Investigations into the regulation of lipid biosynthesis in *Brassica napus* using antisense down-regulation

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Abstract

We have generated antisense plants of *Brassica napus*, targeting either the cytoplasmic Type I acetyl-CoA carboxylase or the \( \beta \)-ketoacyl-acyl carrier protein reductase of the Type II dissociable fatty acid synthase, in order to investigate the importance of these components in regulating lipid metabolism in plants. Using a range of down-regulated plants, it has become clear that down-regulation of these genes also causes down-regulation of other components of lipid metabolism, at the activity, translational and transcriptional levels. These plants exhibit effects, not immediately predicted, on oil yield and carbon resource allocation in seeds.

Nature of the metabolic machinery

The de novo biosynthesis of fatty acids in plants involves two main enzymic components, acetyl-CoA carboxylase (ACCase), which generates malonyl-CoA from acetyl-CoA, and fatty acid synthase (FAS), which catalyses the progressive addition of carbon units to an acetyl-CoA primer, eventually giving rise to a long-chain fatty acid (Figure 1).

In plants, the plastids contain a Type II, freely dissociable, FAS [1]. This is similar in composition to the FAS from *Escherichia coli* [2] and most other bacteria. There are three different condensing enzymes [ketoacyl-acyl carrier protein (ACP) synthases (KASs)] involved in de novo fatty acid biosynthesis: KAS III catalyses the initial condensation step between acetyl-CoA and malonyl-ACP, KAS I is involved with condensation reactions up to \( C_{16:0} \) and uses acyl-ACP substrates, and KAS II elongates \( C_{16:0} \) to \( C_{18:0} \). Desaturation of fatty acids from \( C_{18:0} \) to \( C_{18:1} \) is catalysed by the soluble stearoyl-ACP desaturase, located in the plastid, and chain termination is catalysed by acyl-ACP thioesterase. Fatty acids, up to a chain length of C18, are synthesized in the plastid and subsequently exported into the cytoplasm, where they can be further elongated by a membrane-associated fatty acid elongase. For the synthesis of very-long-chain fatty acids (over 18C) there is a requirement for malonyl-CoA in both the cytoplasm and the plastid. ACCase exists in two different forms: Type I ACCase consists of a single polypeptide containing three functional domains, the biotin carboxyl carrier protein (BCCP), carboxyl transferase (CT) and biotin carboxylase (BC); Type II ACCase consists of four separate polypeptides, BCCP, BC, CT\(_\alpha\) and CT\(_\beta\) [3]. In *Brassica napus* (oil seed rape), Type I ACCase is located exclusively in the cytoplasm while Type II is located in the plastid. The regulation of lipid biosynthesis in plants has a number of different components that include provision of substrate, accumulation of products, metabolite channeling, the control of the biosynthesis and activity of enzymes of the metabolic

Figure 1

Reactions of de novo fatty acid biosynthesis in plants

DH, hydroxyacyl-ACP dehydrase; MCAT, malonyl-CoA-ACP transacylase; SD, stearoyl-ACP desaturase; TE, acyl-ACP thioesterase.

Key words: acetyl-CoA carboxylase, fatty acid synthase, \( \beta \)-ketoacyl-ACP reductase, lipid, pleiotropy.

Abbreviations used: ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; BKR, \( \beta \)-ketoacyl-ACP reductase; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CaMV, cauliflower mosaic virus; CT, carboxyl transferase; DH, hydroxacyl-ACP dehydrase; ENR, enoyl-ACP reductase; FAS, fatty acid synthase; KAS, \( \beta \)-ketoacyl-ACP synthase; nptII, neomycin phosphotransferase; WT, wild-type.

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pathway. All of these factors are liable to interact, in some way, with each other.

**Fatty acid synthesis is both a luxury and an essential metabolic function**

The biosynthesis of fatty acids can be viewed as serving both a luxury and an essential metabolic function. The biosynthesis of membranes, signalling molecules and intermediates in metabolism can all be viewed as essential functions. Luxury functions are mainly concerned with storage reserve accumulation, in that lipids do not have to be the major form of storage product. In oil seed rape, lipid reserves are stored as triacylglycerols in oil droplets. An oleosin coat that limits the size of the oil particles surrounds these droplets. The level of storage lipids can, and does, vary between plants; in oil seed rape, the major reserves are oil and protein, while in peas, the main reserves are starch and protein.

