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# Antisense Expression and Overexpression of Biotin Carboxylase in Tobacco Leaves[1]

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**The plastid acetyl-coenzyme A carboxylase (ACCase) catalyzes the first committed step of fatty acid synthesis and in most plants is present as a heteromeric complex of at least four different protein subunits: the biotin carboxylase (BC), the biotin carboxyl carrier protein, and the α and β subunits of the carboxyltransferase. To gain insight into the subunit organization of this heteromeric enzyme complex and to further evaluate the role of ACCase in regulating fatty acid synthesis, BC expression was altered in transgenic plants. Tobacco (*Nicotiana tabacum*) was transformed with antisense-expression and overexpression tobacco BC constructs, which resulted in the generation of plants with BC levels ranging from 20 to 500% of wild-type levels. Tobacco plants containing elevated or moderate decreases in leaf BC were phenotypically indistinguishable from wild-type plants. However, plants with less than 25% of wild-type BC levels showed severely retarded growth when grown under low-light conditions and a 26% lower leaf fatty acid content than wild-type plants. A comparison of leaf BC and biotin carboxyl carrier protein levels in plants with elevated and decreased BC expression revealed that these two subunits of the plastid ACCase are not maintained in a strict stoichiometric ratio.**

Although much is known about the biochemistry involved in the synthesis of fatty acids, comparatively little is understood about the regulation of this pathway. Recently, several in vivo studies have implicated the plastid-localized ACCase as a primary regulatory enzyme in fatty acid synthesis. Light activation of fatty acid synthesis in chloroplasts is controlled through the ACCase reaction (Post-Beittenmiller et al., 1991, 1992), and this enzyme was also identified as a regulatory step in the feedback inhibition of fatty acid synthesis in tobacco (*Nicotiana tabacum*) suspension cultures (Shintani and Ohlrogge, 1995). Furthermore, Page et al. (1994) demonstrated that the plastid ACCase of maize (*Zea mays* L.) and barley (*Hordeum vulgare*) leaves exerts a high degree of flux control over fatty acid synthesis. These findings extend the observations from animal, fungal, and bacterial systems that implicate ACCase as the major rate-determining enzyme involved in the regulation of fatty acid synthesis (Kamiryo and Numa, 1973; Goodridge, 1985; Magnuson et al., 1993).

Because of its role in regulating plant fatty acid synthesis, the plastid ACCase has become the subject of intense interest. This has led to the discovery that in dicot and nongramineous monocot plants, the plastid ACCase exists as a heteromeric enzyme complex composed of four independent polypeptides corresponding to BCCP, BC, and the α and β subunits of CT (αCT and βCT) (Alban et al., 1994; Sasaki et al.,1994; Choi et al., 1995; Shorrosh et al., 1995, 1996). Recently, the genes or cDNA coding for all of the known subunits of ACCase have been cloned from plants (Sasaki et al.,1994; Choi et al., 1995; Shorrosh et al., 1995, 1996). These achievements have now made it possible to further examine the structure of ACCase and its regulatory role in plant fatty acid synthesis.

BC catalyzes the first half-reaction required for the carboxylation of acetyl-CoA to malonyl-CoA. During protein purification this subunit co-purifies with BCCP, suggesting a close association between BC and BCCP in vivo. We have previously reported the cloning of a tobacco cDNA clone corresponding to the BC subunit of the plastid ACCase (Shorrosh et al., 1995). In the present study the BC cDNA was expressed in the sense and antisense orientation behind a strong constitutive promoter in transgenic tobacco plants. The goals of this work were to determine if the expression of other subunits of the plastid ACCase would be influenced by altering levels of BC and to study the effect of the altered BC level on the production of fatty acids.

