ORIGINAL PAPER

Lipids in hairy roots and non-Agrobacterium induced roots of *Crambe abyssinica*

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Abstract Hairy root cultures of *Crambe abyssinica* were obtained through infection of leaves with two wild-type agropine strains of *Agrobacterium rhizogenes*. The efficiency of transformation was about 16 %. The presence of T-DNA from *A. rhizogenes* in the hairy roots genome was confirmed by PCR using specific primers for *rol*B and *rol*C genes. Selected clones of hairy roots and non-Agrobacterium induced roots from sterile cultures were used for analyses of acyl-lipids. The total amount of acyl-lipids per mg of dry weight was similar in both the non-Agrobacterium induced roots and the hairy roots in good physiological condition, and ranged from 38 to 53 nmol. However, in the clones which showed symptoms of ageing, the lipid content was severely reduced. Also the lipid composition of hairy roots appears to be similar to the composition of non-transformed roots. Polar lipids were the dominant class of lipids in both types of roots (about 75 %). Furthermore, we found diacylglycerols, free fatty acids (FFA), triacylglycerols, sterol esters, and an unidentified lipid class. The dominant fatty acids in the lipids of both types of roots were α-linolenic acid, palmitic acid, and linoleic acid (over 12 % of total FA). Among the lipids of both hairy roots and non-Agrobacterium induced roots of *C. abyssinica*, an unidentified FA was found (over 16 % of total FAs). The present study is the first example of establishment of hairy roots cultures of *C. abyssinica*. It also includes the first analysis of the lipids in hairy roots and non-Agrobacterium induced roots of this species.

Keywords *Agrobacterium rhizogenes* · *Crambe abyssinica* · Lipid · Transformation

Abbreviations

AC	Acetosyringone
DAG	Diacylglycerols
DW	Dry weight
FA	Fatty acids
FFA	Free fatty acids
FS	Free sterols
GC	Gas chromatography
HEAR	High erucic acid rape
LEAR	Low erucic acid rape
PVP	Polyvinylpyrrolidone
PUFA	Polyunsaturated fatty acid
SE	Sterol esters
TLC	Thin layer chromatography
TAG	Triacylglycerols

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Introduction

Crambe abyssinica (Abyssinian mustard) is a Mediterranean native oilseed crop belonging to the Brassicaceae family. Its oil contains a high amount of erucic acid (55–60 %) and is, therefore, used almost exclusively for industrial purposes. Present uses of crambe oil include lubricants, corrosion inhibitors, surfactants, coating agents,

and anti-sticking agents. Its oil is more interesting to the chemical industry than high erucic acid rape (HEAR) oil, due to lower amounts of polyunsaturated fatty acids (PUFAs) (Nath et al. 2007). *C. abyssinica* is also an ideal species for genetic manipulations aimed at improving its industrial suitability. It does not cross-breed with other cultivated plants, or with the majority of related wild species (Wang and Peng 1998). Currently, *C. abyssinica* is cultivated only in a few countries, on very limited areas. Therefore, a potential new, transgenic variety of *C. abyssinica* would probably be spatially separated from classic varieties. However, even if mixing occurred by chance, it would not have any effect on the consumers, as crambe's oil is not edible.

All the above-mentioned unique characteristics of *C. abyssinica* contribute to its growing popularity among plant biotechnologists. *C. abyssinica* transformation methods developed so far use *Agrobacterium tumefaciens* as the vector (Li et al. 2010; Engler and Montanez 2010). Still, these methods were shown not to be very efficient. The aim of this study was to develop an alternative transformation method using *Agrobacterium rhizogenes*. This bacterium is an etiological agent of the hairy root disease, which is caused by incorporation of a bacterial-derived segment of transferred-DNA (T-DNA), containing root loci (*rol*) genes, into the chromosomes of plant cells (Hunter and Neill 1990). However, laboratory strains without these genes are available that can be used to introduce other genes into *C. abyssinica* genome (Mankin et al. 2007). Hairy root cultures can produce a spectrum of secondary metabolites that are not present in the parent plant (Tepfer 1984; Tiwari et al. 2008). This technique has become a useful tool for studying biochemical properties of plants (Cai et al. 2012). Laboratory protocols for hairy root induction involve cultivation of a wounded, sterile explant that has been either directly inoculated, or co-cultivated with an *A. rhizogenes* suspension, and then treated with antibiotics to eliminate the bacteria. Recent advances in hairy roots induction include introduction of a new technique—sonication-assisted *A. rhizogenes*-mediated transformation (SAArT)—which is particularly suitable for inducing hairy roots in recalcitrant plant species (Georgiev et al. 2012). Due to their fast growth rates and biochemical stability, hairy root cultures remain unsurpassed as a model root system and seem to be a promising bioprocess system (Cai et al. 2012).

