

Engineering Cytoplasmic Male Sterility via the Chloroplast Genome by Expression of β -Ketothiolase¹

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While investigating expression of the polyhydroxybutyrate pathway in transgenic chloroplasts, we addressed the specific role of β -ketothiolase. Therefore, we expressed the *phaA* gene via the chloroplast genome. Prior attempts to express the *phaA* gene in transgenic plants were unsuccessful. We studied the effect of light regulation of the *phaA* gene using the *psbA* promoter and 5' untranslated region, and evaluated expression under different photoperiods. Stable transgene integration into the chloroplast genome and homoplasmy were confirmed by Southern analysis. The *phaA* gene was efficiently transcribed in all tissue types examined, including leaves, flowers, and anthers. Coomassie-stained gel and western blots confirmed hyperexpression of β -ketothiolase in leaves and anthers, with proportionately high levels of enzyme activity. The transgenic lines were normal except for the male-sterile phenotype, lacking pollen. Scanning electron microscopy revealed a collapsed morphology of the pollen grains. Floral developmental studies revealed that transgenic lines showed an accelerated pattern of anther development, affecting their maturation, and resulted in aberrant tissue patterns. Abnormal thickening of the outer wall, enlarged endothecium, and vacuolation affected pollen grains and resulted in the irregular shape or collapsed phenotype. Reversibility of the male-sterile phenotype was observed under continuous illumination, resulting in viable pollen and copious amount of seeds. This study results in the first engineered cytoplasmic male-sterility system in plants, offers a new tool for transgene containment for both nuclear and organelle genomes, and provides an expedient mechanism for F₁ hybrid seed production.

Male-sterility-inducing cytoplasm have been known for more than 100 years. Bateson and Gairdner (1921) reported that male sterility in flax (*Linum usitatissimum*) was inherited from the female parent. Chittenden and Pellow (1927) observed that male sterility in flax was due to an interaction between the cytoplasm and nucleus. Jones and Clarke (1943) established that male sterility in onion (*Allium cepa*) is conditioned by the interaction of the male-sterile cytoplasm with the homozygous recessive genotype at a single male-fertility restoration locus in the nucleus. The authors also described the technique used today to exploit cytoplasmic-genic male sterility (CMS) for the production of hybrid seed. CMS inbred lines have been widely used for hybrid production of many crops. The use of CMS in hybrid seed production has recently been reviewed by Havey (2004).

Naturally occurring CMS has been reported for maize (*Zea mays*; Kriete et al., 1996), oilseed rape (*Brassica napus*; Kriete et al., 1996), rice (*Oryza sativa*), and Beta beets (Kriete et al., 1996), but such a natural

occurring system is not available for most other crops used in agriculture. A natural source of nuclear male sterility was identified in leek (*Allium porrum*; Smith and Crowther, 1995). The CMS phenotype is a maternally inherited trait caused by incompatibility between the nuclear and cytoplasmic genomes. CMS systems are used to produce commercial F₁ hybrid lines, but full advantage of this system may be obtained if a nuclear restorer gene is available to suppress the male sterility in the hybrid plant. A few such nuclear fertility-restorer (*Rf*) genes have been isolated, including the maize *rf2* gene that encodes an aldehyde dehydrogenase (Cui et al., 1996; Liu et al., 2001) and the petunia (*Petunia hybrida*) *Rf* gene, which encodes a pentatricopeptide repeat-containing protein (Bentolila et al., 2002). Recently, the pentatricopeptide repeat protein gene *orf687* was shown to restore fertility in cytoplasmic male-sterile Kosenia radish (*Raphanus sativus* cv Kosenia; Koizuka et al., 2003). Unfortunately, introgression of *Rf* genes to restore fertility of rapeseed led to the deleterious effects and to the loss of genetic information (Delourme et al., 1998).

Engineered sources of nuclear male sterility have been developed in model systems (Mariani et al., 1990; Hernould et al., 1993; Perez-Prat and van Lookeren Campagne, 2002). A problem with these nuclear transformants is that they segregate for male fertility or sterility and must be over planted and rogued by hand or sprayed with herbicides to remove male-fertile plants. Male-sterility systems have been created by different mechanisms, most of these affect tapetum and pollen development (Kriete et al., 1996; Yui et al., 2003; Zheng et al., 2003). Unfortunately, additional

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severe phenotypic alterations that were due to interference with general metabolism and development had precluded its use in agriculture (Hernould et al., 1998; Napoli et al., 1999; Goetz et al., 2001).