## MATERIALS AND METHODS

### Biotin Carboxylase Plant Expression Constructs and Transformation of Tobacco

The tobacco (*Nicotiana tabacum* var SR71) BC cDNA clone (Shorrosh et al., 1995) was subcloned in the sense and antisense orientation as a *Pst*I fragment in place of the *adh*I intron I *Pst*I fragment in the plant gene expression vector p1079. The p1079 vector contained the promoter for the 35S transcript of the CaMV that was engineered to contain two copies of the CaMV 35S enhancer element and the nopaline

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Abbreviations: ACCase, acetyl-CoA carboxylase; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CaMV, cauliflower mosaic virus; CT, carboxyltransferase; FAMEs, fatty acid methyl esters.

synthase 3′ terminator sequences. The double CaMV 35S promoter of p1079 was constructed by subcloning the *Hin*cII *Eco*RV fragment corresponding to the CaMV 35S enhancer region of the CaMV 35S promoter into the *Hin*cII site of the CaMV 35S promoter. The BC sense and antisense expression constructs were subcloned as *Xba*I fragments into the *Xba*I site of pBIN19 (Bevin, 1984). The resulting overexpression and antisense expression/*Agrobacterium tumefaciens* binary vector constructs were designated as p0719S and p0719A, respectively. These constructs were introduced into the *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) by direct transformation, as described by An et al. (1988). Tobacco leaf discs were transformed as described by Rogers et al. (1986).

### Affinity Purification of Biotin Carboxylase Antibodies

Antibodies previously prepared to the C-terminal sequence of castor bean BC (Roesler et al., 1995) were affinity-purified using immobilized tobacco BC. The coding sequence of mature BC protein of the tobacco BC cDNA clone, described by Shorrosh et al. (1995), was amplified by PCR and subcloned into pET15b (Novagen, Madison, WI). The BC cDNA was expressed in *Escherichia coli*, and BC protein was purified based on the His tag and a nickel affinity column.

Approximately 4 mg of the purified *E. coli*-expressed BC protein in 100 mM Mes, pH 5.8, was reacted with 1 mL of Affigel-10 (Bio-Rad) for 30 min at 4°C on a rocking platform, after which the gel matrix was collected by centrifugation at 10,000*g* for 10 min. The supernatant was then reacted a second time with 1 mL of Affigel-15 (Bio-Rad) overnight at 4°C on a rocking platform. The reacted Affigel-10 and Affigel-15 were then combined and washed sequentially with 10 column volumes of 10 mM Tris, pH 7.5, 10 column volumes of 100 mM citrate, pH 2.5, 10 column volumes of 10 mM Tris, pH 8.8, and 10 column volumes of TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.02% sodium azide).

Five milliliters of the rabbit antiserum against castor bean BC (Roesler et al., 1995) was adjusted to 50% ammonium sulfate saturation. After centrifugation, the pellet was resuspended in 2.5 mL of TBS (10 mM Tris, pH 8.0, 150 mM NaCl, 0.02% sodium azide), desalted on a PD-10 desalting column (Pharmacia) equilibrated with TBS, and then bound to the BC affinity column overnight at 4°C on a rocking platform. The affinity column was washed with 10 column volumes of TBST and the antibody was eluted with 10 column volumes of 100 mM citrate, pH 2.5. The pH of the eluted fractions was neutralized by adding 1 M Tris, pH 8.8.

### Immunoblot Analysis of Tobacco Leaf Extracts

Tobacco leaves were immediately frozen in liquid nitrogen after being harvested. Approximately 1 g of leaf material was ground to a fine powder in liquid nitrogen and homogenized in 3 mL of 50 mM Hepes-KOH, 5 mM DTT, 1 mM PMSF, and 1 mM benzamidine using a polytron homogenizer (Brinkmann) at the highest setting. SDS was added to the homogenate to a final concentration of 1% and the mixture was incubated in a boiling water bath for 5 min. The homogenate was clarified by centrifuging at 10,000*g* for 20 min at 15°C. The protein concentration of each leaf extract was determined as described by Bradford (1976). One hundred-microgram protein aliquots of each leaf extract were fractionated on 10% SDS-PAGE gels as described by Laemmli (1970), after which proteins were transferred to nitrocellulose filters as described by Post-Beittenmiller et al. (1989). Filters were probed with either a 1:250 dilution of the affinity-purified BC polyclonal antibodies or a 1:2000 dilution of the biotin antibodies (Sigma), as described by Roesler et al. (1995). Bands on the western blots were quantitated by densitometry. At least three independent western blots were performed on extracts isolated from different individual leaves from each of the transformants. Although the total BC level varied slightly between different leaves, in each case the levels of BC relative to the wild-type controls were indistinguishable between the replicate blots.