In addition to the development of a new protocol of crambe transformation, another goal of this research was cultivation of stable hairy roots cultures of *C. abyssinica*. Hairy roots cultures offer genetic stability and dynamic growth without growth hormones (Hamill et al. 1987).

They can be used to produce different kinds of chemicals for industrial and pharmaceutical applications. Moreover, the potential of hairy roots of *Echium acanthocarpum* for production of polyunsaturated fatty acids (PUFAs) was investigated. Total amount of lipids obtained in different hairy root lines ranged from 29.75 to 60.95 mg/g dry weight (DW) and PUFAs accounted for approximately 55 % of total FAs (Cequier-Sánchez et al. 2011). It may be possible to use hairy roots cultures in bioreactors for oil production, which would make them a viable alternative to oil seed crops. Nevertheless, the knowledge about lipids in hairy roots remains limited (Cequier-Sánchez et al. 2008, 2011; Toivonen et al. 1992). The results we present may be considered a first step in revealing the lipid metabolism of hairy roots and non-transformed roots of *C. abyssinica*.

Materials and methods

Plant material

Seeds of *C. abyssinica* cv. Mayer were surface-sterilised for 15 min in a 5 % solution of $CaCl_2O_2$, mixed (1 drop/100 ml) with Tween 80 (POCh, Poland), rinsed four times with sterile, distilled water and placed in glass jars (capped with polypropylene lids) with agar-solidified medium (7.5 g agar l^{-1}; Sigma) containing 2 % (w/v) of sucrose (Sigma-Aldrich, Germany). The pH of the media was adjusted to 5.8 prior to autoclaving (114 °C; 105 kPa). The cultures were incubated at 20–22 °C under a 16-h photoperiod (white cool fluorescent light, Philips, TLD 58 W/84o, 30–35 μmol m^{-2} s^{-1}), in a growth chamber, for 4 weeks. During this time, the seeds germinated and the seedlings reached several centimetres in length.

A. rhizogenes agropine strains: A4 (ATCC 31798)—obtained from the Botanical Garden of Wroclaw, Poland), and ATCC 15834 (obtained from the Laboratoire Agronomie et Environnement, Ecole Nationale Superieure d'Agronomie et des Industries Alimentaires, Nancy, France)—were grown on a YEB agar-solidified medium at 26 °C, in the dark. Before transformation, the bacteria were inoculated to liquid YEB medium (Miller 1972) or agar-solidified YEB medium supplemented with 200 μM acetosyringone (AC), and cultured at 26 °C in the dark, for 24 h. Liquid cultures were grown on a gyratory shaker at 260 rpm to a final $OD_{600} = 0.6$.

Establishment of hairy root cultures

Transformation of *C. abyssinica* was obtained using two different protocols. In both of them, two wild-type

agropine strains: A4 and 15834, were used with explants of leaves and cotyledons (100 pieces of each).

1. *C. abyssinica* explants (cotyledons and leaves) were inoculated using inoculating needles containing *A. rhizogenes*. After inoculation, the tissues were put on MS medium containing 200 µM AC and incubated in the dark, at 20–22 °C. After 3 days of co-culture, they were transferred to fresh MS medium containing 500 mg l^{-1} carbenicillin (Polfa, Tarchomin, Poland) and 500 mg l^{-1} claforan (Hoechst, M. Roussel, Germany).

2. Overnight *A rhizogenes* culture which grew in the presence of 200 mM acetosyringone was diluted to $OD_{600} = 0.6$. Into the resulting suspension, we put explants of *C. abyssinica* (cotyledons, leaves) which were previously wounded with a needle. They were then sonicated (frequency 40 kHz, Polsonic, Poland) for 3 s at 20 °C. Next, the co-cultures were rinsed with sterile, distilled water and put on a solid MS medium. After 3 days, they were transferred to a solid MS medium containing carbenicillin and claforan (500 mg l^{-1} each).

After 1 month, numerous, fine roots appeared on the leaves. After cutting off, they were put into liquid MS medium containing 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA), carbenicillin and claforan (500 mg l^{-1} each). Individual clones were cultured in the dark, at 22 °C, with shaking (110 rpm). The hairy root cultures were passaged on a new MS medium every 2–3 weeks. Antibiotics were added during the first 5–6 passages, until *A. rhizogenes* was eliminated from the cultures.

Hairy root cultures, free from bacteria, were maintained in the dark at 20 ± 2 °C, on a rotary shaker (110 rpm, amplitude 9) in 250-ml flasks containing 35 ml of liquid MS medium (without antibiotics) supplemented with 0.1 mg l^{-1} NAA and 0.1 % polyvinylpyrrolidone (PVP, Sigma-Aldrich, Germany). Subcultures were made every 3 weeks. The results were expressed as percentage transformation frequency (% transformation frequency = [number of explants producing hairy roots/total number of explants infected with *A. rhizogenes*] × 100). All the experiments were carried out in triplicate and the results were expressed as mean ± SD. Statistical analysis was performed according to Tukey (1953).