In plants, three modes of inheritance of plastids and mitochondria are known: uniparental maternal, biparental, or uniparental paternal organelle inheritance. The majority of angiosperms exhibit the maternal mode of plastid inheritance, whereas in gymnosperms the paternal or the biparental modes are prevailing (Hagemann, 2004). The uniparental maternal inheritance characteristics of cytoplasmic organelles (plastid and mitochondria) are achieved through organelle exclusion from the generative cell during the first haploid pollen mitosis; organelles are distributed into the vegetative cell and not the generative cell, and, therefore, the sperm cell is free of any organelles. If the generative cell acquires a few organelles, they degenerate during maturation (Hagemann, 1992). However, rare exceptions to uniparental maternal inheritance have been reported (Hagemann, 2004).

Polyhydroxybutyrate (PHB) synthesis takes place by the consecutive metabolic action of β -ketothiolase (*phaA* gene), acetoacetyl-CoA reductase (*phaB*), and PHB synthase (*phaC*). Poirier et al. (1992) reported the expression of PHB in plants for the first time by expressing the *phaB* and *phaC* genes in the cytosol via nuclear transformation, taking advantage of available cytosolic acetoacetyl-CoA. This approach yielded very low levels of PHB, but severe pleiotropic effects were observed in the transgenic plants. In an attempt to increase the PHB yield in plants, Nawrath et al. (1994) introduced the *phbA*, *phbB*, and *phbC* genes in individual nuclear *Arabidopsis* (*Arabidopsis thaliana*) transgenic lines and reconstructed the entire pathway, targeting all enzymes to the plastids. This approach resulted in PHB expression up to 14% leaf dry weight, and no pleiotropic effects were evident. This suggested that the depletion of metabolites from essential metabolic pathways in the cytoplasm might have caused the pleiotropic effects, and that by targeting the enzymes to chloroplast, which is a compartment with high flux through acetyl-CoA, the adverse effects were overcome (Nawrath et al., 1994). With expression of optimized gene constructs, PHB yield increased up to 40% leaf dry weight, but this was accompanied by severe growth reduction and chlorosis (Bohmert et al., 2000), indicating that targeting the PHB pathway to the chloroplast can result in pleiotropic effects, at higher concentrations of polymer synthesis (Bohmert et al., 2000). Lossl et al. (2003) reported the expression of PHB in tobacco (*Nicotiana tabacum*) by expressing *phaA*, *phaB*, and *phaC* via plastid transformation. The expression of PHB resulted in severe growth reduction, and the authors concluded that in tobacco significant levels of PHB could only be achieved if a sufficient pool of acetyl-CoA precursor is generated (Lossl et al., 2003). Additionally, they observed that when the transgenic plants were grown autotrophically, PHB levels significantly decreased,

which overcame the stunted phenotype, but male sterility was still observed. It was not known whether the polymer or other metabolic factors were responsible for the male-sterile phenotype (Lossl et al., 2003).

In an attempt to address the role of *phaA* expression in the pleiotropic effects observed in transgenic plants expressing PHB, Bohmert et al. (2002) expressed the *phbA* gene constitutively and under inducible promoters via the nuclear genome. Constitutive expression of the *phbA* gene led to a significant decrease in transformation efficiency, inhibiting the recovery of transgenic lines, and prevented analysis of plants expressing the β -ketothiolase gene (Bohmert et al., 2002). Such toxic effect exerted by *phbA* expression was speculated to be the result of PHB biosynthesis intermediates or its derivatives, the depletion of the acetyl-CoA pool, or of interaction of the β -ketothiolase with other proteins or substrates (Bohmert et al., 2002).