### Fatty Acid Analysis

Approximately 50 mg of fresh leaf tissue was weighed and then homogenized in 1 mL of 50 mM Hepes, 5 mM DTT, 1 mM PMSF, and 1 mM benzamidine plus 25 μg of heptadecanoic acid. Total lipid was extracted from the homogenate as described by Bligh and Dyer (1959). The extracted lipids were concentrated under nitrogen and FAMEs were prepared at 90°C for 30 min in 1 mL of 10% boron trichloride/methanol (Sigma). The methylation reaction was stopped by adding 1 mL of water and FAMEs were extracted two times with 2 mL of hexane. Leaf FAMEs were analyzed by GC with a Hewlett-Packard 5890 using a column (30 m × 0.25 mm i.d., DB 23, J&W Scientific, Rancho Cordova, CA) with an oven temperature programmed from 180°C (10-min hold) to 230°C at 3°C/min with a column head pressure of 200 kPa of helium.

## RESULTS AND DISCUSSION

### Overexpression and Antisense Expression of Biotin Carboxylase Resulted in the Generation of Transgenic Tobacco with a Wide Range of Leaf Biotin Carboxylase Expression Levels

The full-length tobacco BC cDNA (Shorrosh et al., 1995) was subcloned in the sense and antisense orientation behind the CaMV 35S promoter. After *A. tumefaciens*-mediated transformation of tobacco leaf discs, 24 independent kanamycin-resistant plants were recovered from each construct.

The levels of BC protein expression in the leaves of the sense and antisense transformants were determined by immunoblots probed with affinity-purified antibodies to BC. A broad range of BC expression levels was observed in the leaves of both the sense and antisense transformants (Fig. 1, A and B). Leaf BC protein levels were significantly greater than the wild-type levels in several of the plants transformed with the BC overexpression construct. Based on scanning densitometry, these transformants contained

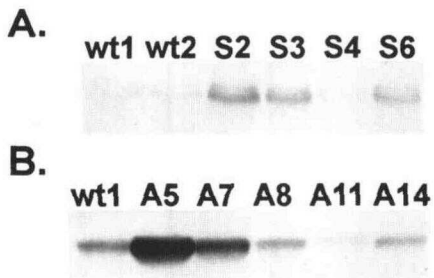


**Figure 1.** BC levels in transgenic tobacco leaves. One-hundred-microgram aliquots of leaf protein extract, isolated from plants transformed with the p0719S overexpression construct (A) and the p0719A antisense construct (B), were fractionated on 10% SDS-PAGE gels and blotted to nitrocellulose. BC was detected using affinity-purified antibodies to the tobacco BC.

BC levels that ranged from 1.5- to 2.5-fold greater than the wild-type levels (Fig. 1A, S2 and S6). Northern analysis of RNA isolated from the leaves of plants transformed with the overexpression construct indicated that BC mRNA levels also increased in correlation with the observed increase in the BC protein (data not shown). Twenty-four independent BC overexpression transformants were analyzed and none were found to have more than a 2.5-fold increase in leaf BC levels.

Decreased BC protein levels were observed in approximately 30% of the plants transformed with the antisense construct. Of these plants, most showed decreases in leaf BC levels that ranged from 25 to 40% of the wild-type levels (Fig. 1B, A8 and A14). Of the 24 independent antisense transformants, one plant (A11) was severely deficient in leaf BC, with an 80% decrease in leaf BC protein relative to wild-type plants (Fig. 1B). Northern analysis of RNA isolated from the leaves of plants containing decreased levels of BC protein indicated that the BC mRNA levels were almost undetectable, whereas a large accumulation of antisense RNA was observed (data not shown).

