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BIOCHEMICAL CONTROL OF HIGH PALMITIC ACID BIOSYNTHESIS

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Summary. Two high palmitic acid sunflower (*Helianthus annuus* L.) mutants, CAS-5 and CAS-12, have been biochemically characterised. The enzymatic activities found to be responsible for the character are the β-keto-acyl-acyl carrier protein synthetase II (KASII; EC 2.3.1.41) and the acyl-acyl carrier protein thioesterase (EC 3.1.2.14). Our data suggest that the high palmitic acid phenotype observed in both mutant lines is due to the combined effect of a lower KASII activity and a higher thioesterase activity on palmitoyl-acyl carrier protein (16:0-ACP). The level of the last one appeared to be not high enough to hydrolyse the produced 16:0-ACP completely. As a consequence of this, three new fatty acids appear: palmitoleic acid (16:1 Δ9), asclepic acid (18:1 Δ11), and palmitolinoleic acid (16:2 Δ9 Δ12). These fatty acids should be synthesised from palmitoyl-ACP or a derivative by the action of the stearoyl-ACP desaturase, fatty acid synthetase II and oleoyl-PC desaturase, respectively.

Besides their food technological applications, seed fatty acid mutants are very useful to study the biochemical pathway of fatty acid biosynthesis. Seeds fatty acid biosynthesis occurs in the plastids (Garwin et al. 1980), by the action of a type II dissociable fatty acid synthetase (FAS). The sequential action of FAS III and I produces palmitoyl-acyl carrier protein (16:0-ACP), which is later elongated by the action of the FAS II complex to stearoyl-ACP. These three enzymatic FAS complexes differ only in one enzyme, the β-keto-acyl-ACP synthetase (KAS). The stearoyl-ACP would be finally desaturated to oleoyl-ACP.

The palmitic acid could be exported to the cytoplasm after hydrolysis by the action of an acyl-ACP thioesterase. The relative activity of the thioesterases over the different substrates inside the plastid (mainly palmitoyl-ACP, stearoyl-ACP and oleoyl-ACP) would determine the amount of each fatty acid that is exported to the cytoplasm and then influence the final fatty acid composition of the oilseed. Any of these two enzymatic activities, KASII or acyl-ACP thioesterase, could be responsible for or necessary to accumulate more palmitic acid than in the normal line.

High palmitic sunflower mutants CAS-5 and CAS-12 were selected after X-ray irradiation of dry seeds (Osorio et al. 1995; Fernández-Martínez et al. 1997), CAS-12 being on a high oleic background. Taking into account that both parental lines had a similar genetic background, that 2 or 3 genes are needed to control the high palmitic acid character and that these genes are the same in both mutants (Pérez-Vich et al. 1998), more than one enzymatic activity was expected to be involved in them. Consequently, we have focused our work on KASII and acyl-ACP thioesterase activities, the rate of "in vivo" fatty acid synthesis and the different fatty acids patterns in the mutants.

β-keto-acyl-ACP synthetase II. Data obtained from 10 independent triplicate KASII assays with different substrate concentrations ranging from 0.15 to 1.25 μM were used to estimate the apparent kinetic parameters K_m and V_{max} (Table 1). There are not important differences between the K_m values, the V_{max} of control lines being similar to that of one another and three times that of mutants. This provides direct evidence that at least one of the biochemical lesions in these high palmitic sunflower lines is indeed a reduction in KASII activity. The enzymatic activities implied in this phenotype should be β-keto-acyl-ACP synthetase II, as the enzyme directly related to the elongation of palmitoyl-ACP to stearoyl-ACP, and the corresponding acyl-ACP thioesterase, which liberates the palmitoyl group, this thioesterase activity should be a FatB type (Jones et al. 1995) that prefer acyl-ACP having saturated acyl

groups. Other FAS enzymes that are not directly related to the elongation to stearoyl-ACP (FASII complex) should be discarded, their activities being likely to provoke disorder in those of the FASI and FASIII enzymatic complex, lowering the total activity and bringing about an accumulation of precursors like myristoyl-ACP.

Table 1. β-keto-acyl-ACP synthetase II (KASII) apparent kinetic parameters of mutants CAS-5 and CAS-12, and control lines RHA-274 and HA-OL9.

