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Substrate specificity of acyl-Δ^6-desaturases from Continental versus Macaronesian *Echium* species

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Abstract

Echium (Boraginaceae) species from the Macaronesian islands exhibit an unusually high level of γ-linolenic acid (18:3n-6; GLA) and relatively low content of octadecatetraenoic acid (18:4n-3; OTA) in the seed, while the amounts of both fatty acids in their Continental (European) relatives are rather similar. We have tested the hypothesis of whether a different specificity of the acyl-Δ^6-desaturases (D6DES) towards their respective usual substrates, linoleic acid (18:2n-6; LA) for GLA and α-linolenic acid (18:3n-3; ALA) for OTA, was partly responsible for this composition pattern. To this aim we have expressed in yeast the coding sequences of the *D6DES* genes for the Continental species *Echium sabulicola*, and the Macaronesian *Echium gentianoides*. When the yeast cultures are supplemented with the two fatty acid substrates (LA and ALA), a similar utilization of both compounds was found for the D6DES of *E. sabulicola*, while a preference for LA over ALA was observed for the enzyme of *E. gentianoides*. This substrate preference must contribute to the increased accumulation of GLA in the seeds of the Macaronesian *Echium* species. Comparison among the amino acid sequences of these desaturases and other related enzymes, allowed us the discussion about the possible involvement of some specific positions in the determination of substrate specificity.

Keywords: *Echium gentianoides*; *Echium sabulicola*; Boraginaceae; Acyl specificity; Gene cloning; Δ^6-Desaturase

1. Introduction

Polyunsaturated fatty acids are receiving considerable attention due their involvement in human health (Gunstone, 1998). Consequently, efforts have been addressed to the obtaining of improved new sources of those compounds. Genetic engineering of oilseed crops using appropriate heterologous genes from the lipid biosynthetic pathway has become a strategy that is giving promising results (Thelen and Ohlrogge, 2002; Sayanova and Napier, 2004; Surinder et al., 2005). One of the key genes encodes the acyl-Δ^6-desaturase (D6DES) an enzyme that catalyses the first committed step in the biosynthesis of essential fatty acids acting as eicosanoid precursors (Napier et al., 1997; López Alonso and García-Maroto, 2000). This enzyme has two preferred substrates in plants, the linoleic acid (LA, 18:2n-6) and the α-linolenic acid (ALA, 18:3n-3), giving rise to either γ-linolenic acid (GLA, 18:3n-6) or octadecatetraenoic acid (OTA, 18:4n-3), respectively. GLA and OTA are unusual in most plant species but they accumulated at a high level by the seeds of some families such as the Boraginaceae. Among them, *Borago officinalis* and several *Echium* species have been used as a source for cloning of *D6DES* genes, and the enzymatic activity of their products has been assayed by using heterologous expression systems (Sayanova et al., 1997; García-Maroto et al., 2002). Though substrate availability (e.g., LA/ALA content)

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seems to be an important determinant in the relative synthesis of GLA/OTA in the seeds of Boraginaceae species (Guil-Guerrero et al., 2001a), there are evidences that other factors such as substrate (i.e., acyl) specificity could also play a significant role. Thus, a distinct substrate preference has been demonstrated for the D6DES enzymes of two Primulaceae species (Sayanova et al., 2003). This is an interesting characteristic that could be usefully exploited to channel the synthesis to a particular product. However, finding of the molecular determinants of this specificity, by comparison of the amino acid sequences, was not possible due to the considerable divergence among them.

A survey of the fatty acid composition in seeds of *Echium* species revealed that taxa from the Macaronesian archipelago (Canary, Cape Verde and Madeira islands) show an unusually high level of GLA and low OTA content, as compared to Continental (European and North African) species where similar amounts of both fatty acids are accumulated (Guil-Guerrero et al., 2000, 2001a,b, 2003). We decided to investigate if different properties of the D6DES enzymes leading to a different utilization of LA/ALA as substrates could be responsible, at least to some extent, for the observed bias in the GLA/OTA ratio. To this aim we have cloned a gene encoding the D6DES from a Continental representative, *E. sabulicola* Pomel and tested its substrate specificity in a heterologous yeast expression system, as compared to the D6DES from *E. gentianoides*, an endemic from the Canary islands for which the gene was available (García-Maroto et al., 2002). Comparison among the amino acid sequences of these desaturases, allowed us a discussion about the possible involvement of specific positions in the determination of substrate specificity.

