Identification and functional analysis of the genes encoding Δ6-desaturase from *Ribes nigrum*†

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GenBank accession numbers: *RnD8A*, GU198924; *RnD8B*, GU198925; *RnD8C*, GU198926; *RnD8D*, GU198927; *RnD8E*, GU198928.

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Abstract

Gamma-linolenic acid (γ-linolenic acid, GLA; C18:3 Δ6,9,12) belongs to the omega-6 family and exists primarily in several plant oils, such as evening primrose oil, blackcurrant oil, and borage oil. Δ6-desaturase is a key enzyme involved in the synthesis of GLA. There have been no previous reports on the genes encoding Δ6-desaturase in blackcurrant (*Ribes nigrum* L.). In this research, five nearly identical copies of Δ6-desaturase gene-like sequences, named *RnD8A, RnD8B, RnD8C, RnD8D, and RnD8E*, were isolated from blackcurrant. Heterologous expression in *Saccharomyces cerevisiae* and/or *Arabidopsis thaliana* confirmed that *RnD8C/D/E* were Δ6-desaturases that could use both α-linolenic acids (ALA; C18:3 Δ9,12,15) and linoleic acid (LA; C18:2 Δ9,12) precursors in vivo, whereas *RnD8A/B* were Δ8-sphingolipid desaturases. Expression of GFP tagged with *RnD6C/D/E* showed that blackcurrant Δ6-desaturases were located in the mitochondrion (MIT) in yeast and the endoplasmic reticulum (ER) in tobacco. GC-MS results showed that blackcurrant accumulated GLA and octadecatetraenoic acids (OTA; C18:4 Δ6,9,12,15) mainly in seeds and a little in other organs and tissues. RT-PCR results showed that *RnD6C* and *RnD6E* were expressed in all the tissues at a low level, whereas *RnD6D* was expressed at a high level only in seeds, leading to the accumulation of GLA and OTA in seeds. This research provides new insights to our understanding of GLA synthesis and accumulation in plants and the evolutionary relationship of this class of desaturases, and new clues as to the amino acid determinants which define precise enzyme activity.

Key words: Δ6-desaturase, Δ8-sphingolipid desaturase, functional analysis, *Ribes nigrum*, subcellular localization.

Introduction

Gamma-linolenic acid (γ-linolenic acid, GLA; C18:3, Δ6,9,12) is an essential fatty acid (EFA) of the omega-6 family and exists primarily in a few plant-based oils, such as evening primrose oil, blackcurrant oil, and borage oil (Sayanova et al., 1997; Das, 2006). GLA was also found in considerable quantities in *Spirulina*, a cyanobacterium (Subudhi et al., 2008), some species of *Primula* (Sayanova et al., 1999a), and fungi (Sperling et al., 2000; Na-Ranong Abbreviations: GLA, γ-linolenic acid; EFA, essential fatty acid; ALA, α-linolenic acids; LA, linoleic acid; OTA, octadecatetraenoic acids; MIT, mitochondrion; ER, endoplasmic reticulum; FAME, fatty acid methyl esters; GC-MS, gas chromatography-mass spectrometry; Cyt b5, cytochrome b5; PMS, premenstrual syndrome; LCBs, long chain bases; OPA, o-phthaldialdehyde; GFP, Green fluorescence protein tagged in C-terminal; -GFP, Green fluorescence protein tagged in N-terminal. © 2010 The Author(s).

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et al., 2005). However, there is no GLA in the major oil crops (Reddy and Thomas, 1996; Sayanova et al., 1999b), such as oilseed rape, soybean, peanut, and palm. GLA may be helpful in the prevention and/or treatment of diabetes, eye disease, osteoporosis, premenstrual syndrome (PMS), eczema, allergies, rheumatoid arthritis, high blood pressure, and heart disease (Horrobin, 1992).

Δ⁶-desaturase is classified as an acyl-lipid desaturase that introduces a double bond between the pre-existing double bond and the carboxyl (front) end of the fatty acid (Qi et al., 2004). It is critical for the metabolic conversion of linoleic acid (LA; C18:2 Δ⁹,12) and α-linolenic acids (ALA; C18:3 Δ⁹,12,15) into GLA and octadecatetraenoic acids (OTA; C18:4 Δ⁶,9,12,15), respectively (Horrobin, 1992; Reddy and Thomas, 1996). The cytochrome b₅ (Cyt b₅) domain is essential for the borage Δ⁶-desaturase (Sayanova et al., 1999b), and three conserved His boxes of the enzyme also are proved to be essential for its function (Sayanova et al., 2001).