	KASII	
	K_m (μM)	V_{max} (pmol · mg prot^{-1} · min^{-1})
RHA-274	2.72	7.05
CAS-5	2.53	1.92
HA-OL9	3.55	6.76
CAS-12	1.77	1.95

Acyl-ACP thioesterase. Another enzymatic activity possibly involved in the high palmitic mutant character is the acyl-ACP thioesterase. We have measured it using major intraplastidial acyl-ACPs (palmitoyl-ACP, stearoyl-ACP and oleoyl-ACP) as substrates and calculating the apparent Km and Vmax from data obtained thenceforth (Table 2). Like in previously studied acyl-ACP thioesterases (McKeon and Stumpf 1982; Hellyer et al. 1992), Km values were in the same order. The Km values for palmitoyl-ACP did not correlate to the mutant phenotypes. Vmax values, as in KASII determinations, show important differences. They were similar to one another in the mutants and around 6 times those from control lines. Data obtained with stearoyl-ACP and oleoyl-ACP, showed, as could be expected, a higher V_{max} with oleoyl-ACP.

The apparent K_m obtained from the "in vitro" assays of the acyl-ACP thioesterase were of the same order for the three substrates assayed in all lines, a similar result to those obtained in previous works (McKeon and Stumpf 1982; Hellyer et al. 1992). Although the highest thioesterase activity was on oleoyl-ACP in all cases, mutant lines had nevertheless a higher V_{max} for palmitoyl-ACP than controls.

All these data suggest that the new mutant character is due to a lower KASII activity in mutant seeds, which will produce an intraplastidial accumulation of palmitoyl-ACP and a more efficient hydrolysis of palmitoyl-ACP by specific thioesterases before it is exported outside the plastid. These results are in accordance with genetic studies of mutant

lines CAS-5 (Perez-Vich et al. 1998) and CAS-12 (unpublished), which showed that at lest two loci are related with the character.

Table 2. Acyl-ACP thioesterase apparent kinetic parameters of mutants CAS-5 and CAS-12, and control lines RHA-274 and HA-OL9

		K_m (μM)	V_{max} (nmoles · mg $prot^{-1}$ · min^{-1})
RHA-274	16:0-ACP	0.19	0.53
	18:0-ACP	0.39	4.49
	18:1-ACP	0.45	8.69
CAS-5	16:0-ACP	0.21	2.93
	18:0-ACP	0.61	4.86
	18:1-ACP	0.24	9.13
HA-OL9	16:0-ACP	0.14	0.64
	18:0-ACP	0.48	1.30
	18:1-ACP	1.87	13.55
CAS-12	16:0-ACP	0.21	3.95
	18:0-ACP	0.12	2.98
	18:1-ACP	0.99	17.34

Proposed biosynthetic pathway. Many mutant lines and also some lines obtained by genetic engineering have side effects on the fatty acid biosynthesis; in this case three fatty acids which were absent or detectable in minor amounts in normal sunflower oil have an increased content. These fatty acids have been identified by GC-MS as palmitoleic acid (16:1 Δ9), asclepic acid (18:1 Δ11) and palmitolinoleic acid (16:2 Δ9 Δ12). They could be used to test and support the actual hypothesis of biosynthesis of these fatty acids and enzyme specificities for chain length and double bond position. This is possible because these new fatty acids must derive from palmitic acid, which is several times more concentrated in the mutants than in normal lines, and by the action of the normal enzymes found in the seed, no new enzyme activity have been produced by the mutagenesis.

The higher intraplastidial concentration of palmitoyl-ACP allowed the stearate desaturase to transform it into palmitoleoyl-ACP, introducing (see review by Heinz 1993) a

