene in those lines and to give an estimate of the copy number

Plant code	PCR-positive for Δ^6-desaturase	PCR-positive for *npt*II	Copy no. of Δ^6-desaturase from Southern analysis
R01Δ	+	+	2
R03Δ	–	+	nd
R07Δ	+	+	> 6
R08Δ	+	+	3
R16Δ	–	+	nd
R20Δ	–	+	nd
R25Δ	+	+	5
R27Δ	+	+	nd
R54Δ	+	+	4
R93Δ	+	+	3
R94Δ	+	+	nd
R99Δ	+	+	nd
R101Δ	+	+	nd
R102Δ	+	+	3
R107Δ	+	+	nd

nd, not determined.

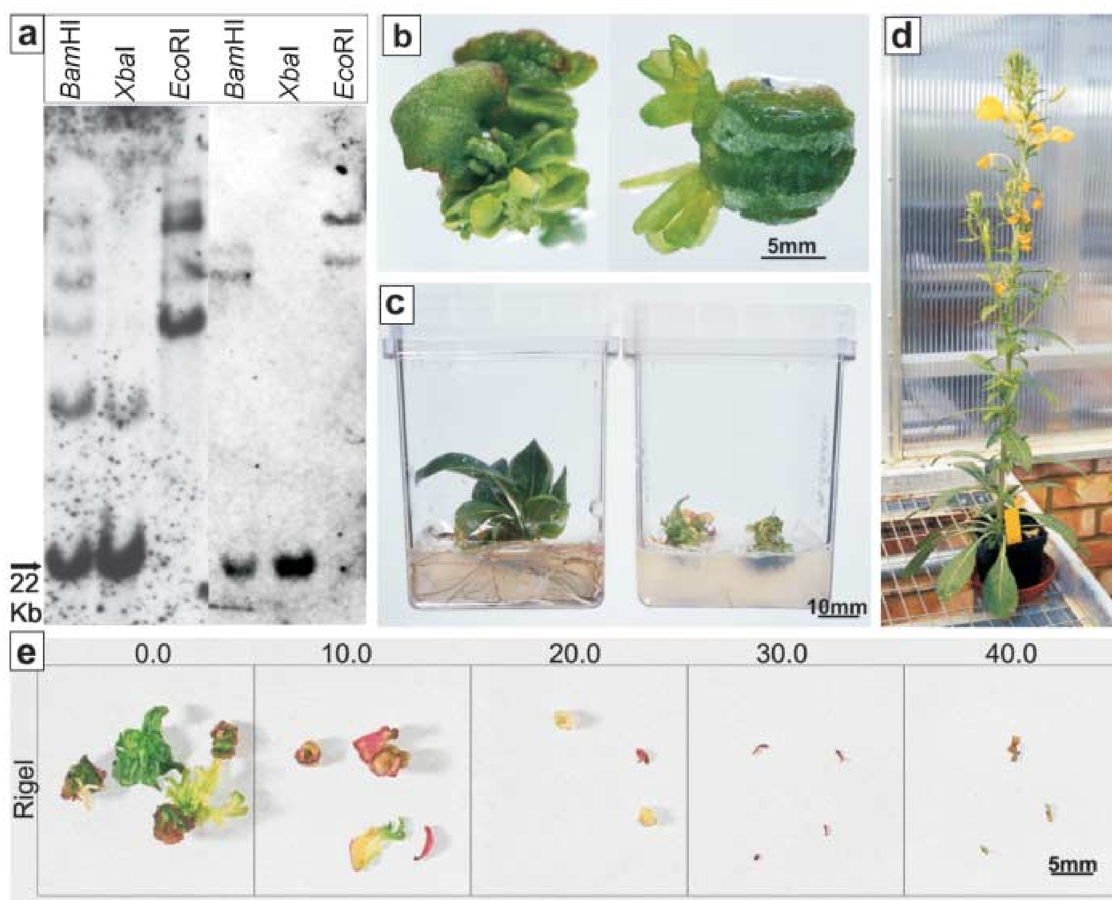

Figure 1 (a) Southern hybridization of transgenic lines R25 (left) and R01. Genomic DNA was digested with *Xba*I, which released the borage Δ^6-desaturase cDNAs as a 2.2 kb fragment, *Bam*HI, which also released one of the two Δ^6-desaturase cDNAs as a 2.2 kb fragment and left the other as part of a larger genomic fragment, and *Eco*RI which cut once within the T-DNA. Incomplete digestion resulted in a 4.4 kb band in the R25 *Xba*I track. (b) Multiple shoot regeneration from cotyledon explants on induction medium. (c) Selection of putative transgenic plants growing on 10 mg/L kanamycin (left) and non-transformed controls (right). (d) Transformed evening primrose (*Oenothera biennis*) plant flowering in containment glasshouse. (e) Effect of increasing kanamycin concentration (from 0 to 40 mg/L) on the early regeneration of non-transformed cotyledon explants.

were confirmed by the *Eco*RI digest, which was expected to give the same number. The T-DNA copy number in the transgenic lines ranged from one to more than six (Table 1 and Figure 1a). No obvious morphological differences were observed between transgenic and control plants at any stage.

Analysis of fatty acids in transgenic plants

