Chloroplast Transformation with Modified accD Operon Increases Acetyl-CoA Carboxylase and Causes Extension of Leaf Longevity and Increase in Seed Yield in Tobacco

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Acetyl-CoA carboxylase (ACCase) in plastids is a key enzyme regulating the rate of de novo fatty acid biosynthesis in plants. Plastidic ACCase is composed of three nuclear-encoded subunits and one plastid-encoded accD subunit. To boost ACCase levels, we examined whether overexpression of accD elevates ACCase production. Using homologous recombination, we replaced the promoter of the accD operon in the tobacco plastid genome with a plastid rRNA-operon (rrn) promoter that directs enhanced expression in photosynthetic and non-photosynthetic organs, and successfully raised the total ACCase levels in plastids. This result suggests that the level of the accD subunit is a determinant of ACCase levels, and that enzyme levels are in part controlled post-transcriptionally at the level of subunit assembly. The resultant transformants grew normally and the fatty acid content was significantly increased in leaves, but not significantly in seeds. However, the transformants displayed extended leaf longevity and a twofold increase of seed yield over the control value, which eventually almost doubled the fatty acid production per plant of the transformants relative to control and wild-type plants. These findings offer a potential method for raising plant productivity and oil production.

Keywords: Acetyl-CoA carboxylase (EC 6.4.1.2) — Fatty acid synthesis — Leaf longevity — Post-transcriptional regulation — Seed yield — Tobacco.

Abbreviations: ACCase, acetyl-CoA carboxylase; NEP, nuclear-encoded RNA polymerase; PEP, plastid-encoded RNA polymerase.

Introduction

Plant oil is one of the most important products of photosynthetic carbon assimilation and has a variety of industrial applications. One possible way to boost oil production is to manipulate a key enzyme involved in fatty acid biosynthesis. In plants, de novo fatty acid synthesis occurs in plastids, and plastidic acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyses the first committed, rate-limiting step of fatty acid biosynthesis: the formation of malonyl-CoA. Therefore, increasing ACCase levels may result in an increase in fatty acid production. Plants have two forms of ACCase: a heteromeric ACCase in plastids that is similar to Escherichia coli-type ACCase, and a homomeric ACCase in the cytosol that is similar to animal-type ACCase (Sasaki et al. 1993, Konishi and Sasaki 1994). Because membranes are impermeable to acyl-CoAs including malonyl-CoA (Jacobson and Stumpf 1972, Banhegyi et al. 1996), and the plastidic ACCase plays an exclusive role in the biosynthesis of malonyl-CoA in plastids. A recent attempt to target Arabidopsis animal-type ACCase into the plastids of transgenic oilseed rape achieved an approximately 5% increase in the total seed-oil content (Roesler et al. 1997). However, successful manipulation of the E. coli-type plastidic ACCase is yet to be achieved.

Plastidic ACCase is composed of biotin carboxylase, the biotin carboxyl carrier protein, and the alpha and beta subunits of carboxyltransferase, the genes of which are designated accC, accB, accA, and accD, respectively (Sasaki et al. 1995, Ohlrogge and Browse 1995). The accD gene is located in the plastid genome and the other three genes occur in the nuclear genome. Neither antisense-expression nor overexpression of accC significantly affects ACCase levels, although a moderate decrease and an elevation in accC mRNA levels were achieved with these techniques, respectively (Shintani et al. 1997). We hypothesized that the expression of accD in plastids might limit the total levels of plastidic ACCase, and that the overexpression of accD might increase ACCase expression, and hence fatty acid production, in plants. Recent progress in plastid transformation by homologous recombination (Svab and Maliga 1993, Bock 2001, Maliga 2002) provided us with an opportunity to examine our hypothesis in tobacco.

In plastids, two types of RNA polymerases are involved in transcription: nuclear-encoded RNA polymerase (NEP) and plastid-encoded RNA polymerase (PEP) (Hajdukiewicz et al. 1997). In general, housekeeping genes such as accD have NEP promoter(s), whereas photosynthetic genes have PEP promoter(s). Interestingly, the rRNA operon (rrn), which is abundantly expressed in both photosynthetic and non-
Metabolic engineering of acetyl-CoA carboxylase has both PEP and NEP promoters (Vera and Sugiura 1995). In the plastid genome of tobacco, accD forms an operon with psaI, ycf4, cemA, and petA (Shinozaki et al. 1986) and these genes are polycistronically transcribed under the control of the NEP-type promoter of accD (Shinozaki et al. 1986, Nagano et al. 1991, Hajdukiewicz et al. 1997). Therefore, to overexpress accD in all tissues, we replaced the promoter of accD with that of rrn by homologous recombination, and examined effects of the modification on ACCase level and fatty acids. Our results show that the promoter replacement not only increased the expression of accD but also enhanced the level of ACCase and fatty acid content in leaves of the resultant transgenic tobacco. Furthermore, the transformants displayed significantly extended leaf longevity, and improved seed production and hence seed oil per plant.