Transformation of *C. abyssinica* was confirmed, at the molecular level (presence of T-DNA fragment in the DNA of hairy roots), with PCR using primers shown in Table 1, according to the procedure described by Krolicka et al. (2010).

The non-Agrobacterium-induced roots of *C. abyssinica* used for the analysis came from a 1-month-old seedlings cultured in vitro on a MS medium solidified with agar.

Lipid analysis

In order to reduce the risk of oxidation, exposure of lipids to oxygen was minimised using nitrogen atmosphere whenever possible, in all operations. Extraction of lipids was done according to the modified method of Bligh and Dyer (1959). Apart from *C. abyssinica* hairy roots clones, non-transformed roots of *C. abyssinica* from in vitro cultures were analysed. Depending on the physiological condition of the analysed roots, different amounts of tissue were collected (from 300 to 800 mg). The tissues were homogenised in a glass homogenizer with the addition of 3.75 ml of chloroform and fresh methanol mixture (ratio 1:2), and 1 ml of 0.15 M acetic acid. The homogenate was moved into a test tube and 1.25 ml of chloroform and 1.25 ml of water were added (in order to obtain phase separation). The mixture was shaken intensively and centrifuged at circa $1,000 \times g$ for 3 min. The chloroform phase was transferred into a new tube and vaporised in a nitrogen atmosphere at 50 °C. The resulting residue was dissolved in 2 ml of chloroform and stored at −20 °C, or used immediately for further analysis.

To determine the total content and composition of fatty acids in the acyl-lipids of the analysed roots, aliquots of the obtained chloroform fractions were vaporised under a nitrogen stream and the sediments were incubated at 90 °C for 40 min with 2 ml of 2 % sulphuric acid in dry methanol. Next, we added an internal standard (17:0-Me—methyl ester of heptadecanoic acid; Sigma), 3 ml of hexane (Merck, Germany), and 3 ml of water. The mixtures were shaken intensively. After centrifugation (at $1,000 \times g$ for 3 min), hexane fractions were retrieved, vaporised, and dissolved in a small amount of hexane. The analyses of methyl esters of fatty acids contained in the extracts were carried out on a Shimadzu gas chromatograph with a flame ionisation detector and a BPx70 SGE capillary column. Fatty acids were identified based on the retention times of standards (Larodan, Sweden). Determination of their content was carried out through comparison of their peak areas with the peak area of the internal standard (done automatically by a computer program).

Lipid composition of the analysed roots was determined through separation of chloroform extracts on TLC plates (silica gel 60; Merck) developed in hexane:diethyl ether:acetic acid (70:30:1, v/v/v—separation of neutral lipids). The lipids were localised through short exposure of the plates to iodine vapour and identified according to the standards co-chromatographed with the samples. The areas of silica gel containing the separated lipid classes were sprayed with distilled water and transferred into test tubes. Water was removed from the gel (small amounts of methanol were added and vaporised in a nitrogen atmosphere, at 50 °C), and the lipids were methylated and analysed using GC as described above.

Table 1 Primers used in the study

Primer name	Primer sequence 5′ → 3′	Product length (bp)
rol B1	GCT CTT GCA GTG CTA GAT TT	423
rol B2	GAA GGT GCA AGC TAC CTC TC	
rol C1	CTC CTG ACA TCA AAC TCG TC	626
rol C2	TGC TTC GAG TTA TGG GTA CA	
vir G1	ACT GAA TAT CAG GCA ACG CC	273
vir G2	GCG TCA AAG AAA TAG CCA GC	

Double bonds of unidentified FAs were determined by silver ion thin layer chromatography. TLC plates were prepared by steady pouring of a silver ion solution (2.5 g of silver nitrate diluted in 2.5 ml of water with addition of 11 ml of acetone) on a silica gel 60 plate (20 × 20 cm), evaporation of the solvents for 15 min at room temperature, and a 10-min activation at 100 °C. The methylated lipid extract of the hairy roots clone which contained the highest amount of unidentified fatty acids was separated on such plate (development in hexane:diethyl ether:acetic acid—70:30:1, v/v/v). The six separated fractions of fatty acids methyl esters from the analysed sample (as well as the co-chromatographed standards—methyl esters of palmitic, stearic, oleic, linoleic, and α-linolenic acid) were visualised by spraying with water and scraped from the plate into the test tubes. Lipids were then extracted from the scraped silica gel by a 15-min sonication with addition of 3.75 ml of chloroform:methanol (1:2) mixture. After centrifugation (2,000×g for 5 min), the extracts were moved to new test tubes, internal standard (17:0-Me) was added, and fatty acid methyl esters were extracted to the chloroform phase (addition of 1 ml of 0.15 M acetic acid, 1.25 ml of water and 1.25 ml of chloroform; vortexing, and centrifugation for 3 min at 1,000×g). After evaporation of the chloroform (nitrogen atmosphere, 50 °C), residues were solubilised in a small amount of hexane and analysed by gas chromatography. All the results of the analyses of lipid composition and content are presented as arithmetic means ($n \geq 3$) with standard deviation.