Fatty acid methyl esters (FAMEs) of leaf extracts from five independent transgenic lines were analysed by gas chromatography (GC), and the levels of C18 PUFAs were quantified. This analysis revealed an altered profile of C18 PUFAs in all transgenics, but no significant difference in the total PUFA content between transgenics and controls. Two additional fatty acids (GLA and OTA) were present in the leaves of all the transgenic lines tested, but were absent in the untransformed controls (Figure 2). The accumulation of GLA and OTA was variable between lines (8.2–21.2% and 5.2–15.3%, respectively) and, in all lines, there was a reduction in the probable substrates linoleic acid (LA, 18:2$\Delta^{9,12}$) and α-linolenic acid (ALA, 18:3$\Delta^{9,12,15}$). We also determined whether cauliflower mosaic virus (CaMV) 35S-driven expression of the borage Δ^6-desaturase resulted in the accumulation of GLA and/or OTA in seeds and older leaves. One line (R01) which had good fertility, a 3 : 1 transgene segregation ratio and showed marked changes in fatty acid levels was selected for further characterization. Analysis revealed that the increased levels of GLA and OTA in the young leaves of this line were unchanged as the leaves matured (Table 2). We also examined the levels of GLA in the seeds of transgenic evening primrose line R01. There was no increase in the levels of GLA in the seeds (Table 2). This was likely to be a consequence of the reduced activity of the 35S promoter in seed tissues, rather than any intrinsic lack of function of the borage enzyme in this tissue.

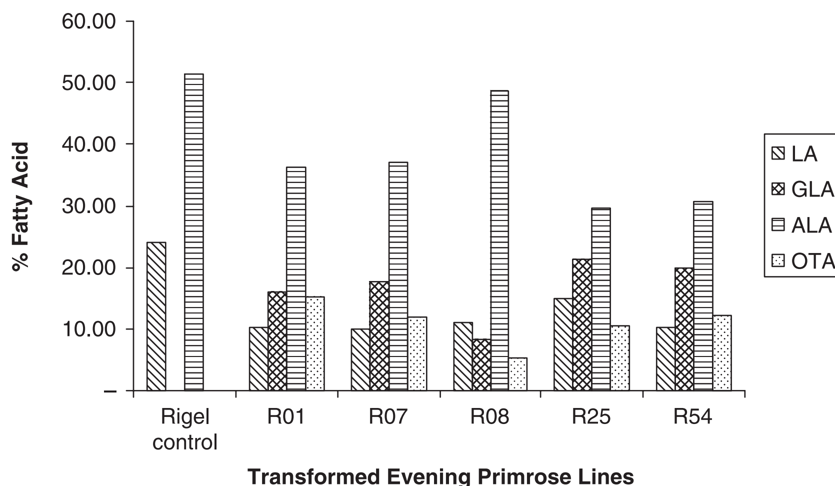

Figure 2 Distribution of selected fatty acids in leaves from control and transgenic evening primrose (var. Rigel) lines: LA (linoleic acid, 18:2$\Delta^{9,12}$); GLA (γ-linolenic acid, 18:3$\Delta^{6,9,12}$); ALA (α-linolenic acid, 18:3$\Delta^{9,12,15}$) and OTA (octadecatetraenoic acid 18:4$\Delta^{6,9,12,15}$). The values represent the average of four independent samples and are presented as mol.% of total fatty acids. The standard error for all measurements was < 3%.