While investigating expression of the *phb* operon encoding the PHB pathway in transgenic chloroplasts, we proposed to address the specific role of β -ketothiolase (*phaA* gene). Therefore, we expressed the β -ketothiolase gene by inserting the transgene into the chloroplast genome and studied the effect of light regulation using the *psbA* promoter and 5' untranslated region (5' UTR), and evaluated the effects of *phaA* expression in the chloroplast transgenic plants under a specific photoperiod or continuous illumination. Fully regenerated transgenic plants facilitated evaluation of the specific effect of β -ketothiolase in the transgenic lines for the first time. Detailed characterization revealed that severe pleiotropic effects, such as stunted phenotype and chlorosis observed during PHB expression (Bohmert et al., 2000, 2002), were not observed during *phaA* expression via the chloroplast genome, with the exception of complete (100%) male sterility. Such elimination of pleiotropic effects was not surprising because the chloroplast genetic engineering system has been able to overcome the toxic effects (stunted growth, chlorosis, and infertility) associated with nuclear expression of the trehalose (Lee et al., 2003), xylanase (Leelavathi et al., 2003), and cholera toxin B subunit (Daniell et al., 2001a) genes producing normal plants that were healthy and fertile. In this article, we characterize *phaA* transgene site-specific integration into the chloroplast genome, transcription, translation in different tissue types, β -ketothiolase activity under different photoperiods, microscopic analysis of different stages of anther development, and electron microscopic analysis of pollen grains, and attempt to correlate these activities with metabolic activities. This study resulted in the first engineered CMS system in plants.

RESULTS

Chloroplast Transformation Vector

The *Acinetobacter* sp. (accession no. L37761) gene *phaA* (1,179 bp) coding for β -ketothiolase was amplified

by PCR and cloned into the chloroplast transformation vector (pLD-ctv) to finally produce the pLDR-5'UTR-*phaA* vector (Fig. 1A). The vector contains chloroplast DNA sequences (flanking sequences), which allow site-specific integration by homologous recombination into the inverted repeat region of the chloroplast genome, in between the *trnI* (tRNA Ile) and *trnA* (tRNA Ala) genes (Daniell et al., 1998). Site-specific integration of the transgene cassette containing the *aadA* (aminoglycoside 3'-adenylyltransferase) gene conferred spectinomycin resistance. Transcription of the *aadA* and *phaA* genes is driven by the constitutive 16S ribosomal RNA gene promoter (*Prrn*), located upstream of the *aadA* gene, and this should produce a dicistron (*aadA-phaA*). In addition, the *psbA* gene promoter and 5' regulatory sequence (5'UTR), known to enhance translation of foreign genes in the light (Eibl et al., 1999; Fernandez-San Millan et al., 2003; Daniell et al., 2004c; Dhingra et al., 2004; Watson et al., 2004), were inserted upstream of the *phaA* gene, and this should produce the *phaA* monocistron. At the 3' end of the gene construct is the 3' *psbA* untranslated region (3'UTR), which is known to be involved in mRNA abundance and stability (Schuster and Gruissem, 1991; Sugita and Sugiura, 1996; Monde et al., 2000).

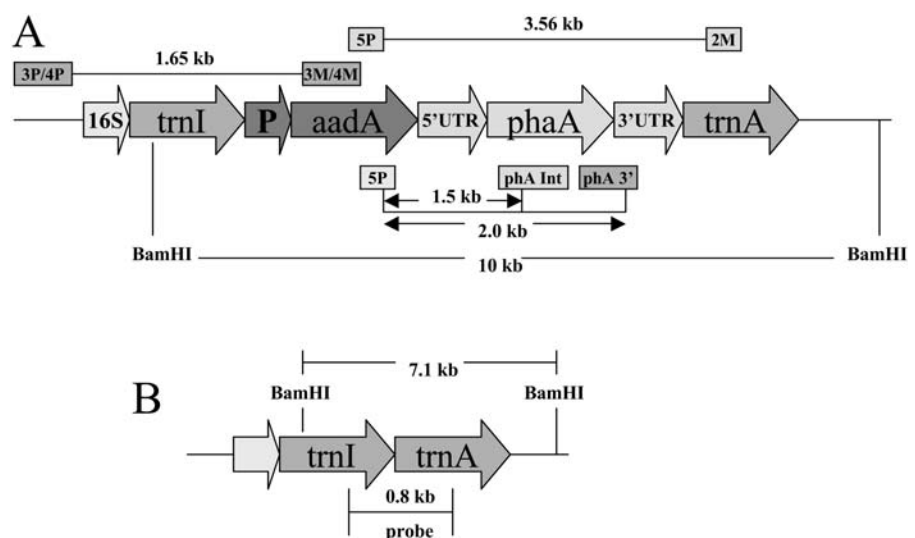
Transgene Integration into Chloroplast Genomes