## Results and discussion

Hairy roots

During 4 weeks of sterile culture, more than 90 % of seeds of *C. abyssinica* germinated and produced few centimetre long seedlings. Leaves and cotyledons of 4-week-old plants were then inoculated with two agropine strains of *A. rhizogenes*: A4 and 15834, using two different methods. After 1 month, numerous frail hairy roots appeared at the points of injection on the leaf fragments (Fig. 1), but not on

cotyledons. If the leaves were inoculated (using a needle) with A4 strain, the frequency of transformation was 16 %. Another *A. rhizogenes* strain, 15834, which was used in this method showed only 2 % of effectiveness. In turn, the use of sonication for transformation provided 5 % of effectiveness for 15834 strain and 1 % for A4 strain. The choice of an appropriate *A. rhizogenes* strain had an important impact on the transformation efficiency. Agropine strains of *A. rhizogenes* (also used in this study) are regarded as the most virulent, due to the fact that their cells contain two T-DNA sequences on Ri plasmid, called the left (TL-DNA) and right (TR-DNA) region (David and Tempe 1988). According to Porter (1991), strain 15834 is more virulent than A4. However, in our experiments, application of A4 strain gave better results. Thus, it seems that the method of introduction of *A. rhizogenes* to the transformed plant tissues is as much important (or perhaps even more important) as the utilised bacterial strain.

The choice of plant material is crucial for successful transformation with *A. rhizogenes*. Usually, transformation of young tissues gives the best results (Bonhomme et al. 2000). Sometimes, only a defined part of the plant can be used. In the case of *Ammi majus*, for example, only the use of the first stem node allows to obtain hairy root cultures (Królicka et al. 2001). Tissue regeneration ability could be the key factor for a successful transformation process. For example, it was shown that all stem tissues of young *Nicotiana tabacum* plants were able to produce hairy roots, while in the case of mature tissues, hairy roots developed only as a result of cambium transformation (Olszowska 1992). However, the ability to form embryogenic callus by cotyledons and leaf explants of *C. abyssinica* seedlings was proven to be similar (Furmanek and Banaś 2011). Nevertheless, in the presented experiments, only the leaf explants produced hairy roots. Therefore, it seems that other factors than the regeneration ability could affect the efficiency of *A. rhizogenes* transformation process.

In the present research, we implemented acetosyringone (4-acetyl-2,6-dimethoxyphenol) to *A. rhizogenes* growth medium, as well as to the culture media used for the growth of inoculated plant explants. It was probably the crucial factor for obtaining high transformation efficiency of *C. abyssinica* leaves. Such assumption could be drawn from earlier studies showing that application of acetosyringone significantly increases the efficiency of plant transformation with bacteria from the *Agrobacterium* genus (Kumar et al. 2006). Sheikholeslam and Weeks (1987) showed, for example, that incubation of *A. tumefaciens*, in the presence of 20 μM acetosyringone, just before inoculation of *Arabidopsis thaliana* plants, resulted in a 60 % efficiency of transformation, whereas the use of bacteria not pre-incubated with acetosyringone resulted in only 2–3 % efficiency. Pre-incubation of *A. rhizogenes* with