In the past 20 years, a number of Δ⁶-desaturase genes, from cyanobacterium (Reddy et al., 1993), fungi (Sakuradani and Shimizu, 2003), plants (Sayanova et al., 1997; 1999a; Sperling et al., 2000; Whitney et al., 2003), and animals (Napier et al., 1998; Seiizc et al., 2001), were isolated and characterized. Accumulation, function, and expression of Δ⁶-desaturase genes had been investigated in only a few higher plants synthesizing GLA. In Borago officinalis, GLA is accumulated up to 23.1% of the total fatty acids in mature seeds, and more than 8.0% in other tissues. The Δ⁶-desaturase gene is expressed but at different levels in all tissues: higher in seeds, young leaves, and roots, and lower in mature leaves and flowers (Sayanova et al., 1999c). In Anemone leevillei, GLA and OTA are absent in seeds, but are readily detectable in leaves. The Δ⁶-desaturase gene is expressed at the highest level in leaves, a moderate level in seeds, and is completely absent in roots (Whitney et al., 2003). In Primula, different species accumulated varying levels of GLA and OTA. In the species with a high level of Δ⁶-fatty acids, GLA and OTA accumulated mainly in the leaves and seeds, and relatively little in roots (Sayanova et al., 1999a).

It was recently reported that there were two nearly identical copies of the Δ⁶-desaturase gene, which differ by nine silent mutations, in the marine microalga Glossostamix chrysoplasra (Hsiao et al., 2007). While, in the higher plants that had been investigated, there were no instances of several different active Δ⁶-desaturase genes being found in the same organism.

Δ⁶-desaturases from some organisms exhibited substrate selectivity. The Δ⁶-desaturase of moss (Ceratodon purpureus) preferred C16:1 Δ⁶ over C18:1 Δ⁶ (Sperling et al., 2000), whereas the Δ⁶-desaturase from Mucor rouxii had a strong preference for the C18:1 Δ⁹ (Na-Ranong et al., 2005). In Boraginaceae, the Δ⁶-desaturase from Macaronesian Echium gentianoides preferred the LA substrate over the ALA substrate (Garcia-Maroto et al., 2006). Δ⁶-desaturases from different Primula species had different substrate preferences; for example, the Δ⁶-desaturase from Primula vialii had a very strong preference for LA compared with LA (Sayanova et al., 2003), whereas that from Primula cortusoides mainly desaturated LA (Sayanova et al., 2006).

The fatty acid-synthesizing and desaturation system had been widely known to exist in chloroplasts and the endoplasmic reticulum (ER) in higher plants. It had been confirmed by using the [¹⁴C]linoleoyl phosphatidylcholine that the microsomal membrane, prepared from the developing cotyledons, contained an active Δ⁶-desaturase, which catalysed the conversion of linoleate into γ-linolenate (Stymne and Stobart, 1986). Since then, front-end desaturases were generally thought to locate in the ER (Napier et al., 1999). To date, however, there has been no direct evidence for subcellular localization to locate plant Δ⁶-desaturases.

Blackcurrant seed oil, typically containing 15–20% GLA, is known to be one of the available natural sources of GLA (Traftier et al., 1988). However, until now, the Δ⁶-desaturase blackcurrant gene has not been reported.

In this research, GLA was detected mainly in seeds and rarely in other organs of blackcurrant. Three genes were cloned (RnD6C, RnD6D, and RnD6E) encoding Δ⁶-desaturases and two genes (RnD8A, RnD8B) encoding Δ⁶-sphingolipid desaturases in blackcurrant. RnD6C, RnD6D, and RnD6E could use both LA and ALA as substrate. RnD6C/D/E was located in the mitochondrion (MIT) in yeast and the ER in tobacco. RnD6C and RnD6E were expressed in all the tissues at a low level, whereas RnD6D was expressed at a high level but only in seeds. This research should provide a useful addition to the previously identified orthologues from other plants and may well prove to be useful for biotechnological applications. Furthermore, this research may also provide not only new insights into the evolutionary relationship of this class of desaturases but also some new clues as to the amino acid determinants which define the precise enzyme activity (i.e. either Δ⁶-desaturase or Δ⁶-sphingolipid desaturase).

**Materials and methods**

**Plant materials**

Blackcurrant (Ribes nigrum L. var. BRÖDTORP), a Finnish variety (Wassenaar and Hofman, 1966) was used in this research.