Whereas the expected increases and decreases in the leaf BC levels were observed in the majority of the plants transformed with the overexpression and antisense expression constructs, respectively, a small proportion of the transformants gave unexpected patterns of BC expression. For example, of the 24 plants transformed with the overexpression construct, one plant (Fig. 1A, S4) contained decreased levels of leaf BC protein. The decrease in BC expression in these plants transformed with an overexpression construct was most likely the result of transgene inactivation (Finnegan and McElroy, 1994, and refs. therein).

More surprisingly, two of the plants transformed with the antisense BC construct contained elevated levels of leaf BC. The A5 and A7 (Fig. 1B) transformants were found to contain BC levels that were 5- and 2-fold, respectively, higher than those measured in the wild-type leaves. Furthermore, the BC level observed in the leaves of the A5 plants surpassed that observed in the highest-expressing plants transformed with the BC overexpression construct (Fig 1A, S2 and S6). BC overexpression was also observed at a low frequency in tobacco suspension cells independently transformed with the p1079A antisense construct (data not shown). At present, we do not have an explanation for the overexpression of BC occurring from plants transformed with an antisense construct. However, because rearrangements of the T-DNA have been reported to occur upon integration during *A. tumefaciens*-mediated transformation, one possible explanation could involve the rearrangement of the antisense construct. Alternatively, integration of the antisense T-DNA could have occurred in such a way that the transgene was positioned in the sense orientation behind a strong endogenous promoter.

### Altered Biotin Carboxylase Levels Do Not Affect Expression of the BCCP Subunit of ACCase

Previous studies of chloroplast multisubunit complexes have indicated that the expression of individual subunits is coordinately regulated in an interdependent manner. For example, the expression of the Rubisco small subunit was found to be dependent on the levels of the Rubisco large subunit such that the uncomplexed subunits were rapidly degraded (Schmidt and Miskind, 1983). Furthermore, antisense expression of the small subunit in tobacco resulted in the coordinate decrease of the large subunit (Rodermel et al., 1988). It has also been reported that in chlorophyll biosynthetic mutants, light-harvesting chlorophyll *a/b* proteins are rapidly degraded because of their inability to assemble the correct pigment-protein complex (Plumley and Schmidt, 1995).

The stoichiometric ratios of Rubisco and light-harvesting complex subunits have been reported to be maintained by posttranslational mechanisms in cases in which excess subunits are proteolytically degraded. To determine if the subunits of the plastid ACCase are coordinately regulated in a similar fashion, BCCP levels were estimated in the leaves from wild-type plants and the two transgenic plants containing the highest and lowest levels of leaf BC (A5 and A11). As shown in Figure 2, the BCCP levels were approximately equivalent in the A5 and A11 leaf extracts, but were slightly higher in the wild-type leaves. Although it is not clear why the wild-type plants expressed BCCP levels at slightly higher levels than either of the transgenic lines, these results indicated that BCCP levels remained largely unaltered by either large increases or large decreases in BC levels. Similar results were observed when the same blots

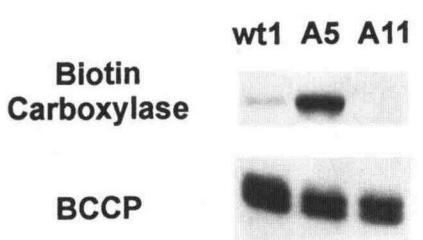


**Figure 2.** Comparison of leaf BCCP and BC levels in transgenic tobacco leaves containing altered levels of BC. One-hundred-microgram aliquots of leaf protein extract, isolated from wild-type, A5, and A11 plants, were run in duplicate on 10% SDS-PAGE gels and blotted to nitrocellulose. BC and BCCP were detected on separate blots using affinity-purified antibodies to the tobacco BC and antibodies to biotin.

were probed with antibodies specific to αCT (data not shown). Therefore, we conclude that BC levels are not coordinately maintained in a strict stoichiometric ratio with BCCP or the αCT subunits of the plastid ACCase. The presence of excess BC subunits in overexpressing plants without a coordinate increase in BCCP and/or αCT levels indicates that the plastid is able to tolerate uncomplexed ACCase subunits and, unlike Rubisco and the light-harvesting complex, excess subunits are not degraded.