Fig. 1 Growth of hairy roots of *C. abyssinica* on MS medium + 2 % sucrose: **a** MS + 500 mg 1^{-1} Claforan + 500 mg 1^{-1} Carbenicillin—4 weeks after infection, **b** MS + 0.1 mg 1^{-1} NAA—6-week-old culture; **c** MS + 0.1 mg 1^{-1} NAA—10-week-old culture, **d** MS + 0.1 mg 1^{-1} NAA + 0.1 % PVP—14-week-old culture
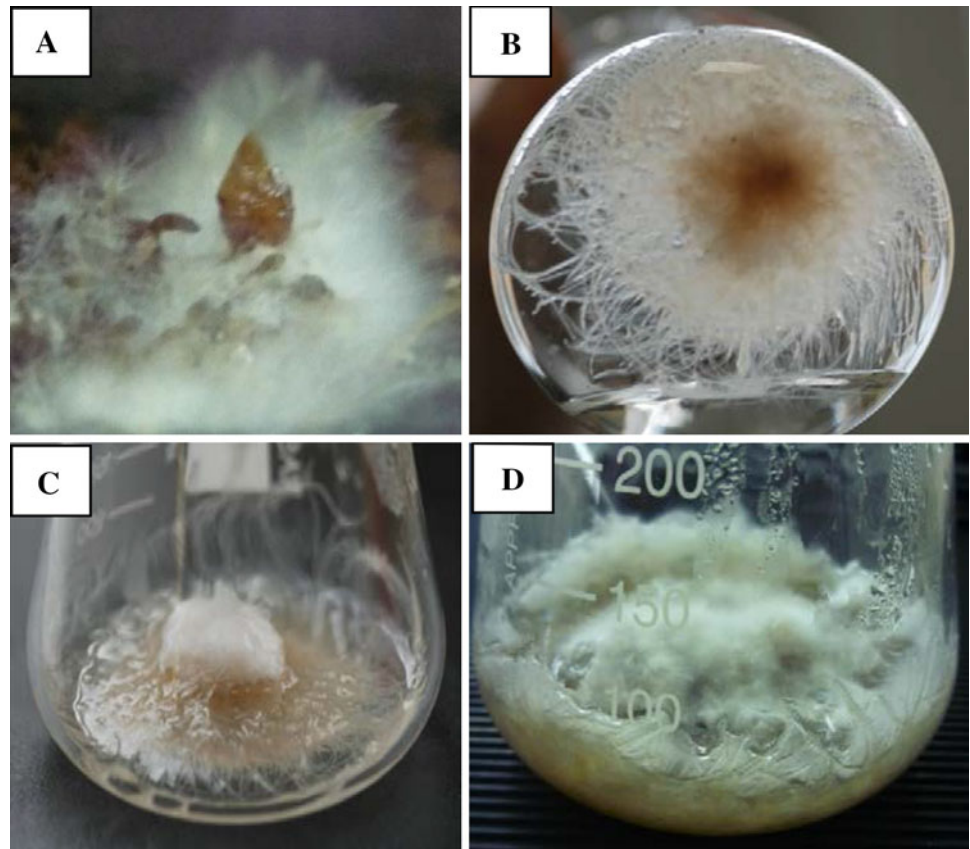
100 μM acetosyringone before transformation of *Alhagi pseudoalhagi* resulted in a fivefold increase in the transformation efficiency, compared to a protocol with untreated bacteria (Wang et al. 2001). These results can be explained by the fact that acetosyringone is excreted by wounded plant tissues and induces vir genes expression in the cells of *A. rhizogenes*, as well as takes part in chemotaxis of these bacteria (Flores and Medina-Bolivar 1995; Melchers et al. 1989). Therefore, addition of acetosyringone to the medium could stimulate these processes.

The data published so far indicate that the efficiency of transformation of the plants from Brassicaceae family (Crucifere) with *A. rhizogenes* varies from 1 % (*Brassica oleracea*) to 43 % (*Brassica campestris*) (Christey et al. 1997). Thus, the results obtained in the presented research (16 % efficiency) place *C. abyssinica* in the middle and can be considered satisfactory, especially in comparison to the transformation efficiency of *C. abyssinica* with *A. tumefaciens*. The protocols of *C. abyssinica* transformation developed and patented so far concern only the use of *A. tumefaciens* with efficiencies of 1.3–2.1 % (Li et al. 2010; Engler and Montanez 2010). Similar to our results, transformation of other crucifers with *A. rhizogenes* was much more efficient, when leaves, rather than cotyledons, were inoculated with the bacteria (Christey et al. 1997).

Hairy root cultures can be grown on a medium without growth hormones, however, addition of 1-NAA leads to higher biomass accumulation (Shen et al. 1988). Roots are more sensitive to auxins than shoots, and a relatively low auxin concentration inhibits their growth. Thus, it is likely that transformation with *A. rhizogenes* changes the sensitivity of the transformed cells to this hormone. In the present research, hairy roots were cultured on a medium containing 0.1 mg 1^{-1} NAA, and this concentration resulted in proper development of hairy root cultures (Fig. 1).
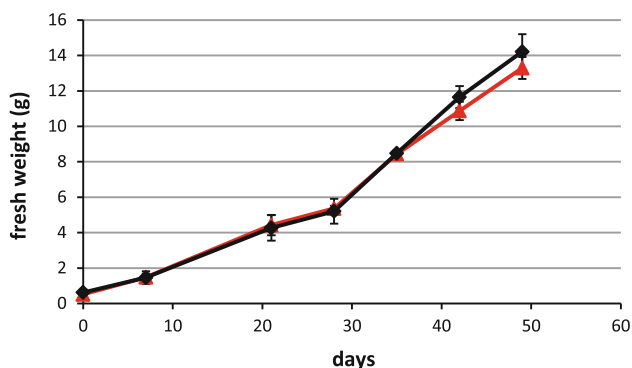
At the beginning of the study, hairy roots were grown in a liquid medium without polyvinylpyrrolidone (PVP). In such conditions, we observed intensive browning of the medium, as well as of the hairy roots culture. When the cultures were not passaged for a period longer than 3 weeks, the transformed roots showed serious signs of ageing (lack of growth and dying out of the culture). Addition of PVP to the medium significantly reduced the afore-mentioned symptoms. It is highly likely that the observed browning could result from intensive excretion of phenolic compounds. The added PVP was probably sequestering the phenolics from the media (PVP is commonly used for this purpose; Siegeland and Enns 1979; Maliyakal 1992) and thus, the cultures could intensify their

development. After 50 days of culture, the biomass of hairy roots increased 27-fold, both when PVP was added to the medium, and when it was not (Fig. 2). The differences in growth rate were not significant, though it is noteworthy that the hairy roots cultured with PVP were brighter. They could be passaged on fresh medium after 5–6 weeks of growth. We observed no darkening of the medium by phenolic compounds, or as a result of dying out of the hairy root culture.