**Blackcurrant genomic DNA extraction and full-length Δ⁶-desaturase gene cloning**

Genomic DNA was extracted by a DNA extraction kit (Biomedtech, Beijing, China) from young leaves of blackcurrant. The DNA fragments of putative cytochrome b₅ fusion desaturase genes were amplified from the genomic DNA by using degenerated primers designed according to the sequence of conserved histidine boxes of known Cyt b₅ fusion desaturases (Reddy and Thomas, 1996; Whitney et al., 2003; Sayanova et al., 2003). The primer sequences were: PL, 5′-TTGGGTGAAAGACCATCCA(T)GGT(A)GG-3′; R, 5′-GGGAAACAAA(G)TGA(G)TGCTCAACCTG-3′. Five amplified products (about 1032 bp) were cloned into the pEASY-Blunt vector (TransGen Biotech, Beijing, China) and sequenced. The 5′ and 3′ extension of these fragments were obtained by the genomic walking method using the BD GenomeWalker™ Universal Kit (BD Biosciences, Palo Alto, CA, USA). Conversed genomic
walking primers were: 5GSP1 (480), GACATTGCTCTG(A/G)TAGTCACCGAATGC; 5GSP2 (270), CCTAATCAATTTTGAGATACTTC; 5GSP3 (702), AGGAA(A/G)TTGGAGTTTTATGCTTTTGATCTAGGTTC; 3GSP2 (1078), CG(T/C)CTTGGATTGGTTTCTAGTGTTG. The PCR products were cloned into the pEASY-Blunt vector (TransGen Biotech) and sequenced. Five sequences similar to ‘front-end’ 6-desaturase or \( \Delta^6 \)-sphingolipid desaturase genes were obtained and named RnD8A, RnD8B, RnD6C, RnD6D, and RnD6E.

Yeast expression plasmid construction and transformation

The five sequences similar to ‘front-end’ \( \Delta^6 \)-desaturase or \( \Delta^6 \)-sphingolipid desaturase genes were purified from 1% agarose gel, digested with the restriction enzymes HindIII and SacI (the restriction sites of the enzymes had been designed in the primers), cloned into the corresponding sites of the yeast expression vector pYES2 (Invitrogen, UK), and sequenced. The five construct plasmids (pRnD8A, pRnD8B, pRnD6C, pRnD6D, and pRnD6E) were transformed into \( S. cerevisiae \) strain INV Sc 1 (Invitrogen) by using the lithium acetate method.

Characterization of genes with \( \Delta^6 \)-sphingolipid desaturase function in \( S. cerevisiae \)

The RnD8A/B-transformed yeasts were grown in SC-U, containing 1% raffinose and 1% (w/v) ter gitol NP-40 (Sigma, Germany), and induced with 2% (w/v) galactose. The yeast cells were cultivated for an additional 72 h at 20 °C, and then harvested and dried at 50 °C. 100 mg dried cells were used to prepare the long chain bases (LCBs) from glycerolipids for subsequent analysis by HPLC as previously described (Markham et al., 2006) and phyto stigmane (18:0) (Sigma) was used as the standard. Briefly, induced yeast cells (100 mg, dried weight) were ground into a fine powder, and subjected to strong alkaline hydrolysis in 2 ml of 10% (w/v) aqueous Ba(OH)\(_2\) and 2 ml of dioxane for 16 h at 110 °C. After hydrolysis, 2 ml of 2% (w/v) ammonium sulphate was added, and the liberated sphingolipid long chain bases were extracted with 2 ml of diethyl ether. The upper phase was removed to a second tube, dried under nitrogen, and derivatized with \( \alpha \)-phthalaldehyde (OPA) (Invitrogen) as previously described (Merrill et al., 2000). Individual LCBs were separated by reversed phase HPLC (Waters 600E, USA) using Penomenex C18 column (Merrill et al., 2006) and fluorescence was excited at 340 nm and detected at 455 nm.

Characterization of genes with \( \Delta^6 \)-desaturase function in \( S. cerevisiae \)

Heterologous expression of \( RnD6C/D/E \) was induced under transcriptional control of the yeast \( GALL \) promoter. Yeast cultures were grown to logarithmic phase at 30 °C in SC-U containing 1% (w/v) raffinose, 0.67% (w/v) yeast nitrogen and 0.1% (w/v) tergitol NP-40 (Sigma), supplemented with 0.5 mM LA (Sigma). The cells were induced by the addition of 2% (w/v) galactose and cultivated for an additional 72 h at 20 °C. Subsequently, cells were harvested by centrifugation, and washed three times with sterile distilled water. The cells were dried and ground into a fine powder. Then the fatty acid analysis was performed using the following method.

Plant plasmid construction and transformation of \( Arabidopsis thaliana \)

\( RnD6C/D/E \) cDNA under the control of the duplicated \( CaMV 35S \) (D35S) promoter and nos terminator were constructed in the binary vector pGI0029 (John Innes Centre, UK). The inserts were sequenced to ensure that undesired mutations were not introduced during the PCR reactions. \( Agrobacterium tumefaciens \) strain LBA4404 containing the helper plasmid pSoup (John Innes Centre, UK) was used as the host of the pGI0029 vectors containing the \( \Delta^6 \)-desaturase gene. \( Arabidopsis thaliana \) (ecotype Columbia) was used for plant transformation with the floral dip method (Clough and Bent, 1998). \( T_0 \) seeds were plated out on selective media containing kanamycin (50 mg l\(^{-1}\)) and then the selected transformed plants were transferred to soil. The harvested seeds were selected for another two generations, and the fixed transformants were selected.