**Considerations of the approaches used to study regulation**

There are a number of approaches that can be used to study regulation of lipid metabolism in plants and different things can be learned from using different approaches. In the first instance, the importance of a gene can be established by a gene knockout. Provided that there is only one metabolic pathway operational and one gene encoding an enzyme activity, then such knockouts provide valuable information on the metabolic route. However, if knockouts are used as the basis for genetic screens, they can give rise to startling results on the importance of fatty acid biosynthesis to cellular functions. For example, the mosaic death 1 mutant, *mod1*, which results in premature death in multiple organs, has been shown to be a result of severe down-regulation of enoyl-ACP reductase (ENR) [4], and the *hic* mutants, involved in high CO₂ response associated with an increased number of stomata, are due to disruption of the β-ketoacyl-CoA synthase gene involved in the synthesis of very-long-chain fatty acids. [5]. Gene knockouts become more difficult when there is more than one gene coding for an enzyme in a metabolic pathway, as all copies may need to be knocked out to show an effect. This is the case for many of the genes in commercial crops, such as oil seed rape, where the genetic background gives rise to genes from two different sources; for example ENR from oil seed rape is coded for by four genes [6]. Problems like this can be overcome by using antisense technology that facilitates the down-regulation of an entire gene family at once. The degree of down-regulation may also be of importance, as severe or complete down-regulation may give rise to other effects. The use of metabolic inhibitors is another way to down-regulate enzyme activity, but this may be limited by pathways of entry of the inhibitors into whole tissue; this is an approach which has been used successfully in studying metabolic flux [7]. Recent advances in whole genome sequencing projects, coupled with the availability of new technologies in DNA array and proteomics, should enable investigation of the spectrum of changes caused by down-regulation of a metabolic enzyme, at both the proteome and transcriptome level. Such investigations have already been performed on *Saccharomyces cerevisiae* [8] and could be performed on higher eukaryotes.

**Generation of antisense lines to Type I ACCase and β-ketoacyl-ACP reductase (βKR)**

In order to down-regulate lipid metabolism, antisense constructs to Type I ACCase and βKR were generated using either a seed-specific ACP or 35 S CaMV (cauliflower mosaic virus) promoter. These two promoters were chosen as they gave the opportunity to observe both specific down-regulation in the seed, using the ACP promoter, and non-specific down-regulation, using the CaMV promoter. A typical construct is shown in Figure 2.

**Generation of homozygous single-insert lines**

Single-insert antisense lines were identified in the T1 generation by hybridization of a neomycin phosphotransferase (*nptII*) sequence to *XbaI*-digested DNA via Southern blotting. Each line containing a novel insertion event was screened in the T2 and T3 generations by kanamycin selection and PCR for the presence of the transgene.

**Figure 2**

*Generalized schematic of antisense sequences introduced into *B. napus* plants*

Promoters were either 35 S CaMV or ACP sequences. LB, left border sequence; RB, right border sequence.
Zygosity and stability of the inserts was confirmed by the heritability of phenotypic traits over five generations.

**Analysis of phenotype**

Several phenotypic differences from wild-type (WT) plants were observed in many antisense lines, irrespective of the gene targeted for down-regulation. The antisense $\beta$KR plants have been examined in most detail and some of the results with these plants are described below.

During vegetative development, predominantly in lines driven by the 35 S CaMV promoter, curling of the leaves was evident from emergence through to maturity. Curling appeared to be associated with localized differences in leaf expansion; microscopic examination showed disruption of the palisade and mesophyl layers. In developing leaves, microscopy indicated reduced wax deposition in antisense plants with a curly phenotype; biochemical analysis of this material showed a $> 70\%$ reduction in surface-extractable lipids. Analysis of total leaf fatty acids from the antisense lines showed reductions at all stages of development, with a $> 60\%$ reduction in fully mature leaves. This partially reflected the smaller size of leaves in the antisense plants. Complex lipid profiles showed significant differences in the abundance of lipid classes, the most marked example of which was a decrease in digalactosyldiacylglycerol and increase in monogalactosyldiacylglycerol; these differences were generally in the order of 10-20\% compared with WT plants.

Post-flowering phenotypic alterations were seen in lines with either 35 S CaMV or seed promoter-driven antisense insertions. Severely affected lines often had abnormal seeds, with poor seed fill, altered pigmentation, precocious germination, or a combination of these features.

We screened $\beta$KR and ACCase antisense lines, under the control of the ACP promoter, to identify plants with altered seed storage lipid content. In the two homozygous single-insert ACCase I lines examined, the lipid content was 71\% and 93\% respectively of the WT. Several $\beta$KR lines showed reduced lipid content, down to 42\% of WT. Some of the $\beta$KR antisense lines under the control of the 35 S CaMV promoter also showed reduced lipid in seeds, down to 54\% of WT.