### Plants Containing Very Low Levels of Biotin Carboxylase Exhibit a Stunted Phenotype When Grown under Low-Light Conditions

Plants exhibiting increased or moderately decreased levels of leaf BC were phenotypically indistinguishable from wild-type plants when grown under a variety of conditions. In contrast, the growth and development of the A11 transformant, which contained very low levels of leaf BC, was severely retarded when grown in growth chambers with relatively low light intensities (approximately 45 $\mu E/m^2$) (Fig. 3). In addition to the stunted growth, the leaves of the A11 plant were slightly chlorotic and less succulent than the leaves of wild-type plants grown under the same conditions. However, when the A11 plant was transferred to higher-light conditions (i.e. >250 $\mu E/m^2$), it grew vigorously and caught up developmentally with the wild-type plants.

Although it is not certain why the A11 plants were stunted only when grown under low-light conditions, there are at least two possible explanations. First, the demand for fatty acid synthesis per chloroplast may be higher in low-light plants to meet the demand for the formation of additional light-harvesting membranes needed during low-light conditions. Our fatty acid analysis indicated that under low-light conditions, the leaf fatty acid content of the wild-type plants was higher than that of plants grown under high-light conditions (Fig. 4, A and B). This observation suggests that wild-type plants respond to lower-light conditions by increasing their leaf fatty acid content, perhaps to increase the amount of the light-harvesting apparatus. In support of this hypothesis, it is known that shaded leaves compensate for low-light conditions by increasing the synthesis of light-harvesting antennae complexes.

A second possible explanation for the stunting of A11 plants under low-light growth conditions could relate to the light activation of ACCase. The activity of the plastid ACCase is increased severalfold upon the illumination of leaves or isolated chloroplasts (Post-Beittenmiller et al., 1991, 1992). Therefore, it is possible that, although A11 plants were deficient in ACCase activity under low-light conditions, under higher-light conditions the ACCase was activated such that the lower concentration of the enzyme was sufficient to maintain wild-type growth rates.

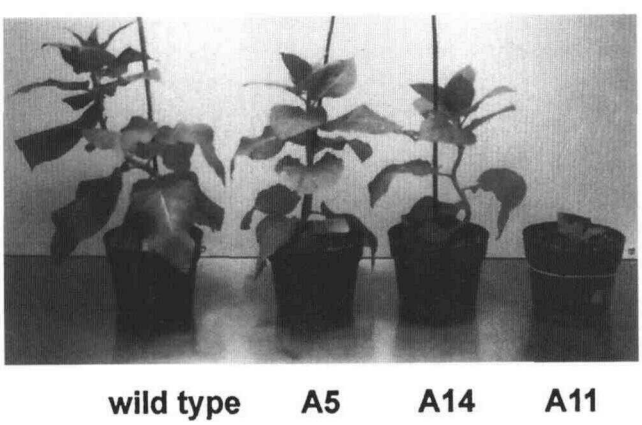


**Figure 3.** Growth of wild-type plants and plants expressing increased and decreased levels of BC under low-light conditions. A wild-type plant and primary p0719A and p0719S transformants were grown in a growth chamber at 25°C under constant illumination of 45 $\mu E/m^2$.