Fatty acid analysis

Cellular fatty acid was extracted by incubating 50 mg yeast powder or 200 mg fresh leaves of transformed \( A. thaliana \) or different black currant tissues (young root, young stem, leaves, flowers, ripening exocarp, and ripening seeds) in 3 ml of 7.5% (w/v) KOH in methanol for saponification at 70 °C for 3 h. After the pH was adjusted to 2.0 with HCl, the fatty acid was subjected to methyl-esterification with 2 ml 14% (w/v) boron trifluoride in methanol at 70°C for 1.5 h. Then 1 ml of 0.9% (w/v) NaCl was added and mixed well. Subsequently, fatty acid methyl esters (FAME) were extracted with 2 ml hexane. The upper phase was removed to a second tube, dried under nitrogen and dissolved in acetic ether. Qualitative analysis of FAME was performed by GC-MS (gas chromatography-mass spectrometry, TurboMass, PerkinElmer, USA) with the capillary column (BPX-70, 30 m x 0.25 mm x 0.25 μm). FAME were analysed and identified by the comparison of their peaks with those of standards: LA, ALA, and GLA (Sigma).

Subcellular localization of \( \Delta^6 \)-desaturase in yeast

To determine its subcellular location, \( RnD6C/D/E \) had been cloned in-frame at the 5′ end and 3′ end with the GFP gene in the pYES2

Table 2. Fatty acid composition of different black currant tissues

| Young root | Young stem | Leaf | Flower | Ripening exocarp | Ripening seeds |
|------------|------------|------|--------|-----------------|---------------|
| 16:0       | 32.3±0.7   | 30.4±0.7 | 17.2±0.6 | 34.7±1.5       | 36.0±0.2 | 10.1±0.0 |
| 16:1       | 23.4±0.9   | –     | 1.7±0.0 | –               | –            | –          |
| 16:2       | –          | –     | 1.2±0.0 | –               | –            | –          |
| 16:3       | –          | –     | 5.0±0.2 | –               | –            | –          |
| 18:0       | 14.3±0.6   | 11.2±0.8 | 2.5±0.3  | 11.1±0.6       | 19.5±1.0   | 3.9±0.0   |
| 18:1       | 6.5±0.2    | 2.9±0.3 | 1.7±0.1 | 9.9±0.5        | 4.6±0.3    | 12.6±0.2  |
| LA         | 16.7±0.3   | 30.8±1.2 | 10.7±0.4 | 24.6±1.3       | 22.2±0.8   | 42.7±0.1  |
| GLA        | 0.8±0.0    | 1.1±0.0 | 0.4±0.0 | 0.3±0.1        | 12.7±0.2   | –          |
| ALA        | 3.6±0.0    | 20.7±0.5 | 57.1±1.3 | 16.9±1.2       | 16.7±0.7   | 13.4±0.1  |
| OTA        | 0.4±0.0    | 0.9±0.0 | 0.4±0.0 | 0.3±0.0        | –           | 2.6±0.1   |

Table 1. Primers (from 5′ to 3′) used in RT-PCR

| Primer name | Sequences |
|-------------|-----------|
| RDL94       | GGTAAGGTTTACAACTGTCACCC |
| RTRDR830    | CGGCTGTGAGGAGCAATCAG |
| RTAABL420   | ATG(C/T)/(T/G)TTGGAGGTGCTT |
| RTAABR1077  | AGCATGAACTATCTATAGGCTCACT |
| RTCEL322    | GAAGGAGCGATGTTGTTTTTC |
| RTCEL864    | CA/AAGAAGTTTGAGGTGCTT |
| Actin L     | CATCAGGAGAAGAGCTTGGAGG |
| Actin R     | GATGGGACCTGACTGCTACAT |
vector and expressed in the yeast strain INV Sc 1. Live yeast cells were incubated with MitoTracker Red and/or ER-tracker Blue as described in the experimental protocol of 'MitoTracker/C210 and MitoFluor/C228 Mitochondrion-Selective Probes' and 'ER-Tracker/C228 Dyes for Live-Cell Endoplasmic Reticulum Labeling' (Invitrogen). The localization of RnD6C/D/E-GFP was determined by using a fluorescence microscope (Olympus, Tokyo, Japan). The desaturation function of the fused proteins was identified by GC-MS.