2. Results and discussion

Fatty acid compositions of seed oils from different *Echium* species are shown in Table 1. Continental *Echium*, as represented by five common European taxa, contain similar amounts of GLA (10% average) and OTA (13% average), with GLA/OTA ratios below the unit and ranging from 0.38 to 0.95. Conversely, Macaronesian taxa represented by five endemics from the Canary islands contain a higher level of GLA (21% average) relative to the OTA content (5% average), with GLA/OTA ratios ranging from 3.3 to 5.8. Similar results have been reported for other *Echium* species (Guil-Guerrero et al., 2001a, 2003). Based on the available biochemical data at least two explanations may account for this composition pattern. One of them is a different substrate (LA/ALA) availability that would favor the synthesis of the respective product. This is likely since opposite n-6 to n-3 ratios are found in both groups of plants, probably due to a different activity of the ω3-desaturase that regulates the flux towards n-3 or n-6 fatty acids during seed development. Thus, the elevated n-6:n-3 ratio indicates that LA availability is higher in Macaronesian taxa, therefore resulting in an increased GLA content, and the contrary applies to the Continental *Echium*. Another but not excluding explanation for the observed GLA/OTA ratios is a different specificity of the D6DES enzymes that determines a different utilization rate of the two substrates. This mechanism was previously illustrated in *Primula* species (Sayanova et al., 2003). To explore this possibility we have investigated the specificity of the D6DES enzymes of the Continental representative *E. sabulicola* and the Macaronesian endemic *E. gentianoides*, using the coding sequences from their respective genes in a yeast expression system.

The *D6DES* gene from *E. gentianoides* (*EGD6DES*) was available from a previous work (García-Maroto et al., 2002). Here we have cloned the gene for the D6DES of *E. sabulicola* (*ESD6DES*; see Section 4). As described for other *D6DES* genes (López Alonso et al., 2003; Sayanova et al., 2003) its genomic sequence is intron-less and encodes a 448 amino acids product bearing typical features of plant Δ^6-desaturases such as a cytochrome-b_5 domain an the three histidine boxes (Fig. 1).

Table 1
Fatty acid composition of seed oils from Macaronesian versus Continental *Echium* species

Echium species	Fatty acid							
	16:0	18:0	18:1	LA	GLA	ALA	OTA	GLA/OTA ratio
Continental (Europe)								
E. sabulicola[a]	7.6	3.1	8.3	19.1	11.5	38.2	12.1	0.95
E. lusitanicum[b]	6.7	2.5	16.4	16.3	10.9	33.3	12.3	0.89
E. boissieri[c]	5.5	2.3	14.7	8.6	5.5	47.1	14.3	0.38
E. vulgare[b]	6.5	3.7	9.4	16.9	10.9	39.3	13.3	0.82
E. plantagineum[d]	6.4	2.8	13.0	13.8	9.2	36.6	13.0	0.71
Macaronesian (Canary Islands)								
E. gentianoides[a]	7.4	4.5	10.6	22.2	26.7	21.9	6.7	3.98
E. pitardii[c]	6.8	3.8	13.0	24.0	18.9	25.9	5.7	3.31
E. auberianum[d]	7.0	2.5	19.2	29.1	17.5	18.9	3.0	5.83
E. strictum[d]	6.9	4.2	11.6	28.8	18.8	23.0	4.4	4.27
E. giganteum[d]	7.6	3.5	12.3	23.3	21.7	23.1	5.7	3.81

The 16:1 fatty acid was under 0.3% for all *Echium* species; The data were obtained from: (a) this work; (b) Guil-Guerrero et al. (2003); (c) Guil-Guerrero et al. (2001a); (d) Guil-Guerrero et al. (2000).