Table 2 Total fatty acid composition of leaves and mature seeds of wild-type evening primrose (Rigel) and transgenic line R01 expressing the borage Δ^6-desaturase. The values represent the average of four independent samples and are presented as mol.% of total fatty acids. Standard error for all measurements was < 3%

	Young leaves		Old leaves		Mature seeds	
Fatty acid	Rigel control	R01	Rigel control	R01	Rigel control	R01
16:0	17.0	18.3	14.0	16.5	9.2	8.8
16:1	2.3	2.1	2.4	2.3	0.3	0.2
18:0	1.0	2.4	1.4	2.6	2.0	1.6
18:1	4.6	2.6	2.7	2.5	5.1	6.9
18:2	26.0	10.3	15.8	11.3	68.3	70.5
18:3	46.0	34.3	63.3	36.8	0.5	0.33
GLA	–	16.8	–	17.0	13.9	13.2
OTA	–	10.7	–	11.1	0.14	0.16

GLA, γ-linolenic acid; OTA, octadecatetraenoic acid.

Discussion

It has been demonstrated that biotechnological approaches can be used to produce significant quantities of GLA in high-yielding oil seed crops, such as *Brassica juncea* and *Brassica napus*, and in model species such as tobacco that do not normally synthesize this fatty acid (Hong *et al*., 2002; Liu *et al*., 2001; Reddy and Thomas, 1996; Sayanova *et al*., 1997). However, the use of commodity food crops to produce pharmacologically active compounds has been criticized (Fox, 2003). We investigated the possibility of enhancing the levels of GLA in a non-food species already grown commercially for its pharmaceutically active seed oil. The evening primrose variety Rigel was chosen because, in previous experiments, it demonstrated high vigour in regenerative *in vitro* tissue culture (Mendoza de Gyves *et al*., 2001), and because of its commercial importance. As a proportion of total fatty acids, evening primrose oil from Rigel contains approximately 12% GLA, compared with 8% for other varieties, and it is owned and currently marketed by Efamol Ltd.

Transformation protocols based on *Agrobacterium tumefaciens* were developed and used to generate 12 independent evening primrose lines containing the borage Δ^6-desaturase cDNA under the control of the CaMV 35S promoter. Fatty acids extracted from the leaves and seeds of transformed lines were shown to contain Δ^6-desaturated fatty acids. In control lines, Δ^6-desaturated fatty acids were found only in seed tissues, where they accumulated as a result of endogenous, non-transgene-derived synthesis.

Levels of the Δ^6-desaturated fatty acids varied between the transgenic lines. This was presumably due to differences in expression levels and/or activity of the borage Δ^6-desaturase enzyme in the different transgenic events. In all lines, there was a reduction in the likely substrates for the borage Δ^6-desaturase enzyme (LA and ALA), but the precise relationship between substrate and product was not studied further.

The accumulation of GLA and OTA in leaf tissue indicates that these Δ^6-desaturated fatty acids are non-deleterious to plant cells that do not normally actively accumulate triacylglycerols (the final site of deposition of GLA in evening primrose is the seeds). It is likely that GLA and OTA are incorporated into the membrane lipids of the cells, presumably in phosphatidylcholine (PC), as PC-esterified fatty acids are the normal substrate for microsomal acyl desaturases (such as the

borage Δ^6-desaturase). No phenotypic alteration in transgenic plants accumulating GLA and OTA was observed; this was in contrast with that observed with the ectopic accumulation of euric acid in *Arabidopsis* (Millar *et al*., 1998), which resulted in severe morphological aberrations after ectopic (CaMV 35S-driven) expression of the FAE1 fatty acid elongase. The ectopic accumulation of the Δ^6-desaturated fatty acid GLA has previously been observed to be phenotypically neutral in plant species which do not accumulate this unusual fatty acid (i.e. tobacco, *Arabidopsis*), most probably as a result of it sharing biophysical properties with the endogenous isomer ALA. However, the presence of OTA, which contains an additional double bond, also appears not to convey any perturbation of gross morphology or function.

