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Cloning and molecular characterisation of a Δ^8-sphingolipid-desaturase from *Nicotiana tabacum* closely related to Δ^6-acyl-desaturases

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Abstract Investigation on the absence of Δ^6-desaturase activity in *Nicotiana tabacum* has led to the cloning of a new desaturase gene from this organism (*NTDXDES*) that exhibited unexpected biochemical activity. Cladistic analysis shows clustering of *NTDXDES* together with functional Δ^6-acyl-desaturases of near Solanales plants, such as *Borago* and *Echium*. This group lies apart from that of previously characterised Δ^8-sphingolipid-desaturases, which also includes two putative tobacco members identified in this study. Moreover, strong expression of *NTDXDES* is found in leaves, flowers, fruits and developing seeds of tobacco plants that is highly dependent on the development phase, with transcriptional activity being higher at stages of active tissue growth. This pattern is similar to that showed by Δ^6-acyl-desaturases characterised in Boraginaceae species. However, functional assays using a yeast expression system revealed that the protein encoded by *NTDXDES* lacks Δ^6-desaturase activity, but instead it is able to desaturate sphingolipid substrates by introducing a double bond on the Δ^8-position. These data indicate that *NTDXDES* represent a novel desaturase gene placed in a different evolutionary lineage to that of previously characterised Δ^8-desaturases.

Keywords Desaturase evolution · Tobacco · RNA interference · Long-chain base · Yeast expression

Abbreviations

ACP	Acyl carrier protein
ALA	α-linolenic acid
CaMV	Cauliflower mosaic virus
CSPD	Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′ chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenyl phosphate
CTAB	Cetyl-trimetyl-amonium bromide
DIG	Digoxigenine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLA	γ-linolenic acid
HPLC	High pressure liquid chromatography
IPCR	Inverse PCR
LA	Linoleic acid
LCB	Long-chain base
OTA	Octadecatetraenoic acid
PCR	Polymerase chain reaction
RNAi	RNA interference
SDS	Sodium dodecylsulfate

Introduction

Fatty acid desaturases make up a complex group of enzymes performing essential roles related to

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the production of regulatory compounds called eicosanoids, and to maintenance of the functional characteristics of biological membranes and their components. From an evolutionary perspective, two lineages representing unrelated classes of desaturases are present in plants (Murphy 1999). A first group, integrated by soluble plastidial proteins like the stearoyl-ACP desaturase, exclusively use acyl-ACP as substrates. The second group, represented by membrane-bound desaturases acting on either acyl-CoA or acyl groups bound to glycerolipids, are mainly responsible for the synthesis of polyunsaturated fatty acids. This is, in turn, a heterogeneous group that includes three main evolutionary lineages (López Alonso et al. 2003; Sperling et al. 2003) corresponding to Δ^9-related desaturases, Δ^{12}/Δ^{15}-desaturases, and a third group of enzymes characterised by the presence of an N-terminal cytochrome b_5-domain. Two kind of desaturases have been described in higher plants that contain this particular electron donor domain. Front-end desaturases (Δ^4-Δ^5-, and Δ^6-desaturases) that introduce a double bond between the carboxyl and a pre-existing unsaturation (Napier et al. 1999, 2002). And a second class of enzymes, Δ^8-desaturases, although mechanistically and evolutionary related to the later, using sphingolipids as substrates. These enzymes introduce a double bond on the long-chain base (LCB) moiety of the molecule without a requirement for previous desaturation, with a stereochemistry resulting in cis and trans isomer products (Sperling et al. 1998, 2001). Investigation aimed to the finding of particular regions responsible for the different catalytic properties of these enzymes has been puzzling, with the conclusion that determinants for substrate specificity may reside on amino acids scattered across different locations of the polypeptide chain (Libisch et al. 2000).

The Δ^6-acyl-desaturases catalyse the introduction of a double bond in the Δ^6-position of linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) to produce the unusual γ-linolenic acid (18:3n-6; GLA) and octadecatetraenoic acid (18:4n-3, OTA), respectively. GLA is accumulated at a significant level by plants of certain genera that are particularly well represented within the Boraginaceae and Onagraceae, but it is also occasionally found in some taxa belonging to other plant families (Gunstone 1992; Tsevegsüren and Aitzetmüller 1993; Sayanova et al. 1999a; Tsevegsüren et al. 1999a; Guil-Guerrero et al. 2004). Finding of significant amounts of GLA in tissues of various Solanaceae genera like *Lycium* and *Hyoscyamus* (Tsevegsüren et al. 1999b; our unpublished results) prompted us to investigate the reasons for the absence of this fatty acid in other species of the same family, and particularly in *Nicotiana tabacum*. Tobacco was chosen since GLA is undetectable in any tissue of the plant, and it is also a particularly well-suited organism to conduct molecular biology studies. Moreover, it has been shown that transformation of tobacco plants with genes encoding acyl-Δ^6-desaturases from Boraginaceae species leads to the accumulation of high amounts of GLA in different tissues of the plant (Sayanova et al. 1999a; García-Maroto et al. 2002). This indicated that the lack of GLA in the wild plant was due to the absence of a Δ^6-desaturase activity in tobacco rather than to an active exclusion of this unusual fatty acid from the complex lipids. A main goal of this work was therefore to investigate the molecular mechanisms that account for the lack of that activity in tobacco. This has led to the cloning and functional characterisation of a new desaturase gene (*NTDXDES*) with Δ^8-desaturase activity on sphingolipid substrates. Evolutionary implications of its placement in the group of acyl-Δ^6-desaturases from related species are also discussed.