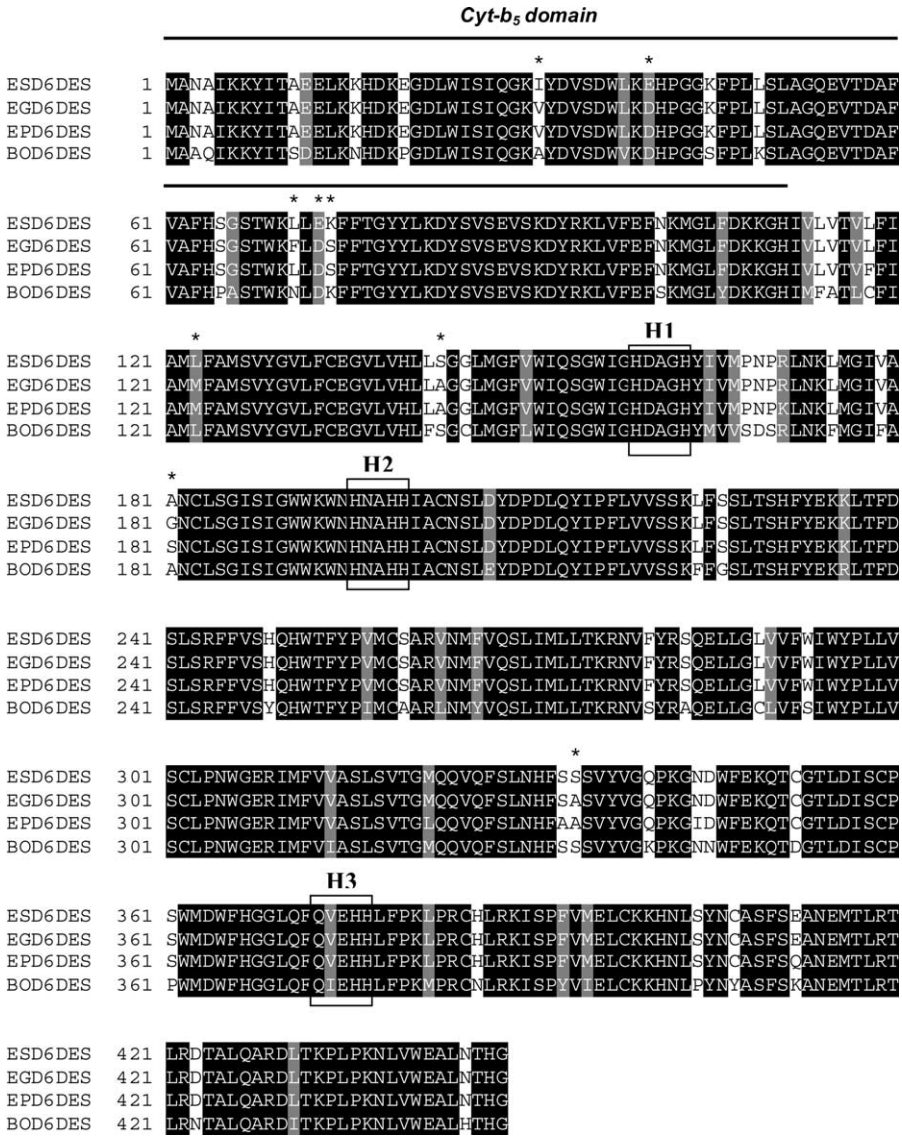

Fig. 1. Amino acid sequences of D6DES enzymes from *Boraginaceae* species. The amino acid sequences of the Δ^6-desaturases of *E. sabulicola* (ESD6DES; acc. no. DQ067612) *E. gentianoides* (EGD6DES, AY055117), *Echium pitardii* (EPD6DES; AAL23581), and *Borago officinalis* (BOD6DES; AAC49700), were aligned with ClustalX (v1.7) (Thompson et al., 1997). The ''Boxshade'' v3.21 software 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 ESD6DES are enclosed in black boxes, and similar residues in gray. Conserved histidine boxes and the cytochrome-b_5 domain are indicated. Amino acid differences among ESD6DES and EGD6DES proteins are marked by asterisks.

The whole coding sequence of *ESD6DES* and *EGD6DES* were cloned in the yeast expression vector pYES-2 under the control of a galactose inducible promoter, and further introduced in the yeast strain INVSc1. The yeast transformed with the empty vector was used in negative controls. Both fatty acid substrates of the D6DES enzymes (LA and ALA) were exogenously supplemented in the culture at equimolar amounts, though ALA was incorporated more efficiently under our experimental conditions (Table 2). Total fatty acids were extracted and analysed from the biomass of the induced cultures, and the amounts of the individual fatty acids were determined by comparison to an internal standard. The conversion rates of LA into GLA (CR_{LA}) and ALA into OTA (CR_{ALA}) were calculated (Table 1). For the D6DES of *E. sabulicola* the average conversion rates into GLA and OTA are similar (18.5 vs. 15.4, respectively) with a slight preference over the LA substrate. However, the conversion rates for the D6DES of *E. gentianoides* ($CR_{LA} = 28.9$ and $CR_{ALA} = 19.2$) indicate a clear preference in the utilization of LA over ALA. These results agree with fatty acid compositions observed in the seeds, and indicate that the higher GLA content in *E. gentianoides* is the result of both a preference in the utilization of LA by the D6DES enzyme and a higher availability of the n-6 substrate as compared to *E. sabulicola*.