Chloroplast transgenic lines were obtained through particle bombardment following the method described previously (Daniell, 1997; Daniell et al., 2004a; Kumar and Daniell, 2004; Dhingra and Daniell, 2005). More than 10 positive independent transgenic lines were obtained. Three independent transgenic lines were characterized, confirming that independent chloroplast transgenic lines show little variation in foreign gene expression, as observed earlier (Daniell et al., 2001a). PCR-based analysis with the primer pairs 3P-3M and 4P-4M was used to test site-specific in-

tegration of the transgene cassette into the chloroplast genome (Daniell et al., 2001b). The 3P-4P primers land on the native chloroplast genome, upstream of the gene cassette, and the 3M-4M primers land on the *aadA* gene, which is located within the gene cassette (Fig. 1A). If site-specific integration had occurred, a PCR product of 1.65 kb should be obtained; this product was detected in transgenic lines (Fig. 2A, lanes 2 and 3). Untransformed plants as well as mutant plants, which had undergone spontaneous mutation of the 16S rRNA gene and acquired resistance to spectinomycin, did not show any PCR product, indicating that these plants are negative for integration (Fig. 2A, lane 1). The integration of the *aadA* and *phaA* genes was further confirmed by the use of primer pairs 5P-2M, 5P-3' *phaA*, and 5P-*phaA* internal, which produced PCR products of sizes 3.56 kb, 2.0 kb, and 1.5 kb, respectively. These primers anneal at different locations within the transgene cassette (Fig. 1A). Results revealed expected-size PCR products in the transgenic lines (Fig. 2A, lanes 4–6), confirming the presence of the *phaA* gene.

Total DNA from T₀ and T₁ generation transgenic lines as well as from untransformed tobacco plants was extracted and used for Southern-blot analysis (Fig. 2, B–E). The flanking sequence probe (Fig. 1B), which hybridizes with the *trnI* and *trnA* genes, was used to analyze heteroplasmy and investigate site-specific integration of the transgene cassette into the chloroplast genome. Additionally, the *phaA* probe was used to confirm the presence of the *phaA* gene. DNA from untransformed plants and transgenic lines was digested with *Bam*HI (Fig. 1, A and B) and probed with either a flanking sequence probe or a *phaA* probe; a 10-kb hybridizing fragment was detected in transformed chloroplast genome (Fig. 2, B–E, lanes 2–4). The detection of a 7.1-kb fragment by the flanking sequence probe in the untransformed line confirmed that these chloroplast genomes lacked foreign genes (Fig. 2, B

Figure 1. Molecular characterization of transgenic lines. A, Schematic representation of the transformed chloroplast genome and the pLDR-5'UTR-*phaA* cassette. Annealing sites for the primer pairs and expected sizes of PCR products are shown. *Bam*HI restriction sites, DNA fragment produced after restriction digestion, and the *phaA* probe used in Southern-blot analyses are shown. B, The map shows the wild-type chloroplast genome, restriction enzyme sites used for Southern-blot analysis, expected fragment sizes after digestion, and probing with the flanking sequence.