To validate the transformation at the molecular level, a PCR analysis of the DNA isolated from the tested clones of hairy roots was conducted. During the DNA extraction (according to the modified method of Bekesiova et al. 1999), sodium sulphite (antioxidant) was implemented to protect DNA. Preliminary results indicated that the protection of DNA against oxidation was absolutely necessary. It is likely that during the cell lysis, phenolics are released, oxidised by cell oxidases, and subsequently bound irreversibly with nucleic acids which results in DNA degradation (Varma et al. 2007). We used the primers for rolB and rolC genes for amplification of genomic DNA of the analysed hairy root cultures and found that both of the tested genes were present in the analysed DNA (product length of 423 and 626 bp, respectively). PCR performed with the primers for virG gene (present on the bacterial Ri plasmid, outside the T-DNA) confirmed the absence of this fragment (product length 273 bp) in the analysed DNA (Fig. 3). Thus, we got a confirmation that the analysed hairy roots clones were free of bacteria and that rolB and rolC genes were present in their genomic DNA.

Root lipids

The acyl-lipid content (shown as the sum of fatty acids) varied significantly between the analysed clones of hairy roots, and oscillated between 15 and 53 nmol FA/mg (4.5 and 14.7 mg/g DW, respectively) (Table 2). The observed differences were probably related to different physiological status of the analysed clones; however, these differences were not always visible. For example, clones C and E did not differ visually (both were cultured during the same time and appeared to be in good physiological condition), but the lipid content in clone E was approximately 40 % higher than in clone C (Table 2). The clones G and H with the lowest lipid content showed, however, clear ageing symptoms. We observed elevated levels of free fatty acids (16 and 30 % of lipids in clone F and G, respectively; Table 3). Considering the fact that all the clones studied were obtained at the same time, it is possible that the variation in lipid content is not associated with the age of the clones but rather is a direct result of the Agrobacterium-mediated transformation. Incorporation of bacterial T-DNA into the plant chromosome is totally random and varies in terms of the number of incorporated fragments and their localisation in the chromosomes (Ambros et al. 1986; Jeon et al. 2000). Thus, it is reasonable to expect that the physiological effect of transformation could also be different. It seems that such variations in transformation effects occurred in the conducted experiments as well. Some of the obtained clones exhibited accelerated lipid catabolism. Nevertheless, from the results we got so far, it is impossible to judge, if it was the cause of accelerated ageing or a result of other effects influencing this process.

Acyl-lipids content in the non-transformed roots of C. abyssinica, cultured in vitro, on MS medium solidified with agar, was somewhat similar to that of the transformed roots (41 nmol FA/mg DW; Table 2). This may suggest that the lipid metabolism in hairy roots (which are in good physiological condition) does not differ significantly from
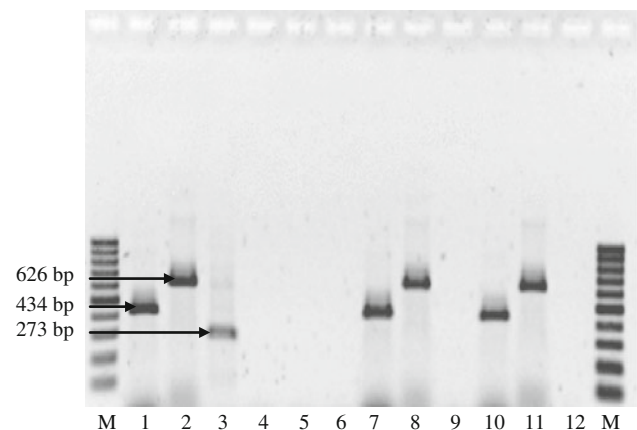
Fig. 3 PCR analysis of Agrobacterium rhizogenes A4 (lanes 1–3, positive control), non-Agrobacterium induced roots (lanes 4–6, negative control), Crambe abyssinica transformed with A. rhizogenes A4 (lanes 7–9) and ATCC 15834 (lanes 10–12). GeneRuler^{TM} 100 bp DNA ladder (lane M). Arrows show the amplified fragments of rolB (423 bp; lanes 1, 4, 7, 10), rolC (626 bp; lanes 2, 5, 8, 11), and virG (273 bp; lanes 3, 6, 9, 12) genes. The PCR results for A. rhizogenes ATCC 15834 were the same as for strain A4