**Results**

**Production of transgenic tobacco plants overexpressing plastidic ACCase**

To replace the promoter of accD with that of rrn by homologous recombination, the plasmid prbcl–aadA–rrn–accD was constructed, in which the accD promoter was replaced with a rrn promoter, and a chimeric gene (aadA*) (Svab and Maliga 1993) was inserted upstream of the accD promoter. aadA* is a selectable marker gene that contains a modified rrn promoter and a pshA terminator, so that only cells with aadA* inserted into the plastid genome can be selected for spectinomycin resistance (Svab and Maliga 1993). A control plasmid was constructed, with an aadA* insertion upstream of the accD promoter. These plasmids were inserted into the tobacco plastid genome by particle bombardment (Fig. 1A).
Metabolic engineering of acetyl-CoA carboxylase (Svab and Maliga 1993, Bock 2001). Because tobacco leaves have 500–10,000 copies of the plastid genome per cell (Bendich 1987), all transformants were made homoplastomic with respect to the transgenes.

Two independent T₀ transformants, in which the aadA*-rrn cassette was inserted into the desired position, were selected by polymerase chain reaction (PCR) and designated A₁ and A₂. These transformants produced no wild-type accD band (data not shown) by PCR with primers Pr₁ and Pr₂ (see Fig. 1A), verifying that they are homoplastomic. Similarly, a control T₀ transformant, in which only the aadA* was inserted into the wild-type genome, was established. All transformants grew normally under our growth conditions and their seeds germinated normally. Southern blot analysis verified that all T₁ progeny inherited the respective transgenes (Fig. 1B). Inheritance of the inserted rrn promoter in the T₂ progeny of the A₁ and A₂ transformants was also confirmed by PCR with primers Pr₃ and Pr₂ (data not shown).

Effects of accD overexpression on ACCase levels

Northern blot analysis of total cellular RNA from T₁ leaves (Fig. 2) showed that the control plants accumulated not only high levels of aadA mRNA but also significant levels of accD mRNA when compared with wild-type plants. Therefore, the insertion of aadA* upstream from accD seems to slightly enhance accD mRNA levels, because the rrn promoter of aadA may affect expression of accD. accD mRNA accumulated significantly more in A₁ and A₂ plants compared with control and wild-type plants, indicating that the replaced rrn promoter functions well. However, the mRNA levels of nuclear-encoded accC were similar in the wild-type and transformant plants, indicating that accC mRNA levels were not affected under these conditions.

Immunoblot analysis showed that the levels of the four subunits comprising plastidic ACCase in total leaf proteins increased significantly in the control, and more extensively in the A₁ and A₂ transformants, compared with the wild-type (Fig. 3). The level of plastid-encoded subunit increased with its transcript level, but the levels of nuclear-encoded subunits increased without a corresponding increase in their transcripts, which suggests involvement of post-transcriptional regulation in expression of the nuclear-encoded genes. The specific activity of ACCase in the chloroplasts of young leaves of A₁ and wild-type plants was 63 and 17 pmol min⁻¹ (mg protein⁻¹), respectively, indicating that the four increased subunits were assembled into an active enzyme in the chloroplasts.

Effects on fatty acid contents in leaves

Lipid analysis showed that the fatty acid content of A₁ and A₂ leaves increased by approximately 5–10% over that of the control (Fig. 4), and these differences are statistically significant according to a t-test (P<0.05). In A₂ leaves, the content of monogalactosyldiacylglycerol, the major chloroplast membrane lipid, increased by about 30% above that of the control.
Fig. 5  Wild-type and T1 plants after 12 weeks of culture. (A) Seeds were germinated and grown for 5 weeks on RMOP medium supplemented with 0.5 mg ml⁻¹ spectinomycin dihydrochloride (only for T1 transformants) at 25°C under a 12-h light/12-h dark regimen at a photon flux density of 40 μmol m⁻² s⁻¹ during the light period. The spectinomycin resistant seedlings were transplanted into soil in pots (700 ml plant⁻¹) and grown for a further 7 weeks in a growth chamber, as described in the Materials and Methods. Plants were fertilized with diluted (1/2,000) Hyponex (Osaka, Japan) every 2 d. Abbreviations are the same as in Fig. 1B. (B) Electron microscopy was carried out on mature green leaves (leaf maximum width 7 cm) harvested from the 12-week-old plant shown in Fig. 5A after illumination with white light for 1 h. Black and white arrows indicate starch granules and lipid droplets, respectively. Bar = 1 μm. Abbreviations are the same as in Fig. 1B.
whereas no other leaf glycerolipid including neutral lipids differed significantly between the A2 and control plants (data not shown). It is noteworthy that the levels of trienoic fatty acids, hexadecatrienoic acid (16:3) and linolenic acid (18:3), increased in A2 leaves by 32% and 14%, respectively, relative to the control, with concomitant decreases in the levels of dienoic fatty acids. Starch analysis showed that the starch content of the control and the transformants decreased compared with those of the wild type.