Table 2
Fatty acid composition (%) and conversion rates of yeast expressing either the empty vector (pYES-2), the D6DES of *E. sabulicola* (*ESD6DES*) or the D6DES of *E. gentianoides* (*EGD6DES*)

Fatty acid	Expression construct[a]		
	pYES-2	ESD6DES	EGD6DES
16:0	20.2 (1.4)	21.3 (0.5)	21.0 (0.3)
16:1n-7	20.2 (1.4)	21.0 (1.4)	19.6 (0.4)
18:0	6.2 (0.6)	6.7 (0.2)	6.7 (0.2)
18:1n-9	11.9 (0.6)	12.0 (0.4)	11.9 (0.1)
LA	15.2 (1.1)	10.9 (0.8)	10.3 (0.4)
GLA	–	2.5 (0.2)	4.2 (0.1)
ALA	25.2 (1.6)	19.9 (1.2)	19.9 (0.2)
OTA	–	3.7 (0.2)	4.7 (0.3)
CR_{LA}	–	18.5 (1.5)	28.9 (1.3)
CR_{ALA}	–	15.4 (1.3)	19.2 (1.0)

[a] Yeast cultures were supplemented with equimolar amounts of both LA and ALA to determine the conversion rates (CR) from each substrate. The conversion rate (CR) of the desaturase substrates is defined as: CR(%) = [molar % of product/molar % of non-transformed substrate + molar % of product] × 100, under our experimental conditions. Total fatty acids were extracted and analysed as indicated in Section 4.3 after washing of cells. Results are the average of three independent experiments with standard error indicated within the brackets.

Previous data obtained for the *Borago* desaturases indicated that the determinants of the substrate specificity were scattered along the whole molecule, so that they remain elusive (Libisch et al., 2000; Sayanova et al., 2003). When the amino acid sequences of EGD6DES and ESD6DES are compared (Fig. 1) nine amino acid changes (four of them being conservative) are recorded, mainly distributed along the first 180 amino acids. Among the non-conservative changes there are three potentially informative sites corresponding to positions 74, 143 and 334. Interestingly, those amino acid replacements in ESD6DES are coincident in the protein from *B. officinalis*, a desaturase that neither shows substrate preference (Libisch et al., 2000), while those positions in EGD6DES are occupied by the same amino acid in the desaturase from *Echium pitardii*, another "GLA-rich" Macaronesian taxa. Site-directed mutagenesis of these amino acids could be used to confirm the involvement of those sites in the determination of the desaturase specificity, and eventually to increase or modify substrate discrimination.

3. Concluding remarks

Our experimental data indicate that both substrate availability and specificity of the Δ^6-desaturase enzyme are responsible for the observed bias in fatty acid compositions of *Echium* species with different geographic distributions. Moreover, comparison of the desaturase sequences suggested possible amino acid positions that could be further investigated to check their involvement on substrate selectivity. This is interesting regarding the possibility of using genetic engineering to obtain more suitable Δ^6-desaturase enzymes.

4. Experimental

4.1. Biological material

Seeds of *Echium sabulicola* Pomel were collected from plants located in their natural habitat at El Alquian (Almería, Spain). The yeast strain INVSc1 (Invitrogen) was used to assay Δ^6-desaturase activities by heterologous expression.

4.2. Cloning of the D6DES gene of Echium sabulicola

Cloning of the *D6DES* gene from *E. sabulicola* (*ESD6DES*) was achieved by PCR amplification using the following flanking primers with sequences derived from *D6DES* genes of other *Echium* species: ESDES-UP (5′-CTACATATGGCTAATGCAATCAAGAAGTACAT-TAC-3′) and ESDES-DOWN (5′-CATAGGATCCAAC-AAGTAGAACCAATGCAAGC-3′). The PCR program consisted of a denaturation step of 3 min at 94 °C, followed by 38 cycles of 15 s at 94 °C, 45 s at 55 °C and 1 min 45 s at 72 °C, ending with a 5 min step at 72 °C. The product was initially cloned into the pGEM-T-Easy® vector (Promega), and fully sequenced on both strands using a Perkin–Elmer ABI-310 DNA automated sequencer and the Big Dye v3.1 chemistry. The sequence of *ESD6DES* was deposited in the GenBank under the accession number DQ067612.

4.3. Functional assays in yeast

The whole *D6DES* coding sequences from *E. gentianoides* or *E. sabulicola* were transcriptionally fused to the GAL1 inducible promoter of the pYES2® expression vector (Stratagene), and the resulting plasmid used to transform *S. 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 two substrates, linoleic acid and α-linolenic 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 lyophilization. Simultaneous lipid extraction and generation of fatty acid methyl esters were performed as described elsewhere (Rodríguez-Ruiz et al., 1998). Fatty acid composition was determined by GC as in García-Maroto et al. (2002).

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