Materials and methods

Biological material

The tetraploid variety Wisconsin-38 of *N. tabacum* was used both as a source of genes, and in *Agrobacterium* transformation experiments. Plants were grown at 25°C under controlled conditions in growth cabinets with a 16 h light/8 h dark photoperiod, and 70% relative humidity. The yeast strains INVSc1 (Invitrogen, Carlsbad, CA, USA), and W303-A1 (Tonon et al. 2005) were used to assay Δ^6- and Δ^8-desaturase activities of the protein encoded by the *NTDXDES* gene by heterologous expression.

Cloning of desaturase genes

Cloning of the *NTDXDES* gene was achieved by PCR amplification of a partial sequence, followed by bi-directional genomic walking through inverse PCR (IPCR). Initially, a 550 bp PCR fragment corresponding to amino acid positions 187–369 (Fig. 2) was obtained using the degenerate oligonucleotides BO-1 (5′-AT(A/C)AG(T/C)AT(T/C)GGTTGGTGGAA(A/G)TGG-3′) and BO-2 (5′-AATCCACC(A/G)TG(A/G)AACCA(A/G)TCCAT-3′) as primers, and tobacco genomic DNA as a template. The PCR program consisted of a denaturation step of 2 min at 94°C, followed by 38 cycles of 10 s at 94°C, 1 min at 48°C and 1 min at 72°C, ending with a 5 min step at 72°C. The product

was cloned into the pGEM®-T Easy vector (Promega, Mannheim, Germany), and a small plasmid library was generated and subsequently screened by plasmid extraction, restriction analysis and Southern blot using Δ^6- and Δ^8-desaturase specific probes from *Echium* sp. (García-Maroto et al. 2002). Selected clones were sequenced on both strands using a Perkin-Elmer ABI-310 DNA automated sequencer and the BigDye® Terminator v3.1 chemistry. Three different sequences were obtained and designated as *NTDXDES*, *NTD8DES1* and *NTD8DES2*. From the partial sequence of *NTDXDES*, two nested upstream primers TAB-1 (5′-CACAGTCATTCATATTGTTGTTGTC CAA-3′) and TAB-3 (5′CATTGCTGTCAACAGTCT TGAATATGAC-3′), and two nested downstream primers TAB-2 (5′-GGTGAAAGGATAATGTTTGT TCTTGCT-3′) and TAB-4 (5′-GGCTCTCTTGACAT ATCATGCCCTAG-3′, were designed to perform the IPCR essentially as described by Ochman et al. (1990). From the several restriction enzymes used to cut the DNA, *Vsp*I resulted most useful, allowing, after circularisation and two nested rounds of PCR amplification, the obtaining of a suitable 3.5 kbp long fragment that was analysed and sequenced as described before. A genomic DNA fragment containing the whole coding sequence for *NTDXDES* was further obtained by PCR amplification using suitable upstream NTUP (5′-AAGCTTTTATGGCAGATTCAAGAAAGTAC ATTAC-3′) and downstream NTDOWN (5′-CGTAG TATTGATAAATTCTAGCAACTGCGGATCC-3′) primers, and the reading-proof polymerase Pfx (Invitrogen). The *NTDXDES* fragment was further cloned and sequenced as indicated before (GenBank accession no. EF110692).

Cladistic analysis

Alignment of nucleotide sequences of the desaturases was performed by using the program Clustal X v.1.7 (Thompson et al. 1994) with the default settings, and was further refined by visual inspection. The alignment output was used to generate a phylogenetic tree based on the Minimum Evolution method (Rzhetsky and Nei 1992), as implemented in the MEGA package v3.1 (Kumar et al. 2001). The Jukes and Cantor metric was used together with the complete deletion option, and confidence of the tree branches was checked by bootstrap generated from 1,000 replicates. Rooting of the tree was acomplished by using the Δ^6-desaturase sequence from the moss *Physcomitrella patens* as outgroup. For sequences selected in Fig. 2, the alignment was visualised using the ''Boxshade'' v. 3.21 software.