Other phenotypes of transgenic plants

The photosynthetic activity of the fully expanded leaves of T1 plants (11th leaves from the bottom in Fig. 5A) did not differ significantly among the A1, control, and wild-type plants (data not shown). The transgenic plants were indistinguishable from the wild-type except that A1 and A2 senesced later than the wild-type and control plants (Fig. 5A). The oldest leaves of 12-week-old wild-type and the control plants (blue arrowheads) were yellow, whereas A1 and A2 leaves of a similar age were green (red arrowheads) and turned yellow 7–10 d later. Indeed, the chlorophyll content of fully expanded leaves (8th leaves from the bottom) in A1 plants was 1.3-fold higher than that of the wild-type plants.

Transformant leaves were compared with those of the wild type by electron microscopy. Both the A1 transformant and the wild-type chloroplasts contained starch granules and opaque droplets, but the transformant appeared to contain more droplets and fewer granules than the wild-type plants (Fig. 5B). Microscopic survey showed that the starch granules became smaller and the droplets became larger in the transformants.

Fig. 6 Fatty acid and starch contents in seeds, and seed yields per plant. (A) All the seeds from a plant were pooled, and fatty acids and dry weight were measured in duplicate for aliquots of 50 seeds. The average value for fatty acid content per dry seed weight was calculated. Data obtained from 2–3 plants were averaged and presented as values relative to the control. Fatty acid and starch contents of 1,000 dry seeds from control plants were 4.6 mg and 0.4 mg, respectively. The weight of 1,000 dry seeds from the control plant was 103 mg. Abbreviations are the same as in Fig. 1B. (B) The number of seeds per plant was counted for 2–3 plants and the value relative to the control was calculated for each experiment. The average values from four independent series of experiments are shown. The seed number per plant for the control group was 1.2×104. Abbreviations are the same as in Fig. 1B.
Thus, apparent alteration of chloroplast constituents was observed.

**Effects on seeds**

The fatty acid content of the seeds (Fig. 6A) and the fatty acid composition (data not shown) were very similar in the wild-type and all transformant plants. The starch content of A1 and A2 plants was less than that of the control plants. Seed dry weight was also similar in the wild-type, A1 and A2 plants, but this value was slightly less than that of the control. However, the number of seeds per plant in the A1 and A2 transformants (Fig. 6B) increased about 2-fold over the control and wild-type plants. The number of seeds per capsule increased considerably in A1 and A2 transformants compared with the wild-type and control plants, although the numbers of flowers and capsules were similar among all plants. Ultimately, the increase in seed yield doubled the fatty acid production per plant.

**Discussion**

**Promoter replacement enhanced accD expression and ACCase levels**

In this work, we found that replacement of the accD promoter with the rrm promoter in the tobacco plastid genome increased the levels of accD transcripts as well as accD protein, in plastids. Therefore, the expression of accD is, at least partly, regulated at the level of transcription. The overexpression of accD increased the three nuclear-encoded subunits of ACCase and increased the activity of ACCase in plastids. This finding supports our hypothesis that the expression of accD in plastids limits total levels of plastidic ACCase, and implies that accD protein level is a determinant for enzyme level. In this way, we succeeded in raising heteromeric ACCase levels.

**Post-transcriptional regulation and the apparent coordination of protein synthesis of nuclei and plastids**

Our finding that the levels of nuclear-encoded subunits increased without a corresponding increase in their transcripts suggests that post-transcriptional regulation is involved in ACCase synthesis, probably at the level of subunit assembly. We infer that a molar excess of nuclear-encoded ACCase subunits is synthesized in the cytoplasm and enters into the plastids, to be assembled with the plastid-encoded ACCase subunit. Conversely, unassembled nuclear-encoded ACCase subunits are probably rapidly degraded for lack of partner accD subunit in plastids. Therefore, only assembled subunits are detected by immunoblotting, and an apparent coordination of nuclear-encoded and plastid-encoded proteins is observed. Most proteins encoded by the plastid genome assemble with the nuclear-encoded proteins to form a functional complex, which was thought to require the coordinated synthesis of nuclear-encoded and plastid-encoded proteins. However, our results suggest that such coordinated synthesis is not always necessary, at least in the case of ACCase. By overexpressing the plastid-encoded subunit, we showed that post-transcriptional regulation is involved in the formation of a functional complex in plastids.