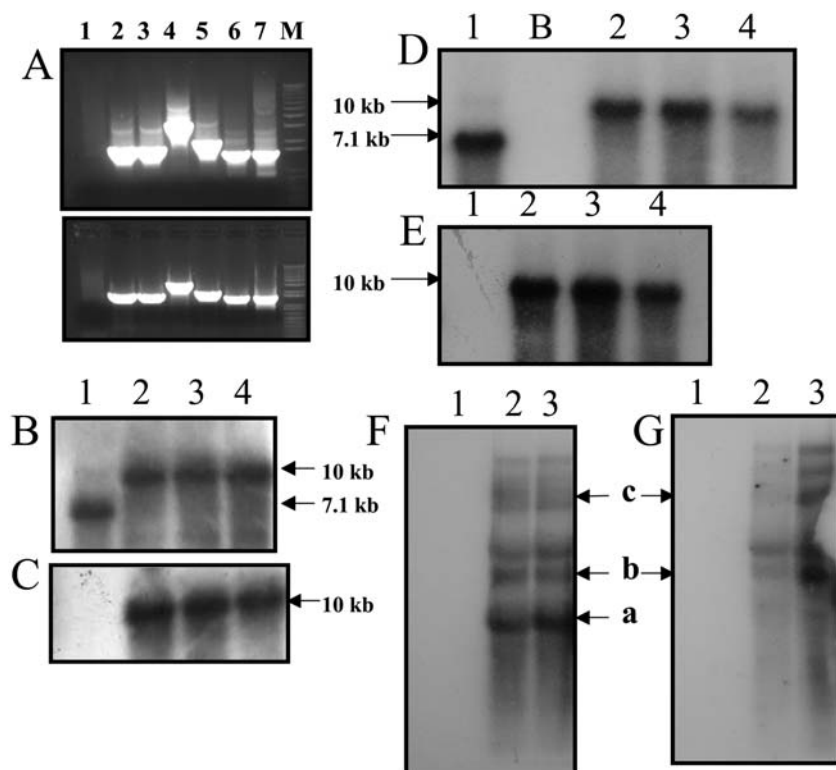


Figure 2. A, PCR analysis of untransformed plants and putative transformants. Top section is transgenic line 4A; bottom section is transgenic line 4A. A, Lane 1, Untransformed plant; 2, 3P-3M (1.65 kb); 3, 4P-4M (1.65 kb); 4, 5P-2M (3.56 kb); 5, 5P-3'phaA (2.0 kb); 6, 5P-phaA internal (1.5 kb); 7, positive control (pLD-5'UTR-*phaA* plasmid DNA) 5P-*phbA* internal (1.5 kb); M, marker. B and D, Southern-blot analysis of T₀ and T₁ generation transgenic lines, respectively, with the chloroplast flanking sequence probe; 10-kb fragment shows integration of transgenes; 7.1-kb fragment shows wild-type fragment. C and E, Transgenic T₀ and T₁ plants, respectively, and untransformed plant probed with the *phaA* gene. The 10-kb fragment is observed in transgenic lines but not in the untransformed plant. F and G, Northern-blot analysis using the *phaA* and *aadA* probes, respectively. Monocistron (a, 5'UTR-*phaA*, 1,384 nt) and polycistrons (b, *aadA-phaA*, 2,255 nt; c, from native 16S *Prrn*, 4,723 nt) containing the *phaA* gene are observed in the transgenic lines when the *phaA* probe is used. Only polycistrons are observed with the *aadA* probe. In B to G: 1, untransformed plant; 2 to 4, chloroplast transgenic lines 4A, 4B, and 4C, respectively; B, blank lane.

and D, lane 1). The fact that no 7.1-kb-size fragment was observed in T₀ generation (Fig. 2B, lanes 2–4) and T₁ generation (Fig. 2D, lanes 2–4) transgenic samples confirmed stable integration of foreign genes within all chloroplast genomes and lack of heteroplasmy (within the limits of detection of Southern blots). The absence of any hybridizing fragment in the untransformed lines when screened with the *phaA* probe confirmed the absence of *phaA* gene (Fig. 2, C and E, lane 1). If any unexpected-size fragment were observed in the transgenic samples when probed with the transgene after prolonged exposure of Southern blots to x-ray film, nonspecific integration into the nuclear or mitochondrial genomes would be indicated. Such nonhomologous recombination was not observed.

Transcription of Transgenes Integrated into Chloroplast Genomes

Transcript abundance and stability in chloroplast transgenic lines were studied by northern-blot analysis using the *phaA* and *aadA* probes on total plant RNA (Fig. 2, F and G). The chloroplast transgenic lines were expected to transcribe a 2,255-nt dicistron (*aadA-phaA*; Fig. 2, F and G) from the upstream 16S promoter (*Prrn*), in addition to a 1,384-nt monocistron (*phaA*) transcribed from the *psbA* promoter, located upstream from the *phaA* gene (Fig. 2F, lanes 2 and 3). As expected, the monocistron was not detected with the *aadA* probe, showing that polycistrons are transcribed from the engineered *Prrn* promoter; less abundant polycistrons