Southern and Northern blot analysis

Genomic DNA was isolated from young tobacco leaves by a CTAB-based extraction procedure (Taylor and Powel 1982). DNA (about 5 μg) was restricted with the appropriate restriction enzymes, separated on a 0.8% agarose gel, and transferred by capillarity onto Hybond® N^+ nylon membranes (Amersham, Freiburg, Germany). Filters were UV-crosslinked, pre-hybridised at 42°C during 5 h in the 50% formamide/high SDS buffer recommended by the DIG manufacturer (Boehringer-Mannheim, Mannheim, Germany), and hybridised at the same temperature and same buffer solution (stringent conditions), containing the digoxigenin-labelled *NTDXDES* specific probe. High-stringency washes were performed twice at 65°C during 15 min in buffer containing 0.5 × SSC, 0.1% SDS, and the luminogenic substrate CSPD® was used for the detection following the instructions provided with the DIG detection kit. Images were obtained by exposure of Biomax® ML films (Kodak, Rochester, NY, USA) for 10–25 min and final developing by standard procedures. The *NTDXDES* probe was obtained by random primed labelling from a PCR fragment spanning 430 bp of the 3′-coding sequence and about 110 bp of the 3′-untranslated region.

Total RNA was extracted from different tissues of tobacco plants, following the method of Chang et al. (1993). About 10 μg per lane of total RNA were loaded onto an agarose/formaldehyde gel, electrophoretically separated, and transferred to Hybond®-N^+ membranes. Filters were hybridised at 50°C (stringent conditions) as described for Southern analysis, and using the same *NTDXDES* specific probe. Stringent washes, accomplished at 68°C, and detection of the DIG-labelled probe were as indicated before. As a control, the filters were re-hybridised with a 900 bp cDNA probe from tobacco, which encodes part of the cytosolic glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (Shih et al. 1986). In this case, hybridisation was done in the same conditions, but the final washes were performed at 65°C.

Desaturase activity assays in yeast

The whole *NTDXDES* coding sequence was transcriptionally fused to the *GAL1* inducible promoter of the pYES2® expression vector (Stratagene, La Jolla, CA, USA), and the resulting plasmid used to transform *Saccharomyces cerevisiae* by the LiAcO method (Elble 1992). Cultures were grown at 28°C in standard

minimal medium supplemented with the auxotrophic requirement of the strain plus 1% (w/v) raffinose, and expression was further induced on a 0.4 OD_{600} culture by the addition of galactose 2% (w/v). To assay Δ^6-desaturase activity, induction was maintained for 48 h at 22°C in the presence of the putative substrate, linoleic acid, at 0.5 mM and 1% Tween-40. Yeast cells were collected by centrifugation, further washed with 1.3% NaCl, and the resulting biomass subjected to lyophilisation. Fatty acid analysis was performed by simultaneous lipid extraction and generation of fatty acid methyl esters as described elsewhere (Rodríguez-Ruiz et al. 1998). Fatty acid composition was determined by gas-liquid chromatography and gas chromatography-mass spectrometry (GC-MS) as in García-Maroto et al. (2006).

The assay for sphingolipid desaturase activity was carried out according to previously described methods (Sperling et al. 1998; Michaelson et al. 2002; Garton et al. 2003; Tonon et al. 2005).

Knockdown of *NTDXDES* in tobacco by RNAi

The construct for *NTDXDES* silencing, by RNA interference, was generated on the pJIT60 plasmid (Guerineau 1995), and consisted of a 1 kb sense genomic fragment of *NTDXDES* (the coding sequence lacking 0.5 kb of the 3′end), followed by the whole *NTDXDES* in antisense orientation. The cassette containing the ''hairpin'' sequence under the control of the 35S CaMV promoter with a duplicate enhancer sequence and the polyadenylation signal was liberated and introduced into pBIN19 (Bevan 1984), as described above, and the resulting construct, used to transform the *Agrobacterium tumefaciens* LBA4404 strain. Tobacco leaf disc transformation was achieved as previously described (García-Maroto et al. 2002). A total of 25 independent transgenic lines were obtained, and *NTDXDES* RNA level was monitored by Northern-blot analysis on total RNA from young developing leaves, against untransformed plants.

LCB analysis in tobacco

Total LCBs were extracted from fresh leaf material (0.5 g) using the barium hydroxide/dioxane method used for yeast. Derivatised LCBs were separated and quantitated on an Agilent 1100 HPLC, with detection at 350 nm. An internal standard of C20 sphingosine was included to allow accurate determination of LCB levels. Identification of individual LCB species was done via co-migration with known standards and APCI-MS as described before (Garton et al. 2003).

Results

Cloning of *NTDXDES*, a Δ^6-desaturase-related gene from *N. tabacum*

The complete genomic sequence of *NTDXDES* was obtained from a partial clone generated by PCR using degenerated oligonucleotides, followed by walking in both directions by IPCR. Degenerated primers, corresponding to highly conserved motives (ISIGWWKW and MDWFHGG) found in front-end desaturases from plants, were used to amplify a 550 bp DNA fragment that was subsequently cloned in a T-vector. An exhaustive screening of the resulting plasmid library was performed allowing the identification of three different sequences. Cladistic analysis revealed that one of them, initially called *NTDXDES*, grouped together with functional Δ^6-desaturases from *Echium* and *Borago* (Fig. 1). The other two sequences,