No additional Δ^6-desaturated fatty acids were found in transgenic seeds. The absence, or very low levels, of additional Δ^6-desaturated fatty acids in the seeds of transgenic tobacco was also observed when the CaMV 35S promoter was used (Reddy and Thomas, 1996; Sayanova *et al*., 1997, 1999), and is probably due to a lack of transgene transcription in this tissue. It has been reported previously that the CaMV 35S promoter is generally not active in immature cotyledons/endosperm and only weakly active in mature seed tissues (Benfey and Chua, 1989; Benfey *et al*., 1990; Sunilkumar *et al*., 2002; Terada and Shimamoto, 1990). When seed-specific promoters were used to direct the expression of a Δ^6-desaturase cDNA in *Brassica juncea* and *Brassica napus*, a significant increase in GLA in the seeds (25–40% and 43%, respectively) was observed (Hong *et al*., 2002; Liu *et al*., 2001). With suitable seed-specific promoters, it may be possible to achieve similar increases in GLA in seeds of transgenic evening primrose.

In summary, we have developed a successful method to transform a commercial evening primrose variety and have demonstrated that over-expression of the borage Δ^6-desaturase cDNA results in an increase in the GLA and OTA content in leaves. The obvious next step is to test whether the same cDNA, under the control of a seed-specific promoter, leads to the significant accumulation of GLA in evening primrose seeds.

Experimental procedures

Plant material

Cotyledons and young leaves from 4-week-old *in vitro*-germinated seedlings of evening primrose (*Oenothera* sp.), variety Rigel (supplied by Scotia Pharmaceuticals Ltd), were used as an explant source for genetic transformation. The variety Rigel is a hybrid between different *Oenothera* species; precise definitions of species in this genera are subject to debate by taxonomists, and hence *Oenothera* sp. is used here (the full provenance of Rigel is given as Supplementary Material). Rigel was chosen specifically because of its commercial importance, its relatively high GLA content, and because, in previous experiments, we found that it demonstrated high vigour in regenerative *in vitro* tissue culture (Mendoza de Gyves *et al*., 2001).

Plasmid and *Agrobacterium* strain

The plasmid pNTdes6, based on pBIN19, contained an *npt*II gene for plant selection (Bevan, 1984) and a tandem repeat of the borage Δ^6-desaturase cDNA under the control of the CaMV 35S promoter and Ω-enhancer (as described by Sayanova *et al*., 1997) within the T-DNA borders. The *A. tumefaciens* hypervirulent strain AGL1 (Lazo *et al*., 1991), previously shown to be effective at transferring T-DNA to recalcitrant tissue such as wheat (Amoah *et al*., 2001; Wu *et al*., 2003), was transformed with pNTdes6 by electroporation according to Shen and Forde (1989). The bacteria were grown in MG/L medium (Tingay *et al*., 1997) with 200 mg/L carbenicillin and 100 mg/L kanamycin.

Plant tissue culture media

Five media formulations were used in the transformation and tissue culture protocols (Table 3). The MS basal salts, sucrose and polyvinylpyrrolidone K30 (PVP) were dissolved in distilled water and the pH was adjusted to 5.8. Agargel was added prior to autoclaving. The growth regulators and selection agents were added after autoclaving to give the appropriate final concentration from concentrated, filter-sterilized stocks.