Fig. 2 Dynamics of growth of C. abyssinica hairy roots during 50 days. Black line growth of hairy roots on MS + 0.1 mg l^{-1} NAA + 0.1 % PVP, red line growth of hairy roots on MS + 0.1 mg l^{-1} NAA (colour figure online)

Table 2 Lipid content (presented as a sum of fatty acids—FA) in hairy roots and non-Agrobacterium induced roots of *C. abyssinica*

Transformed roots of *C. abyssinica* (selected clones)	$\sum$ FA/mg DW (nmol)	$\sum$ FA mg/g DW
A	52.47 ± 0.91	14.7 ± 0.25
B	48.85 ± 0.57	13.55 ± 0.16
C	38.33 ± 0.04	10.73 ± 0.03
D	46.63 ± 1.05	13.1 ± 0.03
E	53.15 ± 0.92	14.7 ± 0.25
F	25.13 ± 1.47	7.23 ± 0.43
G	17.63 ± 0.36	5.2 ± 0.12
H	15.45 ± 1.06	4.5 ± 0.32
I	18.73 ± 0.61	5.5 ± 0.18
Non-Agrobacterium induced roots of *C. abyssinica*	40.97 ± 2.23	11.43 ± 0.62

Values expressed as mean ± standard deviation ($n \geq 3$)

Non-Agrobacterium induced roots of *C. abyssinica* (8-week-old plants with roots grown on MS medium solidified with agar)

A, B, C, D, E hairy roots clones of *C. abyssinica* in good physiological condition, *F, G, H, I* hairy roots clones of *C. abyssinica* exhibiting ageing symptoms, *DW* dry weight

the lipid metabolism of the non-Agrobacterium induced roots. This assumption is also supported by the lipid composition in both types of roots (Table 3). Polar lipids were the dominant lipid class, both in the hairy roots and the non-Agrobacterium induced roots of *C. abyssinica*, and accounted for about 75 % of acyl-lipids. Furthermore, we found diacylglycerols (DAG), free fatty acids (FFA), triacylglycerols (TAG), and sterol esters (SE). Additionally, in all examined samples, an unidentified lipid (X), localised just above the polar lipids on the TLC plate (developed with hexane:diethyl ether:acetic acid: 70/30/1 v/v/v), accounted for about 9 % and about 3 % of acyl-lipids of the hairy roots and of the non-Agrobacterium induced roots, respectively. In contrast to lipid X, no clear tendencies concerning other lipid classes can be observed due to natural variability of hairy roots. The observed differences could be connected with the transformation or with the differences in the physiological status of the analysed

roots. The observed variations in lipid composition between the clones of hairy roots showing and not showing ageing symptoms suggest, however, that, at least partially, these differences could be explained by the condition of the analysed roots.

Polar lipids were the major class of lipids in the hairy roots of *Catharanthus roseus* (Toivonen et al. 1992) and *E. acanthocarpum* (Cequier-Sánchez et al. 2008). These lipids are the main constituents of membranes. However, the precise role of neutral lipids in the roots remains unclear. Further research is necessary to clarify if they serve only as a storage material, or play also a certain role in the function of membranes.

Fatty acids composition of the lipids of both the hairy roots and the non-Agrobacterium induced roots of *C.abyssinica* did not differ significantly. The observed differences were only in terms of quantity, but not in terms of chemical composition. α-Linolenic acid was the dominant fatty acid in the lipids of both types of roots, and accounted for about 27 % of all fatty acids. Palmitic acid content amounted to about 17–20 % and linoleic acid to about 12–15 %. Among the lipids of the non-Agrobacterium induced roots, oleic acid accounted for about 5 % of total fatty acids, but in the hairy roots, only for about 1 %. All other identified fatty acids content ranged from 1 to 4 %, and there were no significant differences between both types of roots (Table 4). Apart from the fatty acids co-chromatographed with known standards, we also found an unidentified fatty acid with retention time between stearic and oleic acid in the lipids of both hairy roots and the non-Agrobacterium induced roots of *C. abyssinica* (about 21 and 16 %, respectively). Separation of fatty acid methyl esters of *C. abyssinica* root lipids using silver ion thin layer chromatography revealed that this unknown fatty acid is co-chromatographed with trans-monoenoic fatty acid methyl esters. Thus, it is probably a trans isomer of oleic acids. However, the position of the double bond needs to be identified. This fatty acid was not present, neither in the lipids of *N. tabacum* roots (data not presented) nor in the root lipids of a few other plants (Banas et al. 2011).