Subcellular localization of Δ⁶-desaturase in tobacco
Wild-type Nicotiana benthamiana was grown as previously described (Ruiz et al., 1998; Voinnet et al., 1998). A. tumefaciens strain LBA4404 with plastids containing RnD6C/D/E-GFP or GFP-RnD6C/D/E and the strain carrying the p19 construct (Voinnet et al., 2003) were grown at 28 °C in YEB supplemented with 50 mg l⁻¹ kanamycin and 5 mg l⁻¹ tetracycline to the stationary phase. Bacterial cells were precipitated by centrifugation.
at 5000 g for 15 min at room temperature and resuspended in 10 mM MgCl₂ and 150 mg l⁻¹ acetosyringone. Cells were left in this medium for 3 h and then infiltrated into the abaxial air spaces of 2–4-week-old N. benthamiana plants. The culture of Agrobacterium was brought to an optical density of 1.0 (OD₆₀₀) to avoid toxicity (Voinnet et al., 2000). Transient co-expression of the RnD₆C/D/E-GFP or GFP-RnD₆C/D/E and the mCherry-tagged ER marker (Nelson et al., 2007) and p19 constructs was at OD₆₀₀ 1.0 (Voinnet et al., 2003). The localization of RnD₆C/D/E tagged with GFP was determined under a confocal microscope (Leica TCS SP5, Leica Microsystems, Bensheim, Germany).

Gene expression analysis
Total RNA from different tissues was extracted using a RNA extraction kit (Biomed-tech, Beijing, China). cDNA was synthesized from total RNA using a reverse transcriptional kit (Toyobo, Osaka, Japan). The RT-PCR primers for detecting our cloned genes from blackcurrant were listed in Table 1. The housekeeping actin gene was used as the control in the RT-PCR reactions. The reaction was denatured at 95 °C for 3 min, followed by 30 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min at 72 °C, and then 7 min at 72 °C.

Cladistic analysis
Nucleotide sequences of the desaturases were aligned by using the program ClustalX 2.0.10 (Thompson et al., 1994) and manual adjustment. The phylogenetic tree was generated using the alignment output based on the Minimum evolution method (Rzhetsky and Nei, 1992), as implemented in the Mega 4.1 (Kumar et al., 2001). The complete deletion option and the Jukes and Cantor metric were used, and confidence of the tree branches was checked by bootstrap generated from 1000 replicates.

Results
Fatty acid composition in different blackcurrant tissues
The fatty acid compositions from young roots and stems, flowers, mature leaves, ripening exocarp, and seeds of blackcurrant were determined by GC-MS. The results showed that all the detected tissues except ripening exocarp contained GLA and OTA. There was very little GLA (<1.1% of total fatty acid) and OTA (<0.4% of total fatty acid) in young roots and stems, flowers and mature leaves, but abundant GLA (>12% of total fatty acid) and OTA (>2.6% of total fatty acid) in ripening seeds (Table 2).

Isolation of putative Δ₆-desaturase genes
Using the genomic DNA walking method, five DNA sequences (1347 bp in length) were cloned and sequenced.
They were very similar in DNA sequences (>85% identity) and encoded five distinct deduced polypeptides of 448 amino acids. All of them had an N-terminal Cyt b5 domain as the heme-binding region and three conserved histidine boxes which exist in all membrane-bound desaturases (Fig. 1) (Shanklin et al., 1994). These five DNA sequences could be grouped into two classes. One included two sequences that showed high identities (>69%) to the DNA sequence of D8-sphingolipid desaturase from borage (Sperling et al., 1998). They were designated as RnD8A and RnD8B and registered in GenBank as GU198924 and GU198925, respectively. The other contained three sequences showing slightly lower identities (>60%) to the DNA sequence of Δ⁸-sphingolipid desaturase from borage (Sperling et al., 1998). They were designated as RnD6C, RnD6D, and RnD6E, which were registered in GenBank as GU198926, GU198927, and GU198928, respectively. The deduced amino acid sequences of the RnD6C, RnD6D, and RnD6E proteins shared a 93% identity to each other and a 70% identity to the sequence of Δ⁸-desaturase from borage. And the predicted amino acid sequences of RnD8A and RnD8B shared a 92% identity to each other and 61% to the sequence of Δ⁸-sphingolipid desaturase from borage (Sperling et al., 2001).

Functional identification of RnD8A, RnD8B, RnD6C, RnD6D, and RnD6E in Saccharomyces cerevisiae

To validate the putative Δ⁶-desaturase activity of RnD6C, RnD6D, and RnD6E, they were expressed in S. cerevisiae. The fatty acid compositions of transformants and the control were analysed by GC-MS, respectively. In the control, no conversion into non-native products was found by feeding potential fatty acid precursors LA (Fig. 2) or ALA (data not shown) to the yeast cells transformed with empty vector pYES2. By contrast, yeast cells transformed with RnD6C, RnD6D or RnD6E could utilize two precursor forms LA (Fig. 2) and ALA (data not shown), to produce a new product GLA (Fig. 2) or OTA (data not shown), respectively. There were no significant difference in Δ⁶-desaturase activity among RnD6C, RnD6D, and RnD6E.
To identify the functions of RnD8A and RnD8B, they were expressed in *S. cerevisiae* under the control of the GAL1 promoter. Besides the phytosphinganine, *cis*- and *trans*-desaturated sphingoid bases were found in the transformed yeast cells with RnD8A or RnD8B by detecting their LCBs with HPLC (Fig. 3) using the method described in the Materials and methods (Markham *et al.*, 2006). The results indicated that RnD8A and RnD8B were Δ⁵-sphingolipid desaturases.