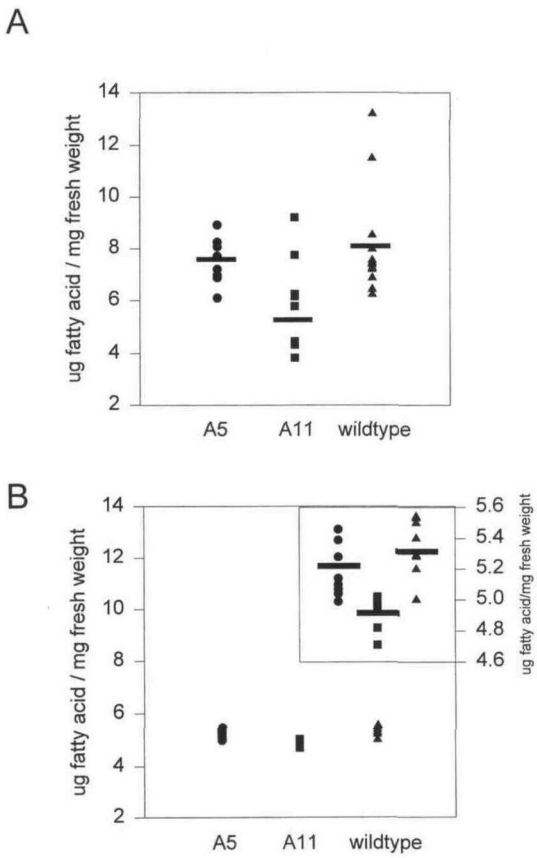


**Figure 4.** Leaf fatty acid content of wild-type, A5, and A11 plants grown under low-light (A) and high-light (B) conditions. Approximately 50 μg of tissue was taken from the leaves of plants grown under illumination of 45 and 300 $\mu E/m^2$. Total lipid was extracted from leaves with 20 or 25 μg of heptadecanoic acid added as an internal standard. The total lipid was then transesterified to FAMEs using $BCl_3$ in methanol. FAMEs were then analyzed by GC. A, Leaf fatty acid content of plants grown under constant illumination of 45 $\mu E/m^2$. B, Leaf fatty acid content of plants grown under constant illumination of 300 $\mu E/m^2$. The inset in B shows the same data expanded on a different scale. Horizontal bars represent the mean value of the combined samples from each plant. A5, A11, and wild-type plants are represented by circles, squares, and triangles, respectively.

The low-light-associated stunted growth observed in A11 plants appeared to be a heritable dominant trait that was correlated with the antisense BC transgene. Southern analysis and segregation patterns of kanamycin resistance revealed that the A11 transformant contained a single transgene locus (data not shown). The stunted phenotype was inherited by both the kanamycin-resistant progeny of the self-crossed primary transformant and the kanamycin-resistant progeny of homozygous A11 $T_2$ plants crossed with the wild-type plants. Furthermore, the degree of stunting correlated directly with the number of antisense gene copies present, so that the stunted phenotype was more severe in the homozygous A11 $T_2$ plants.

Stunted growth was also observed in the progeny of another independent BC antisense transformant, confirming that this phenotype was due to the antisense expression of the BC gene. A portion of the kanamycin-resistant progeny of the self-crossed A14 primary transformant showed severe stunting when grown in low light (data not shown). Thus, whereas the decrease in the BC content we observed in the A14 primary transformant (Fig. 1B) was not severe enough to alter plant growth, the co-segregation of multiple copies of the BC antisense transgene in a population of A14 $T_2$ plants was sufficient to decrease BC levels to a level that resulted in stunting. Western analysis of leaf extracts from these plants revealed that the dwarfed A14 $T_2$ plants contained decreased levels of BC per milligram of protein compared with levels in the nonstunted A14 segregants. These observations strongly support the conclusion that severe decreases in BC levels can prevent plants from supplying sufficient levels of fatty acids to maintain normal growth.

### A11 Plants Grown under Both High- and Low-Light Conditions Have Decreased Leaf Fatty Acid Content

Because of the instability of the ACCase and fatty acid synthase complexes, it was difficult to accurately assess rates of fatty acid synthesis and ACCase activity in isolated tobacco chloroplast or leaf extracts. However, an indirect determination of the impact of changes in the BC levels on leaf fatty acid production could be made from analysis of the leaf fatty acid content. Fatty acid analysis was performed on multiple leaf samples from plants with increased, decreased, and wild-type levels of BC.