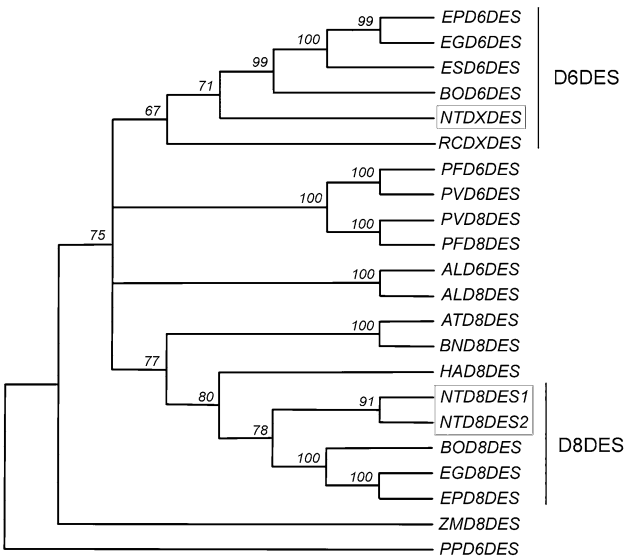

Fig. 1 Minimum evolution tree showing relationships among tobacco *NTDXDES* and related desaturase genes from dicot species. Nucleotide sequences (about 550 nts), corresponding to amino acids located between the second and third histidine boxes of the desaturases were aligned, and the resulting matrix analysed using the Minimum Evolution method, as described in Materials and Methods. Branches with a bootstrap value below 50% were collapsed, and the values represented on the corresponding nodes. The tree was rooted using the *Physcomitrella patens* Δ^6-desaturase (*PPD6DES*) as outgroup. Organism abbreviations stand for: *Echium pitardii* (EP), *E. gentianoides* (EG), *E. sabulicola* (ES), *Borago officinalis* (BO), *Nicotiana tabacum* (NT), *Ricinus communis* (RC), *Primula vialli* (PV), *P. farinosa* (PF), *Anemone lendsquerelli* (AL), *Arabidopsis thaliana* (AT), *Brassica napus* (BN), *Helianthus annuus* (HA) and *Zea mays* (ZM)

Fig. 2 Sequence comparison of NTDXDES with related desaturases of higher plants. The amino acid sequence of NTDXDES (GenBank accession no. EF110692) is aligned, using the ClustalX (v1.7) software, together with those of Δ^6-desaturases of *Echium gentianoides* (EGD6DES, acc. no. AY055117) and *Borago officinalis* (BOD6DES, acc. no. U79010) and Δ^8-desaturases of *B. officinalis* (BOD8DES, acc. no. AF133728) and *Helianthus annuus* (HAD8DES, acc. no. CAA60621). The Boxshade program is used to highlight the homology between protein sequences. Shading is applied when there is agreement for a fraction of sequences above 0.5. Amino acids identical to NTDXDES are enclosed in *black boxes*, and similar residues in *grey*. Conserved histidine boxes are *underlined*

NTD8DES1 and *NTD8DES2*, appeared together in a cluster made up by Δ^8-desaturases, close to the *Borago* Δ^8-desaturase and putative orthologous sequences from *Echium* (Fig. 1).

The whole *NTDXDES* sequence was assembled from two additional clones obtained by IPCR, and a whole genomic DNA fragment encompassing the coding sequence was generated by proofreading PCR using flanking primers. *NTDXDES* lacks intervening sequences (introns), a feature that is also shared by other Δ^6-desaturase genes of plants (García-Maroto et al. 2002). It encodes a 447 amino acids protein that shows a 73% identity (85% similarity) to the *Echium* and *Borago* Δ^6-desaturases (Fig. 2). A deduced polypeptide sequence characteristic of the consensus for Δ^6/Δ^8-desaturases was observed, with the presence of an N-terminal cytochrome b_5 domain, and the three histidine boxes (Fig. 2).

Genomic organisation and expression analysis of *NTDXDES*

The genomic pattern for *NTDXDES* was investigated by Southern-blot analysis. Genomic DNA obtained from tobacco plants (cv. Wisconsin 38) was digested with five different restriction enzymes, followed by hybridisation under stringent conditions using a *NTDXDES* specific probe (see Materials and Methods). Two hybridisation bands of similar intensity were obtained in all cases (Fig. 3). Since none of the restriction enzymes employed cut within the coding sequence, this indicates that two copies of *NTDXDES* are present in the tobacco genome. It is known that cultivated *N. tabacum* is an allotetraploid species, and it is therefore likely that these copies represent homoeologous genes corresponding to the *N. tomentosiformis* and *N. sylvestris* parental genomes.