Table 3 Lipid composition of non-Agrobacterium-induced roots and hairy roots of *Crambe abyssinica*

Analysed roots	Lipid classes (% of total fatty acid)						
	Polar lipids	X	DAG	FFA	TAG	SE	Other
Non-Agrobacterium induced roots	74.5 ± 0.6	2.7 ± 0.1	2.4 ± 2.3	9.5 ± 2.3	8.5 ± 2.2	1.1 ± 0.2	1.4 ± 0.4
Hairy roots in good physiological condition (clones: A, B, C, D, E)	74.8 ± 3.4	8.9 ± 3.2	3.6 ± 1.2	5.1 ± 1.7	5.1 ± 2.4	1.2 ± 0.5	1.3 ± 0.6
Ageing hairy roots—clone F	48.4 ± 1.1	21.4 ± 0.4	2.9 ± 0.3	16.3 ± 2.2	0.7 ± 0.2	2.0 ± 0.1	8.3 ± 1.4
Ageing hairy roots—clone G	40.9 ± 2.3	4.8 ± 1.3	7.0 ± 1.3	30.0 ± 2.3	3.3 ± 0.9	2.5 ± 0.3	11.5 ± 1.2

Mean ± standard deviation presented

X unidentified lipids, *DAG* diacylglycerols, *FFA* free fatty acids, *TAG* triacylglycerols, *SE* sterol esters

Table 4 Fatty acid composition of *Crambe abyssinica* lipids from roots and seeds

Analysed roots	Fatty acids (mol%)											
	16:0	18:0	Y	18:1	18:1Δ11	18:2	18:3	20:0	22:0	22:1	24:0	Other
Non-Agrobacterium induced roots	19.80 ± 1.30	2.30 ± 0.10	15.70 ± 0.40	5.20 ± 0.50	4.20 ± 0.80	15.10 ± 1.30	26.50 ± 4.50	1.50 ± 0.30	2.40 ± 0.50	n.d.	2.40 ± 0.50	4.90 ± 1.30
Hairy roots in good physiological condition (clones: A, B, C, D, E)	17.30 ± 1.40	0.90 ± 0.20	21.20 ± 3.70	1.10 ± 0.20	5.70 ± 1.00	12.30 ± 2.00	27.20 ± 5.80	1.10 ± 0.20	2.60 ± 1.00	n.d.	2.60 ± 1.20	8.00 ± 0.60
Ageing hairy roots (clones F)	20.90 ± 0.30	2.20 ± 0.60	26.10 ± 0.10	1.40 ± 0.20	4.10 ± 0.10	5.10 ± 0.10	6.50 ± 0.10	3.00 ± 0.10	8.40 ± 0.20	n.d.	10.20 ± 0.10	12.10 ± 0.20
Ageing hairy roots (clones G)	24.80 ± 1.20	1.90 ± 0.20	28.30 ± 0.10	1.10 ± 0.20	3.70 ± 0.40	1.20 ± 0.30	0.90 ± 0.10	3.10 ± 0.30	11.30 ± 1.00	n.d.	13.80 ± 0.70	9.90 ± 1.60
Seeds of *C. abyssinica*	3.10 ± 0.20	0.90 ± 0.20	n.d.	17.00 ± 0.20	0.90 ± 0.03	10.40 ± 0.10	6.10 ± 0.10	1.00 ± 0.10	1.60 ± 0.04	51.70 ± 0.10	0.10 ± 0.002	7.20 ± 0.25

Values expressed as mean ± standard deviation ($n \geq 3$)

16:0 palmitic acid, *18:0* stearic acid, *Y* unidentified trans isomer of oleic acid, *18:1* oleic acid, *18:1Δ11* vaccenic acid, *18:2* linoleic acid, *18:3* α-linolenic acid, *20:0* arachidic acid, *22:0* behenic acid, *22:1* erucic acid, *24:0* lignoceric acid, *n.d.* not detected

Nevertheless, at present, it is impossible to conclude, whether it is specific only to crambe or also to other plant species.

No differences in the fatty acid composition of hairy roots and the non-Agrobacterium induced roots were also observed in *C. roseus*. The main fatty acids found in the lipids extracted from both types of roots were palmitic, oleic, linoleic, and linolenic acids, and they accounted for more than 95 % of the total amount of fatty acids (Toivonen et al. 1992).

Erucic acid is used in the manufacture of numerous industrial products, such as lubricants, surfactants, cosmetics and polyesters (Tao and He 2005; Vargas-Lopez et al. 1999). Current studies are focused on the development of high erucic acid oil crops which would substantially reduce the cost of erucic acid production (Li et al. 2012; Nath et al. 2009). Erucic acid was not detected in the hairy roots of *C. abyssinica*, which suggests that this system is not actually suited for production of this fatty acid. The most recent studies on plant oils are focused on investigating alternative methods of producing oil in different organs and tissues such as leaves, tubers or roots (Carlsson et al. 2011). Further research is needed to assess whether these systems will be able to compete with seed oil production.

In conclusion, it seems that hairy roots could be a very useful material for studying both anabolism and catabolism of root lipids. The method of transformation using *A. rhizogenes*, that we developed, is an effective way of obtaining this type of roots.

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