A11 leaves synthesized less fatty acid on a fresh weight basis than the leaves of plants expressing wild-type or elevated levels of BC. The decrease in leaf fatty acid content was observed in plants grown under both high- and low-light conditions. The stunted A11 plants grown under low-light conditions had a 26% decrease in leaf fatty acid content compared with wild-type plants grown under the same conditions (Fig. 4A). Although the A11 plants grown under higher-light conditions were phenotypically indistinguishable from the wild-type plants, a modest but statistically significant 8% decrease in the fatty acid content was measured in the leaves of these plants (Fig. 4B). Although the fatty acid content varied in different leaves sampled from the same plant according to the Wilcoxon-Mann-Whitney statistical test, the leaf fatty acid content of A11 plants grown under both light conditions was significantly different from that of wild-type plants grown under the same conditions (confidence level $> 99\%$). This statistical test also indicated that the leaf fatty acid content of A5, the transgenic plant showing the highest levels of BC, was not significantly different from that of wild-type plants grown under either condition. Additionally, the leaf fatty acid content of plants showing only modest decreases in leaf BC levels was not significantly different from that of the wild-type plants.

### The Biotin Carboxylase Subunit of ACCase May Be Present in Excess in Wild-Type Tobacco Leaves

The low frequency of antisense transformants containing BC levels lower than 40% of the wild-type levels, together with the observation that no transformants were found containing BC levels lower than 20% of the wild-type levels, is consistent with the hypothesis that very low levels of BC are detrimental to the plant, and that BC must be present at a critical minimal level to recover viable transformants. Furthermore, the observation that BC levels could be decreased 40% below wild-type levels without an effect on the growth rate, fatty acid content, or levels of BCCP suggests that BC may be present in excess in the wild-type plants. It was only after BC levels were decreased by 80% in A11 plants that changes in fatty acid content and growth rate were observed. The lack of a phenotype in response to moderate antisense suppression is consistent with results of several other antisense experiments. For example, in studies in which antisense approaches were used to decrease the level of pyrophosphate: Fru-6-P phosphotransferase, Fru-1,6-bisphosphatase, and citrate synthase, a greater than 80% decrease in enzyme levels was needed before changes in metabolism or phenotype were detected (Hajirezaei et al., 1994; Kossmann et al., 1994; Landschutze et al., 1995).

Two interpretations of the lack of phenotype in plants showing moderate reductions in BC levels seem possible. First, as reviewed by Stitt and Sonnewald (1995), regulatory enzymes usually operate well below their maximum activity, thereby allowing these enzymes to respond rapidly to changes in metabolism. In this case, cells may respond to the decrease in BC by increasing the activation of the remaining complete ACCase complex. Alternatively, the BC half-reaction may not be rate-determining for the overall ACCase reaction. If this is the case, a decrease in the level of the CT subunits may have a larger influence on the flux through this regulatory point in plant fatty acid synthesis.

## CONCLUSIONS

Two new insights into the structure and regulation of the plastid ACCase have been revealed through these studies. First, although BCCP and BC function together and copurify, it appears that the levels of BC are not maintained in a strict stoichiometric ratio with those of BCCP, $\alpha$CT, and, presumably, other subunits of the plastid ACCase. As observed in plants expressing high levels of BC, the tobacco

plastids were evidently able to tolerate the accumulation of uncomplexed subunits of ACCase. These results indicate that the formation and maintenance of the plastid ACCase complex differ from the formation and maintenance of other plastid-localized protein complexes such as Rubisco and the light-harvesting complex.

Second, transgenic tobacco plants were able to tolerate at least 50% decreases in BC levels without growth or leaf fatty acid content being affected. These results suggest that the BC subunit of ACCase may be present in excess in wild-type leaves. However, under low-light conditions, severe retardation of growth was observed in the A11 plant and its progeny, which expressed very low levels of leaf BC. Analysis of the leaf fatty acid content of the A11 transformant suggested that the stunted phenotype was associated with the inability of these plants to maintain sufficient rates of fatty acid synthesis necessary for normal development.

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