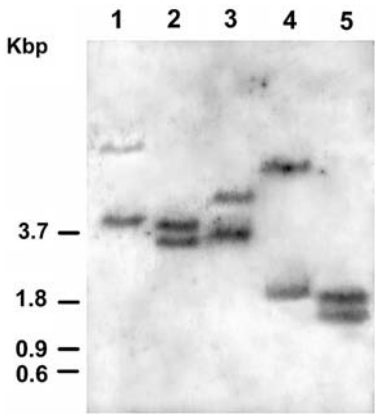

Fig. 3 Southern blot analysis of *NTDXDES* in *Nicotiana tabacum*. Genomic DNA (5 μg) was digested with *Hind*III (1), *Dra*I (2), *Vsp*I (3), *Eco*RV (4) or *Hae*III (5) restriction endonucleases. The resulting fragments were separated on a 0.8% agarose gel in TAE buffer, run at 70 V for 3 h, and transferred to a positively charged Nylon membrane (Hybond® N^+, Amersham). Hybridisation was performed using a *NTDXDES*-specific probe under stringent conditions, as described in Materials and Methods. Size markers (kbp) positions are indicated

The expression pattern of *NTDXDES* was studied by Northern-blot analysis of total RNA obtained from different tissues of tobacco plants (Fig. 4). Hybridisation with the *NTDXDES* probe under stringent conditions revealed strong signals in young Ld and growing fruits (Fr). Lower expression levels are detected in developing seeds (S) and actively growing flowers (F_2), while no detectable expression is observed in roots (Rd, Rm), mature leaves (Lm), small flower buds (F_1) nor in fully developed flowers at anthesis (F_3). A correlation seems to exist between the expression level and an active development of the flower or leaf tissue. Further analysis were performed in leaves taken at different heights from adult flowering plants (Fig. 4b). Signal was undetectable in fully developed leaves ranging from number 7 to 15 (counted from the cotyledons), and became clearly visible around leaf 19 (Fig. 4b). However, strong hybridisation was obtained for intermediate leaves (number 12/13) obtained from young plants in which those leaves were still developing (Fig. 4b). This indicates that expression of *NTDXDES* is not dependent on the position of the leaf in the plant but rather on the developmental stage itself. This pattern closely resembles that of the Δ^6-desaturases from boraginaceae species (Sayanova et al. 1999b; García-Maroto et al. 2002; our unpublished results), rather than the constitutive expression normally associated with sphingolipid Δ^8-desaturases.

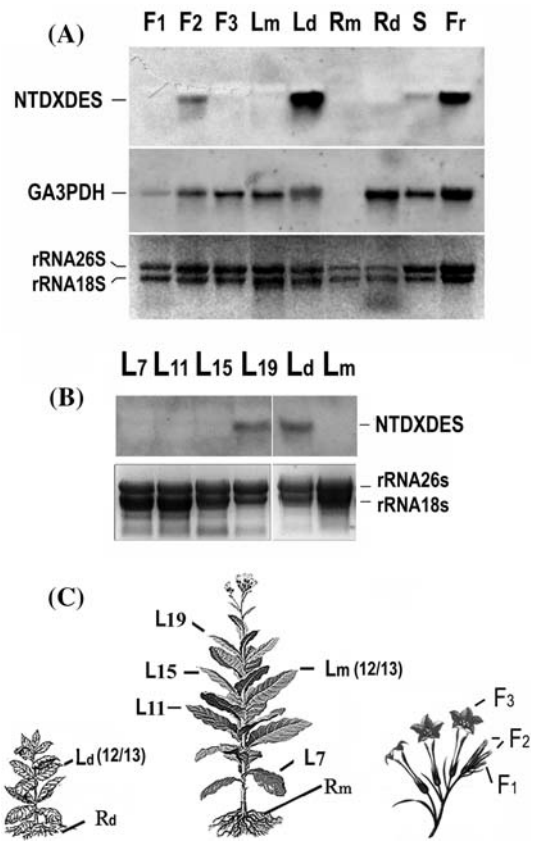

Fig. 4 Expression analysis of *NTDXDES* in tissues of *Nicotiana tabacum*. (**a**) Equivalent amounts of total RNA (10 μg) from developing flowers at different stages: 3–6 mm (F_1), 6–12 mm (F_2) and 2–4 cm (F_3) buds, mature (*Lm*) or young developing leaves (*Ld*), developing seeds (*S*), roots from young (*Rd*) or old flowering (*Rm*) plants and developing fruits (*Fr*), were subjected to electrophoresis in an agarose/formaldehyde gel, run for 1 h at 110 V, blotted and hybridised with a *NTDXDES*-specific probe under high-stringency conditions, as indicated in Materials and Methods. The expression pattern of the cytosolic glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) from tobacco, a constitutive gene used as a control, is shown below besides the ethidium bromide staining of the gel. (**b**) Equal amounts of total RNA from leaves taken at different heights or developmental stages were analysed as in panel a. Leaf numbers (counted from the button of the plant without considering cotyledons): 7 (*L7*), 11 (*L11*), 12–13(*Lm*), 15 (*L15*) and 19 (*L19*) were collected from mature flowering plants, and leaves 12–13 (*Ld*) from a young developing plant. The ethidium bromide staining of the gel is also shown below. (**c**) Drawings depicting the different organs and developmental stages of tobacco plants used in the expression analysis