Co-cultivation, selection and regeneration

Seeds were surface-sterilized by immersing in 70% (v/v) ethanol for 5 min and then in 10% (v/v) sodium hypochlorite for 20 min, and finally washed five times with sterile distilled water. Seeds were germinated in sterile Magenta boxes on medium A. Leaf and cotyledon squares (approximately 10 mm × 10 mm) from 4-week-old seedlings were taken and placed on to medium B to pre-culture for 48 h before co-cultivating with *Agrobacterium*. The explants were placed with the abaxial surface in direct contact with the medium. Liquid medium (MG/L) was inoculated with *Agrobacterium* (AGL1 containing pNTdes6) from glycerol stocks and incubated at 29 °C for 48 h on a rotary shaker at 250 r.p.m. Cells were pelleted by centrifugation at 1411 ***g*** at 4 °C and the supernatant was discarded. The pellet was diluted to an optical density at

Table 3 Composition of the plant tissue culture media used in tissue culture procedures

	A	B	C	D	E
MS basal salts*	1/2×	1×	1×	1×	1×
Sucrose (g/L)	30	30	30	30	30
pH	5.8	5.8	5.8	5.8	5.8
Agargel (mg/L)	5	5	5	5	5
Polyvinylpyrrolidone K_{30} (PVP) (mg/L)	2	2	2	2	2
Thidiazuron (TDZ) (mg/L)	–	0.1	0.1	0.1	–
Indole-butyric acid (IBA) (mg/L)	–	0.5	0.5	0.5	0.5
Cefotaxime (mg/L)	–	–	500	500	500
Kanamycin (mg/L)	–	–	–	10	20

*Murashige and Skoog (1962).

600 nm of 0.1–0.2 with sterile MS liquid medium supplemented with 3% sucrose. The explants were immersed in the bacterial suspension for 25 min and then blotted on to sterile filter paper to remove the excess bacteria. The explants were transferred to fresh medium B (antibiotic free) and were incubated for 48 h in the dark at 25 °C. Infected explants were then transferred on to medium C (with cefotaxime) under diffuse light conditions at 25 °C to eliminate *Agrobacterium* cells. After 1 week, the explants were transferred on to medium D (with kanamycin) to start the selection of transformed plant cells. At this stage, most of the non-transformed tissue became black and died. The explants were subcultured frequently on to the same fresh selective medium D in order to avoid the accumulation of polyphenolic compounds in the medium. Regenerated shoots were transferred on to selection medium E in Magenta boxes for rooting. Individual shoots with good root formation were considered as putative transformants and, 2 months after the original inoculation, were transferred to soil in containment glasshouse facilities.

PCR and Southern analysis

Young leaves (surface area, 10–15 cm^2) from putative transgenic plants were ground under liquid nitrogen using a pestle and mortar. Genomic DNA was isolated and purified using the cetyltrimethylammonium bromide (CTAB) extraction method (Stacey and Isaac, 1994). PCR analysis of putative transformants was performed using unique primers designed to the borage Δ^6-desaturase sequence (Sayanova *et al*., 1997). For Southern analysis, genomic DNA (5 µg) from PCR-positive plants was digested overnight with *Eco*RI, *Xba*I or *Bam*HI and separated by electrophoresis on a 0.7% agarose gel in tris borate EDTA (TBE). The DNA fragments were transferred to a nylon membrane, hybridized to a PCR-derived probe designed to the borage Δ^6-desaturase sequence, and then visualized using the DIG chemiluminescent detection method (Boehringer Mannheim). The hybridization and washing conditions used were those recommended by the membrane manufacturer and/or the DIG kit protocol.

Fatty acid analysis

Lipids were extracted from young and mature leaves and from the seeds of transformed and control plants by homogenization in MeOH–$CHCl_3$ using a modification of the method of Bligh and Dyer (1959). The samples were transmethylated with 1 M HCl in methanol at 80 °C for 90 min. FAMEs were extracted in hexane. The analysis, detection and quantification of individual FAMEs were performed as described previously (Sayanova *et al*., 1997).

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Supplementary material

The following supplementary material is available from http://www.blackwellpublishing.com/products/journals/suppmat/PBI/PBI079/PBI079sm.htm. **Figure S1** Plasmid map of pNTdes6 with the restriction enzyme sites used for Southern analysis. **Appendix S2** Provenance of evening primrose (*Oenothera sp.*) variety Rigel (Scotia Pharmaceuticals Ltd.).

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