Functional identification of RnD6C, RnD6D, and RnD6E in *Arabidopsis thaliana*

The cDNAs of *RnD6C*, *RnD6D*, and *RnD6E* were individually expressed in *Arabidopsis thaliana*. The transgenic plants were verified by PCR using two primers targeting the internal sequence of the genes. The fatty acid profile of lipids from leaves of transgenic plants was determined. Figure 4 showed the fatty acid compositions in leaves of 3-week-old seedling of T₃ *RnD6C* transgenic plants. Compared with the wild type, the leaves of the transgenic plants had an altered fatty acid profile containing two additional peaks corresponding to GLA and OTA. There were no significant differences in the production efficiency of GLA and OTA among transgenic *RnD6C*, *RnD6D*, and *RnD6E* lines (data not shown).

Subcellular localization of RnD6C/D/E in *S. cerevisiae* and tobacco

The Δ⁶-desaturase RnD6C/D/E was tagged with GFP in both the N-terminal and C-terminal, and expressed in *S. cerevisiae* and *N. benthamiana*. RnD6C/D/E led to similar results and the representative results are shown. The results showed that RnD6C/D/E was localized in mitochondria when introduced into yeast cells (Fig. 5) and ER in tobacco epidermal cells (Fig. 6).

Expression profiles of RnD8A, RnD8B, RnD6C, RnD6D, and RnD6E

RT-PCR was used to investigate the expression profiles of RnD8A, RnD8B, RnD6C, RnD6D, and RnD6E. The sequence identity between RnD8A and RnD8B is 91.6%, and that between RnD6C and RnD6E is 97.1%. Thus, the expression of RnD8A and RnD8B, and that of RnD6C and RnD6E, were investigated together. The results showed that RnD8A&RnD8B and RnD6C&RnD6E were expressed in all the tissues at a low level, while RnD6D was expressed only in seeds at a high level (Fig. 8). Using genomic walking, a seed specific expression promoter upstream of *RnD6D* has been cloned and sequenced (data not shown).

Discussion

In a few previously studied higher plants, the profiles of the GLA and OTA accumulation were different. GLA is accumulated in mature seeds and in other tissues in *Borago*...
officinalis at a high level (Sayanova et al., 1999c), but was absent in seeds and was at a high level in leaves in Anemone leveillei (Whitney et al., 2003). In some primula species, GLA and OTA were accumulated mainly in leaves and seeds, and were relatively low in roots (Sayanova et al., 1999a). In blackcurrant, mature seeds (Traifler et al., 1988) and ripening seeds accumulate GLA and OTA at a high level, while other tissues contained very little GLA and OTA.

In Borago officinalis, Anemone leveillei, and some species of primula with high GLA, a single gene encoding Δ⁶-desaturase had been identified and characterized. In this research on blackcurrant, three genes encoding Δ⁶-desaturase (namely RnD6C, RnD6D, and RnD6E) had been cloned. Their desaturase activities were similar in yeast and Arabidopsis. However, their expression profiles were different. RnD6C&RnD6E was expressed in all the tissues at a low level, while RnD6D expressed only in seeds. RnD6D may mainly contribute to the accumulation of GLA and OTA in the seeds of blackcurrant.

It is widely believed that the Δ⁶-desaturases, which were only found in some plants, have evolved from Δ⁸-sphingolipid desaturases that existed in nearly all plants (Libisch et al., 2000; Sperling et al., 2003). Cladistic analysis of Δ⁶-desaturases and Δ⁸-sphingolipid desaturases genes (Fig. 9) revealed that Δ⁶-desaturases and Δ⁸-sphingolipid desaturase genes from the same plants clustered together, rather than being separated into two groups, for example, Ribes nigrum and Anemone lendsquerelli. The Δ⁶-desaturases and Δ⁸-sphingolipid desaturases genes of Primula vialli (Pv), and Primula farinose (Pf) clustered in two groups as the two species belong to the same genus. The only exception is Borago officinalis in which the genes were not clustered together due to the greater difference between the two desaturases than that in other plant species. The sequence identity of Δ⁶-desaturases and Δ⁸-sphingolipid desaturase in Borago officinalis is 58%, suggesting that the Δ⁶-desaturase and Δ⁸-sphingolipid desaturase separated much earlier in Borago officinalis than those in other species. The identity of Δ⁶-desaturases and Δ⁸-sphingolipid desaturases is more than 80% in blackcurrant and the distance between them is much shorter than that in other species. Furthermore, among the plant species with reported Δ⁶-desaturase genes, blackcurrant is the only one in which several functional genes or copies of