Investigation of the desaturation activity of NTDXDES

The Δ^6-desaturase activity of NTDXDES was investigated by heterologous expression in *S. cerevisiae* and supplementation of the culture with either linoleic (LA) or linolenic acid (ALA) substrates. No additional peak was observed after induction with galactose,

thus indicating that NTDXDES does not exhibit Δ^6-desaturase activity. Δ^8-desaturase activity on sphingolipids was also investigated using the same yeast expression system. Induced cells were subjected to strong alkaline hydrolysis to liberate the LCBs from complex sphingolipids, and the extracted LCBs derivatised and subsequently analysed by reverse-phase HPLC as previously described (Sperling et al. 1998). Non-induced control cells show a profile, containing C_{18}- and C_{20}-phytosphingosine as the main compounds (Fig. 5a), that is in agreement to the composition previously reported in yeast (Whitney et al. 2003). When expression of the *NTDXDES* gene was induced, two new peaks corresponding to the Δ^8-desaturation products, t18:1Δ^8 and t20:1Δ^8, were obtained (Fig. 5b) thus indicating that the protein encoded by *NTDXDES* has Δ^8-desaturase activity on sphingolipids. Unresolved doublets that are apparent in the additional peaks indicate that both cis and trans isomers are products of the reaction as it has been shown for other Δ^8-desaturases of plants (Sperling et al. 2001; Michaelson et al. 2002; Whitney et al. 2003).

Silencing of *NTDXDES* in tobacco plants

To investigate the role of *NTDXDES*, gene knockdown by RNA interference was performed in tobacco. A total of 25 independent transgenic lines were initially obtained that were analysed by Northern-blot of total RNA obtained from young Ld, to monitor the expression level of the endogenous *NTDXDES* gene. Most of the lines that were analysed exhibited a

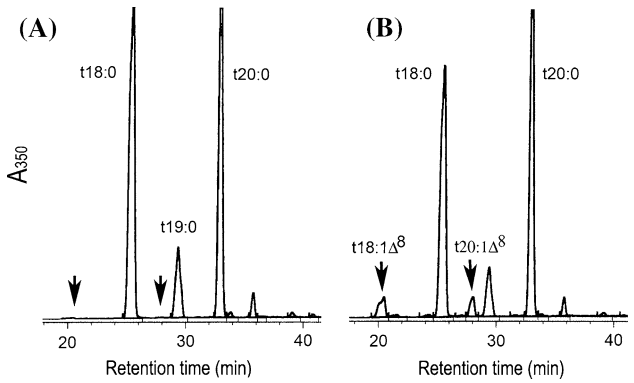

Fig. 5 Identification of the Δ^8-desaturase activity of NTDXDES. The whole coding sequence of *NTDXDES* was expressed in yeast using the galactose-inducible pYES2 expression system. Total LCBs were extracted from uninduced yeast culture (**a**) or from the galactose induced cells (**b**). Derivatised LCBs were analised by reverse-phase HPLC and detected by absorbance at 350 nm. The presence of additional desaturated LCBs after induction of the *NTDXDES* expression is indicated by the *arrows*

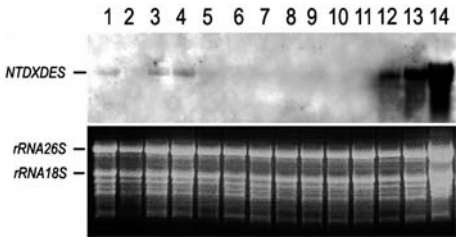

Fig. 6 Silencing of *NTDXDES* in tobacco plants by RNA interference. Eleven RNAi transgenic lines (PTS-1–PTS-11, lanes 1–11) were checked for endogenous *NTDXDES* RNA steady level by Northern blot (*upper panel*) on total RNA (10 μg) obtained from young developing leaves of the T_0 primary lines. As a control the same material from wild type plants was analysed using different RNA loading amounts for better comparison (5, 10 and 20 μg in lanes 12–14, respectively). RNA samples were subjected to electrophoresis in an agarose/formaldehyde gel, run for 1 h at 110 V, blotted and hybridised with a *NTDXDES*-specific probe under high-stringency conditions, as indicated in Materials and Methods. Ethydium bromide staining of the gel (*lower panel*) is shown for loading comparison, as well as the position of the predominant rRNAs

reduced steady state level of the RNA at a developmental stage were the gene is strongly expressed in untransformed plants (Fig. 6). However, none of them, even those were the *NTDXDES* transcript was undetectable showed obvious phenotypic alterations such as in their morphology, growth rate or flowering time (results not shown). To further investigate the impact of the *NTDXDES* gene knockdown on the LCB profile of the plant, four of the silenced lines where the RNA transcript was undetectable (PTS-2, 9, 10 and 11, Fig. 6) were selected to perform LCB analysis from young leaf material. LCB composition in tobacco leaves includes as major compounds t18:0 (4-hydroxysphinganine), t18:1Δ^8 (4-hydroxy-8-sphingenine, mainly the trans isomer) and d18:2$\Delta^{4,8}$ (4,8-sphingadienine), besides minor amounts of d18:0 (dihydrosphingosine) and d18:1Δ^8 (8-sphingenine). Table 1 shows the relative percentage of the three mayor LCBs in the four knockdown lines, as compared to the wild type tobacco plant. A moderate but consistent decrease in t18:1, paralleled by a similar increase in the t18:0 which acts as substrate for the Δ^8-desaturase is observed in the silenced tobacco lines. The difference between t18:0 and t18:1 was statistically significant by the ANOVA test in all silenced lines (Table 1). This agrees with the results obtained in the yeast heterologous system where a Δ^8-desaturation activity on t18:0 was also shown for NTDXDES. As it will be later discussed, the reduction in t18:1 seem to affect mainly to the trans isomer, an observation that is statistically supported at least for two of the silenced lines (PTS-9 and PTS-10).