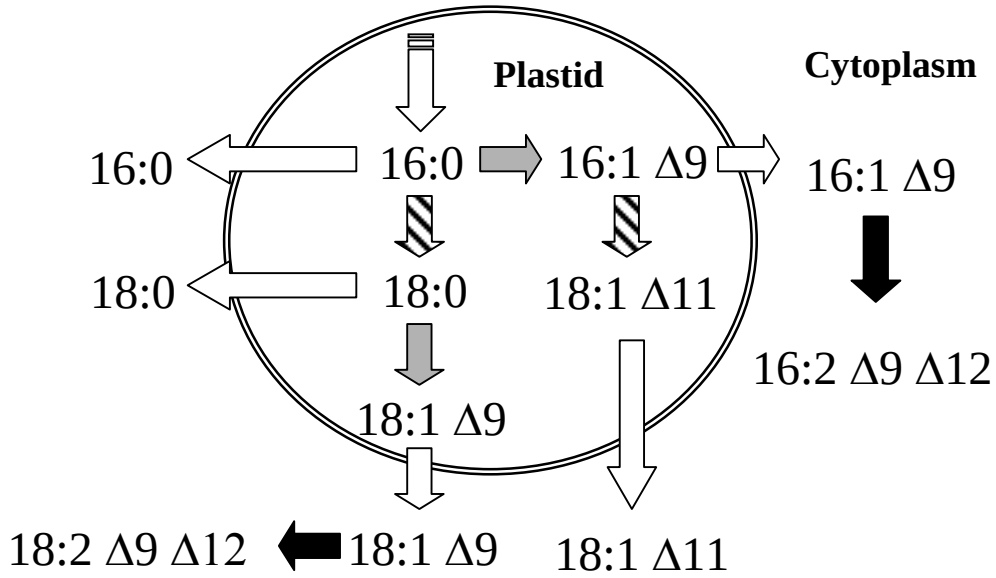
double bond in position 9 counting from the carboxyl group (Δ9). In spite of the low activity that this enzyme displays on palmitoyl-ACP (McKeon and Stumpf 1982; Gibson 1993), the mutants have ten times more palmitoleic acid than controls. This reaction is also enhanced because of an almost two times reduced intraplastidial amount of stearoyl-ACP (that is the substrate of the reaction and acts in this case as a competitive inhibitor) and for the disruption of the channelling proposed for the FAS system.

Another increased fatty acid is asclepic acid, usual in fruit pulp lipids (Shibahara et al. 1987) and found in a small amount-up to 3%-in common vegetable seed oils. In the data it is always added to the amount of oleic acid (Kleiman and Payne-Wahl 1984). Our data suggest that an elongation of palmitoleic acid (16:1 Δ9) mediated by FASII will produce the increased of asclepic acid.

Unlike oleic acid, which is actively desaturated to linoleic acid at a low temperature (Martínez-Force et al. 1998) asclepic acid is not further modified. When plants were grown at 20/10 ºC (day/night) the amount of asclepic acid was 3.7%, being 3.6% at 30/20 ºC. This proves that oleate desaturase does not recognise or is unable to introduce a double bond in asclepic acid position 12 counting from the carboxyl group (Δ12), being another one at position Δ11.

Finally, as a consequence of their higher content of palmitoleic acid outside the plastid, some palmitolenic acid (16:2 Δ9 Δ12) appears in the mutants. The presence of this fatty acid has not been described before in sunflower or in any other oilseed crop.

Figure 1. Proposed biosynthetic pathways of palmitoleic acid (16:1 Δ9), palmitolinoleic acid (16:2 Δ9 Δ12), and asclepic acid (18:1 Δ11) in sunflower mutant seeds. β-keto-acyl-ACP synthetase II, ▨; Δ9 stearate desaturase, ▭ and Δ12 oleate desaturase ■



Palmitolenic acid must be synthesised from palmitoleic acid by the action of the oleate desaturase introducing a double bond in position Δ12 (reviewed by Heinz 1993). This point is corroborated when comparing the palmitolenic acid content between both high-palmitic acid mutant mature seeds (30 DAF). While it reaches 1.5% in mutant CAS-5, it is just 0.1% in the CAS-12 line. The difference between both mutant lines is the high-oleic background of CAS-12, in other words, the lower level of oleate desaturase activity.

A proposal for the biosynthesis pathway of these new fatty acids in sunflower seeds can be found in Fig. 1.

Our data indicate that the high palmitic acid phenotype observed in lines CAS-5 and CAS-12 is due to the combined effect of a lower KASII activity and a higher thioesterase activity on 16:0-ACP. The level of the last one appeared not to be high enough to hydrolyse the 16:0-ACP completely.

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