Fig. 5. The localization of RnD6C/D/E tagged with GFP at the C-terminal end (A–C) and N-terminal end (D–F) in yeast cells and yeast cells INv Sc 1 with pYES2 (G–H) as the negative control. (A, D, G) Fluorescent images of yeast cells that expressed GFP and the negative control under an exciter filter of 485 nm. (B, E, H) Fluorescent images of yeast cells that were stained with MitotrackerRed (Invitrogen) under an exciter filter of 510 nm. (C, F, I) The merged image.
Δ⁶-desaturase have been found. These genes have identities higher than 92% based on the amino acid sequences. The existence of three such genes may have resulted from a recent gene duplication event, as the delta desaturases were thought to be conserved in evolution until now (Libisch et al., 2000). It is speculated that Δ⁶-desaturase genes in blackcurrant may have evolved later than those in other species.

Δ⁶-desaturases from previously reported plants always exhibited different substrate selectivity. Some prefer LA (García-Maroto et al., 2006; Sayanova et al., 2006) and some prefer ALA as the substrate (Sayanova et al., 2003). In the present study, the three Δ⁶-desaturases, RnD6C, RnD6E, and RnD6D from blackcurrant can use both LA and ALA as the substrates, but their substrate selectivity needs to be confirmed by future studies.

Front-end desaturases are generally thought to locate in the ER, although the actual location is unknown (Hsiao et al., 2007). Expression of GFP-tagged RnD6C/D/E in yeast and tobacco could facilitate the visualization of its exact location. The present study provides the unequivocal evidence that RnD6C/D/E is located in the ER in tobacco, but it is located in mitochondria when introduced into yeast.

Fig. 6. The localization of RnD6C/D/E tagged with GFP in tobacco epidermal cells. The results shown are from transient transformed Nicotiana benthamiana epidermal cells. (A–C) RnD6C/D/E fused with GFP at the C-terminal end; (D–F) RnD6C/D/E fused with GFP at N-terminal end. (A, D) Fluorescent images of epidermal cells that expressed GFP under an exciter filter of 485 nm. (B, E) Fluorescent images of epidermal cells that expressed the endoplasmic reticulum marker ER-mCherry under an exciter filter of 510 nm. (C, F) The merged image.

Fig. 7. GC analysis of FAME of total lipids of RnD6C/D/E tagged with GFP in Saccharomyces cerevisiae. The total fatty acid in induced yeast transformed with (A) RnD6C/D/E, (B) GFP-RnD6C/D/E, and (C) RnD6C/D/E-GFP (arrows indicated the novel peak of GLA).
The effect of the fusion of GFP with \( \Delta^6 \)-desaturases on their enzyme activity has not been reported. Results from the present study indicate that the yeast cells containing \( \text{RnD6C/D/E-GFP} \) accumulated a lower level of GLA than those containing \( \text{GFP-RnD6C/D/E} \) or \( \text{RnD6C/D/E} \) and there were no significant differences between \( \text{GFP-RnD6C/D/E} \) and \( \text{RnD6C/D/E} \) (Fig. 7). It is shown that the GFP tagged with \( \Delta^6 \)-desaturases in the N-terminal (\( \text{GFP-RnD6C/D/E} \)) had no effect on the activity of \( \Delta^6 \)-desaturases, but tagged with C-terminal (\( \text{RnD6C/D/E-GFP} \)) could decrease the activity of \( \Delta^6 \)-desaturases. This reduced activity may result from the alteration of the three-dimensional structure of the C-terminal when the protein is tagged with GFP at that end. The results suggested that the three-dimensional structure of the C-terminal of \( \Delta^6 \)-desaturases should be important for their enzyme activity. However, the effect of the three-dimensional structure of the C-terminal of \( \Delta^6 \)-desaturases on their enzyme activity is worthy of further investigation.

In conclusion, three genes encoding \( \Delta^6 \)-desaturases (\( \text{RnD6C/D/E} \)) and two encoding \( \Delta^8 \)-sphingolipid desaturases (\( \text{RnD8A/B} \)) from blackcurrant that were very similar in their sequences have been isolated and characterized. \( \text{RnD6D} \) was expressed in all the tissues at a low level, while \( \text{RnD6E} \) was expressed only in seeds. \( \text{RnD6D} \) could mainly contribute to the accumulation of GLA and OTA in blackcurrant seeds. To the authors’ knowledge, this is the first report of the identification of several \( \Delta^6 \)-desaturases in a single plant species and of finding a \( \Delta^6 \)-desaturase gene expressed specifically in seeds. Blackcurrant \( \Delta^6 \)-desaturases were confirmed to localize in the ER of plant cells and in the mitochondria of yeast cells. It is speculated that the evolution of \( \Delta^6 \)-desaturase genes from \( \Delta^6 \)-sphingolipid desaturase genes in blackcurrant occurred more recently than that in other species.