Table 1 Composition of the main LCBs present in leaves from silenced tobacco lines for the *NTDXDES* gene

Tobacco line	Mean LCB percentage (MSE)					
	d18:2	t18:0	t18:1(t)	t18:1(c)	t18:1(*c* + *t*)	Δ(t18:0-t18:1)[a]
WT	28.54 (3.14)	36.36 (1.35)	32.29 (1.87)	2.81 (0.17)	35.10 (1.88)	1.26
PTS-2	27.90 (3.09)	40.43* (1.04)	28.70 (2.57)	2.98 (0.55)	31.68 (2.63)	8.75*
PTS-9	31.38 (1.15)	37.62 (0.92)	27.98* (2.03)	3.02 (0.12)	31.00* (2.03)	6.62*
PTS-10	30.31 (2.75)	39.20* (1.34)	27.59* (1.55)	2.90 (0.32)	30.50* (1.58)	8.70*
PTS-11	29.66 (1.36)	38.48* (1.98)	29.13 (2.12)	2.73 (0.25)	31.86 (2.13)	6.62*

Relative contains (%) of the main LCBs of young leaves from pools of transgenic T_1 or wild-type plantlets (n = 5–7) of each line were obtained for three independent determinations, and the mean values and standard errors (*MSE*) are expressed. The two t18:1Δ^8 isomers (cis/trans) which are desaturation products of the 18:0 were considered. Differences in LCB contents between wild type and silenced tobacco lines were statistically assessed by the ANOVA test, and significant differences at the 0.05 level are marked by an asterisk

[a] Percentage difference between the unsaturated LCB t18:0 and the Δ^8-desaturation product t18:1(cis and trans isomers)

Discussion

Contrary to some Solanaceae species, GLA is not detected in tissues of tobacco plants. To investigate the existence of a putative silencing mechanism of the *D6DES* gene, we have conducted a genomic-PCR screening looking for tobacco sequences related to Δ^6-desaturases. This has led to the identification of a gene (*NTDXDES*) that groups in cladistic analysis with functional Δ^6-desaturases of Boraginaceae species, like those from *Borago* and *Echium* (71% bootstrap support, Fig. 2). Boraginaceae and Solanaceae are closely related families belonging to the euasterids-I clade according to recent classifications (Angiosperm Phylogeny Group 2003), thus supporting this clustering. It should be noticed that Δ^8-desaturase sequences previously characterised from Boraginaceae, cluster apart from that of Δ^6-desaturases from the same species (Fig. 2). In agreement to this scheme, two other tobacco sequences (*NTD8DES1* and *NTD8DES2*) were detected in our screening that grouped within the Δ^8-desaturase cluster, together with those of *Borago* and *Echium* (78% bootstrap, Fig. 2). Those genes probably represent equivalent orthologues of Δ^8-desaturase genes characterised in this group of plants.

However, we have shown that *NTDXDES* encodes a protein with a Δ^8-sphingolipid desaturase activity. Since Δ^8-sphingolipid desaturases seem to be widely distributed in plants while Δ^6-acyl desaturases appear to be much more restricted, it is commonly assumed that Δ^8-desaturases are ancestral to Δ^6-desaturases in this kingdom. Thus, placement of *NTDXDES* may be interpreted as a relic in the evolutionary lineage leading to Δ^6-desaturases of Boraginaceae species. Basal position of *NTDXDES* in the clade would favour this interpretation, though a more extensive sampling of species within this group would be necessary in order to clarify that point. In this context, the synthesis of GLA has been reported in other Solanaceae such as *Lycium* and *Hyoscyamus* (Tsevegsüren et al. 1999b; our unpublished results), thus indicating that they contain a functional Δ^6-desaturase that might also be closely related to those of Boraginaceae species. If this was the case, it would be possible that *NTDXDES* evolved from a Δ^6-desaturase common ancestor shared by plants from the Solanaceae and Boraginaceae families. Therefore, an alternative evolutionary scenario could involve a recruitment of *NTDXDES* from a *D6DES* gene to perform Δ^8-desaturation on sphingolipid substrates. Functional shift is a recurrent theme in evolution that has been recognised as a key mechanism of evolutionary change (Ganfornina and Sánchez 1999; Ohta 2000; True 2002). Although additional information will be required to ascertain the evolutionary history of *NTDXDES*, its placement clearly indicates a different origin to that of previously characterised Δ^8-desaturases from *Helianthus* and *Borago* (Fig. 1), which is interesting regarding the possibility that different functions could be performed by those enzymes.