Relative amounts of mRNAs for four ACCase are known to co-ordinately change during leaf and seed development (Ke et al. 2000). This result suggests that the four transcript levels are regulated at least in part by coordination between nucleus and plastids during development. However, our results that overexpression of accD did not affect the levels of accC mRNA suggests that no close coordination occurs between the nucleus and plastids in the regulation of the transcript levels. Taken together, we propose that although the rates of synthesis of nuclear- and plastid-encoded subunits are regulated at the levels of transcripts in the cytoplasm and plastids, respectively, the level of each ACCase subunit is ultimately regulated at the level of protein assembly in plastids.

**Effects on leaves**

An increase of ACCase in transgenic tobacco chloroplasts resulted in an increase of total fatty acid content in leaves, and extended leaf longevity. Lipid analysis showed that the major chloroplast membrane lipid, monogalactosyldiacylglycerol, increased and the trienoic fatty acids also increased. These changes probably affect membrane properties, which might affect leaf longevity, although the kinds of membrane properties that influence leaf longevity have not been identified. It is also possible that membrane repair might be enhanced in our transgenic tobacco plants, which might delay leaf senescence, because older leaves approaching senescence are believed to require increased repair activity in the thylakoid membranes (Hellgren and Sandellius 2001).

Alternatively, decreased starch content in A1 and A2 might in turn delay leaf senescence (Miller et al. 1997). Furthermore, we do not exclude the possibility that altered fatty acid composition might play a role in delaying leaf senescence, because fatty acids are important regulators of plant growth and differentiation (Ohlrogge and Browse 1995, Topfer et al. 1995). Our transgenic tobacco plants may provide a useful experimental system for studying the mechanism of senescence in relation to lipid and carbohydrate metabolism.

**Effects on seeds**

We did not observe significant changes in fatty acid composition or content per seed. As described in the legend to Fig. 4 and Fig. 6, the ratio of fatty acid content to starch content is more than 11 in seeds, whereas it is 0.02 in leaves. This suggests that the biosynthesis of fatty acids is more active in developing seed than in leaves in tobacco. Indeed, oil accounts for about 30% of seed dry weight. We are not successful in determining the level of ACCase in developing seeds because the seeds did not synchronously mature in the seed capsule. However, if the biosynthesis of fatty acids in seeds is fully activated in the wild type, further enhancement of the biosynthesis per seed may be difficult by the present method.
To our surprise, a remarkable increase in seed number was observed in transgenic plants. It is reasonable to ascribe the increased seed number per plant to increased leaf longevity, because extended longevity brings about abundant production of photoassimilates. Although further experiments are required to elucidate the mechanisms underlying this phenomenon, the outcome has potential utility in crop improvement. Improvements in seed yield should circumvent the limitation of fatty acid production per seed and provide a basis for developing a new strategy for improving commercially important oil-producing crops, such as oilseed rape and soybean. Enhanced production of seed oil should accelerate our efforts towards engineering plants as factories for industrial materials (Somerville and Bonetta 2001).

The replacement of the accD promoter described here enhanced the primary transcripts of the accD operon that contains accD, psaI, ycf4, cemA, and petA. We did not survey the gene products for psaI, ycf4, cemA, and petA, and do not exclude the possibility that some of these gene products except for accD protein might have positive effects on the prolonged leaf longevity and the increased seed yield.

Promoter engineering

We succeeded in engineering the promoter of the plastid genome, using the rrr promoter that contains the NEP and PEP promoters. There are various possible promoters to direct the expression of a target gene in a specific organ at an appropriate time. Studies of promoter dissection may provide more powerful tools for plastid gene expression. The technique of plastid promoter engineering presented here offers a new method to improve oil production and increase plant yields.

Materials and Methods

Construction of plasmid DNA

PCR was performed with tobacco (cv. Xanthi) chloroplast DNA (Shinozaki et al. 1986) as template. The primers P1–P6, with added restriction sites, were used for PCR and are shown in Fig. 1: P1, 5'-CCCGGCGCCGCGGGGCTCAGATGGAAATAAG-3'; P2, 5'-GTC- GACGTGCTCTACTTGATTTTC-3'; P3, 5'-GTGGCGTCTGCGTGTCC-3'; P4, 5'-CCCGGCGCCGCGGGGCTCAGATGGAAATAAG-3'. The replacement of the accD promoter described here enhances the primary transcripts of the accD operon that contains accD, psaI, ycf4, cemA, and petA. We did not survey the gene products for psaI, ycf4, cemA, and petA, and do not exclude the possibility that some of these gene products except for accD protein might have positive effects on the prolonged leaf longevity and the increased seed yield.

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