**Fig. 8.** Expression profiles of \( \Delta^6 \)-desaturases and \( \Delta^8 \)-sphingolipid desaturases by RT-PCR in different tissues. (A) Expression levels in root, stem, leaf, flower, exocarp, and seed and relative transcript levels (normalized to \( \text{actin mRNA} \)) were determined by reverse transcriptase (RT)-PCR. (B) Seed RNA from different ripening stages: green (G), green and black (G&B), black and green (B&G), black (B) and relative transcript levels (normalized to \( \text{actin mRNA} \)) determined by reverse transcriptase (RT)-PCR. All the RT-PCR experiments were repeated three times, and representative results are shown. The \( \text{actin} \) gene for actin RNA was amplified as a control. The PCR products were separated by 2% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.

**Fig. 9.** Phylogenetic analysis of \( \Delta^6 \)-desaturases and \( \Delta^8 \)-sphingolipid desaturase genes (ORF region) based on the multiple nucleotide sequence alignment program ClustalX and the Minimum evolution method. Organism abbreviations stand for: \( \text{Borago officinalis} \) (Bo), \( \text{Anemone lendosquerelli} \) (Al), \( \text{Camellia sinensis} \) (Cs), \( \text{Nicotiana tabacum} \) (Nt), \( \text{Echium gentianoides} \) (Eg), \( \text{Arabidopsis thaliana} \) (At), \( \text{Brassica rapa} \) (Br), \( \text{Primula vialii} \) (Pv), \( \text{Primula farinosa} \) (Pf). GenBank accession numbers are: \( \text{BoD6/8} \) (U79010/AF133728), \( \text{AID6/8} \) (AF536525/AF536526), \( \text{CsD6} \) (AY169402), \( \text{NtD8} \) (EF110692), \( \text{EgD6} \) (AY055117), \( \text{AtD8} \) (NM_116023), \( \text{BrD8} \) (AJ224160), \( \text{PvD6/8} \) (AY234127/AY234126), \( \text{PvD6/8} \) (AAp23034/AAY234124), \( \text{RnD8A/B} \) (GU198924/GU198925), and \( \text{RnD6C/D/E} \) (GU198926/GU198927/GU198928). Bootstrap values at nodes represent the percentages of times a clade appeared in 1000 replicates.

The effect of the fusion of GFP with \( \Delta^6 \)-desaturases on their enzyme activity has not been reported. Results from the present study indicate that the yeast cells containing \( \text{RnD6C/D/E-GFP} \) accumulated a lower level of GLA than those containing \( \text{GFP-RnD6C/D/E} \) or \( \text{RnD6C/D/E} \) and there were no significant differences between \( \text{GFP-RnD6C/D/E} \) and \( \text{RnD6C/D/E} \) (Fig. 7). It is shown that the GFP tagged with \( \Delta^6 \)-desaturases in the N-terminal (\( \text{GFP-RnD6C/D/E} \)) had no effect on the activity of \( \Delta^6 \)-desaturases, but tagged with C-terminal (\( \text{RnD6C/D/E-GFP} \)) could decrease the activity of \( \Delta^6 \)-desaturases. This reduced activity may result from the alteration of the three-dimensional structure of the C-terminal when the protein is tagged with GFP at that end. The results suggested that the three-dimensional structure of the C-terminal of \( \Delta^6 \)-desaturases should be important for their enzyme activity. However, the effect of the three-dimensional structure of the C-terminal of \( \Delta^6 \)-desaturases on their enzyme activity is worthy of further investigation.

In conclusion, three genes encoding \( \Delta^6 \)-desaturases (\( \text{RnD6C/D/E} \)) and two encoding \( \Delta^8 \)-sphingolipid desaturases (\( \text{RnD8A/B} \)) from blackcurrant that were very similar in their sequences have been isolated and characterized. \( \text{RnD6C} \) and \( \text{RnD6E} \) was expressed in all the tissues at a low level, while \( \text{RnD6D} \) was expressed only in seeds. \( \text{RnD6D} \) could mainly contribute to the accumulation of GLA and OTA in blackcurrant seeds. To the authors’ knowledge, this is the first report of the identification of several \( \Delta^6 \)-desaturases in a single plant species and of finding a \( \Delta^6 \)-desaturase gene expressed specifically in seeds. Blackcurrant \( \Delta^6 \)-desaturases were confirmed to localize in the ER of plant cells and in the mitochondria of yeast cells. It is speculated that the evolution of \( \Delta^6 \)-desaturase genes from \( \Delta^8 \)-sphingolipid desaturase genes in blackcurrant occurred more recently than that in other species.
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