On the other hand, clustering of NTDXDES with D6DES enzymes argues in favour of the high ''plasticity'' of these enzymes, as it has also been described in *Primula* and *Anemone*, where both kind of activities are encoded by closely related genes that have evolved late and independently from the event that separated Δ^6/Δ^8-desaturase genes in Boraginaceae species (Sayanova et al. 2003; Whitney et al. 2003). The similarity between NTDXDES and D6DES proteins prompted us to look for residues responsible for the different activities. However, the number of changes (45 of 448 non-conservative differences between NTDXDES and the D6DES of *Echium*) besides the fact that they are scattered across the whole protein, made difficult to extract reliable conclusions.

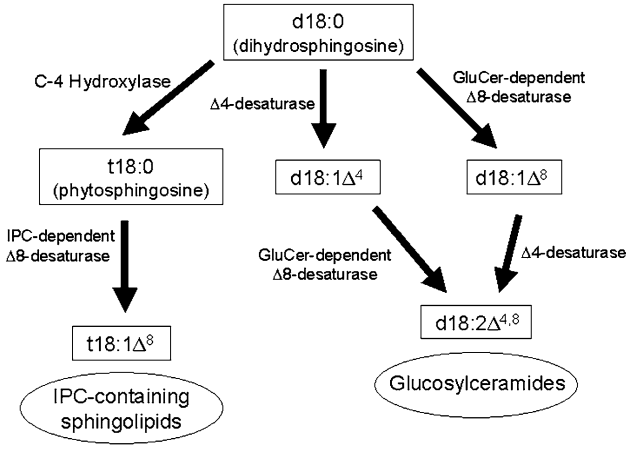

Fig. 7 Long-chain base modifications in complex sphingolipids of plants. The desaturation and hydroxylation reactions responsible for generating different LCBs found in plants are shown in the diagram. The possible existence of Δ^8-sphingolipid desaturases with preference in desaturating either GlcCer or IPC sphingolipid classes is also included as a working hypothesis (see explanation in the text)

As we show, NTDXDES catalyses a reaction on sphingolipids that is similar to that performed by Δ^8-sphingolipid-desaturases previously described in other plants (Sperling et al. 2001; Michaelson et al. 2002; Sayanova et al. 2003; Whitney et al. 2003). Desaturation is performed both on C_{18} and C_{20} LCB substrates, and it is stereo-unselective, rendering a mixture of the cis and trans isomer products. Therefore, it is possible that the biochemical activity of NTDXDES was in addition (i.e. functionally redundant) to that of ''genuine'' Δ^8-desaturases. Results from knockdown experiments performed on tobacco seem to be congruent with this statement, since plants where *NTDXDES* expression was abolished, only showed a small reduction of the desaturation products.

Another possibility that cannot be discarded is that, even though NTDXDES is able to desaturate sphingolipids to some extent, they might not act as the main substrates in the cell. There are some examples in literature of desaturase-related enzymes that function in specialised pathways of secondary metabolism. For instance, it has been shown that a stearoyl-ACP desaturase-related enzyme from *Pelargonium x hortorum* is involved in the synthesis of pest resistant-anacardic acids (Schultz et al. 1996). Similarly, microsomal desaturases of the FAD2 class show considerable functional variation across the Plant Kingdom, including hydroxylation, epoxygenation and acetylation (Sperling et al. 2003). It is therefore possible that NTDXDES carries out a reaction that is distinct from the LCB desaturation previously described for other Δ^8-desaturases.

Equally, an explanation for the reduced impact of the gene knockdown experiments is that desaturation by NTDXDES was carried out specifically over a minor sphingolipid class of molecules. In this context, it is interesting to notice that the moderate impact of the *NTDXDES* silencing on the LCB profile seems to selectively affect desaturation from the trihydroxylated (t18:0) compound, while does not seem to alter the desaturation from dyhidroxylated (d18:1Δ^4, or d18:1Δ^8) LCBs, as inferred by the fact that d18:2 level seem to remain unaffected. It has been noticed that the distribution of LCBs between the two complex sphingolipid classes, glucosylceramides (GlcCer) and derivatives of inositolphospohorylceramide (IPC), which are found in plants is not uniform. Thus, d18:2 LCBs mainly accumulate in the GlcCer fraction, whereas t18:1 is predominantly found in IPC (Dunn et al. 2004; Markham et al. 2006). Therefore, it is tempting to speculate with the existence of distinct Δ^8-desaturases showing preference on either GlcCer or IPC sphingolipids (Fig. 7). Following that reasoning, NTDXDES might exhibit a preference on IPC substrates, thus explaining the selective reduction of t18:1 LCB in the silenced tobacco plants. In agreement to that, the reduction in t18:1 seem to affect mainly to the trans isomer which is also typically much more abundant in the IPC sphingolipid class (Markham et al. 2006).

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