Allometric analysis of seed yield and quality, in antisense $\beta$KR lines, revealed a complex picture and indicated that seed carbon resource allocation was affected in a more global fashion. Some lines had seeds of normal appearance that were reduced in number and increased in mass. Others had decreased numbers of seeds with reduced mass and a higher incidence of abnormal and precociously developed seed. The protein content of dry seed was significantly increased in lines with a severe seed phenotype and reduced seed mass, but was unaffected in lines with normal-type seed.

**Down-regulation of other FAS components brought about by antisense**

One question that we wished to address was if down-regulation of one component of lipid metabolism caused down-regulation of another component in the same pathway and, if so, at what level this was achieved (transcription, translation or post-translational). Studies have been performed that demonstrate the co-ordination of transcripts of FAS components during seed development [9]. Where there is co-ordinated control of gene expression of a metabolic pathway, then alteration of the activity of related components might be an expected outcome of down-regulation of a single component. Such co-ordinated changes occurred with antisense down-regulation of ribulose bisphosphate carboxylase-oxygenase, where down-regulation of other components of the dark reactions of photosynthesis were observed [10]. We have measured the activity of both $\beta$KR and ENR in antisense $\beta$KR and control lines, and have found that both activities are reduced to a similar degree in the antisense lines. The steady-state levels of each of these proteins and their mRNA levels were also co-ordinately reduced when measured using Western and Northern blots. These results are consistent with down-regulation of one component of FAS affecting another member in the biosynthetic pathway. It is not clear what the mechanism of sensing is, but the observation implies that a signal is generated causing a co-ordinated feedback inhibition of transcription.

**Conclusion**

Several stable, independently transformed, down-regulated lines of lipid metabolism have been generated. Antisense to the $\beta$KR component of FAS results in a lowered lipid content; more surprisingly, antisense to the Type I ACCase also results in lower lipid content. Detailed analysis of the lipid composition of the down-regulated $\beta$KR lines has demonstrated that partitioning of fatty acids to different complex lipids could be affected.
It is clear that down-regulation of a specific component of lipid metabolism not only alters seed storage lipid content, but also affects other components of that pathway. The effects go even further in potentially altering the allocation of resources within the seed and lowering the proportion of seeds reaching maturity. It is clear that there must be some cross-talk between signalling pathways within the cell; these pleiotropic effects obviously have great biological significance, as they give a clear indication that cellular events are connected. Future proteome and transcriptome analyses should help to elucidate the potential connections between these different components of metabolism and link metabolism to cell biology. These plants will form a useful resource for future metabolic flux analysis studies that are in progress.

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References

Regulation of mammalian acetyl-CoA carboxylase

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Abstract

Acetyl-CoA carboxylase (ACC) plays a critical role in the regulation of fatty acid metabolism and its two isoforms, ACCα and ACCβ, appear to have distinct functions in the control of fatty acid synthesis and fatty acid oxidation, respectively. They are regulated by similar short-term mechanisms of allosteric activation by citrate, and reversible phosphorylation and inactivation, and there is clearly interaction between these mechanisms. AMP-activated protein kinase is the important physiological ACC kinase for both isoforms and yet there is a potential physiological role for cAMP-dependent protein kinase in the hormonally mediated inactivation of ACCα, and phosphorylation of ACCβ in its unique N-terminus.

Key words: AMP-activated protein kinase, cAMP-dependent protein kinase, fatty acid oxidation, fatty acid synthesis.

Abbreviations used: ACC, acetyl-CoA carboxylase; AlCAR, 5-aminooimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CPT1, carnitine palmitoyltransferase-1; PKA, cAMP-dependent protein kinase.

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Acetyl-CoA carboxylase (ACC) isoform structure and function

ACC catalyses the carboxylation of acetyl-CoA to malonyl-CoA, a major precursor for fatty acid synthesis [1], but it is also a crucial regulator of mitochondrial fatty acid β-oxidation through its inhibition of carnitine palmitoyltransferase-1 (CPT1) [2]. The regulation of ACC, through a complex interaction of short- and long-term mechanisms, plays a key role in the control of both the synthesis and the oxidation of fatty acids.

In mammals, ACC is a multifunctional enzyme with biotin carboxyl carrier protein, biotin carboxylase and carboxyl transferase domains all contained within a single polypeptide chain [1]. A large body of evidence, including purification [3-6], immunological analysis [7] and cDNA cloning [8-11], has revealed two major isoforms of ACC with molecular masses of approx. 265 kDa (ACC1 or ACCα) and 280 kDa (ACC2 or ACCβ). The two isoforms are distinct gene products with the ACCα gene localized to human chromosome 17 [9] and the ACCβ gene localized to human...