transcribed from the native 16S promoter were also detected (Fig. 2G). The monocistron was only detected with the *phaA* probe. These results showed that both the monocistron and dicistron transcripts were abundant in the transgenic lines because of the efficiency of the *psbA* and *Prrn* promoters, which are strong chloroplast promoters. Additionally, larger-size transcripts were detected, one of them correlating with the size of a transcript initiated at the chloroplast native 16S promoter (*Prrn*) and terminating at the 3'UTR *psbA*; the predicted size of this transcript is 4,723 nt (Fig. 2, F and G). Other transcripts detected may be either read-through (because 3'UTR does not terminate transcription efficiently in chloroplast) or processed products.

Translation of the *phaA* Gene Integrated into Chloroplast Genomes

To confirm expression of β -ketothiolase in the chloroplast transgenic lines, untransformed and transformed plants were subjected to western-blot analysis using anti- β -ketothiolase antibody. Chloroplast-synthesized β -ketothiolase, when treated with β -mercaptoethanol (BME) and boiled, appeared mostly as a monomeric form (40.8 kD), or in polymeric forms, which included the homotetrameric form (163 kD; Fig. 3B, lanes 2–6). The homotetramer is the functional form of β -ketothiolase. No β -ketothiolase was detected in wild-type samples (Fig. 3B, lane 1). The appearance of a distinct band at 40.8 kD in the Coomassie-stained SDS-PAGE gel (Fig. 3A, lanes 1–4) but not in the untransformed

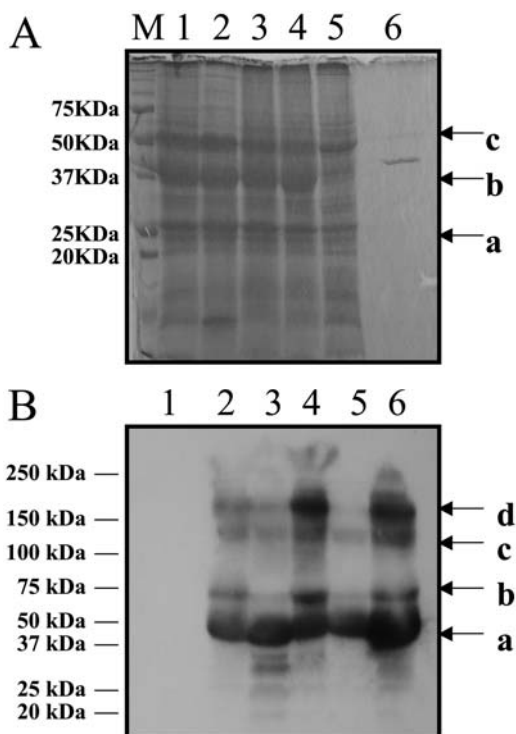


Figure 3. β -Ketothiolase characterization in transgenic lines. A, Coomassie-stained gel showing abundant β -ketothiolase expression in transgenic lines; 15 μ g of total plant protein was loaded per lane (a, D1 protein; b, β -ketothiolase, 40.8 kD; c, Rubisco large subunit, based on molecular range). M, Marker; 1 to 3, transgenic lines 4A, 4B, and 4C, respectively; 4, 4A T_1 generation; 5, untransformed plant; 6, bacterial purified β -ketothiolase. B, Western-blot analysis; 10 μ g of total plant protein from transgenic lines and wild type was loaded per lane; anti- β -ketothiolase antibody was used. Bands corresponding to the 40.8-kD monomers (a), a possible modified version of the monomer (b), trimer (c), or tetramer (d) are observed in transgenic lines. 1, Untransformed plant; 2 to 4, transgenic lines 4A, 4B, and 4C, respectively; 5 and 6, 4A and 4B T_1 generation, respectively.

sample (lane 5) suggests that the chloroplast transgenic plants were expressing high levels of β -ketothiolase.

The activity of the chloroplast-expressed β -ketothiolase was measured in the thiolysis direction (breaking down acetoacetyl-CoA to acetyl-CoA) spectrophotometrically at 304 nm. The chloroplast transgenic lines showed β -ketothiolase activities that were up to 30-fold higher than previous levels reported for nuclear transgenic plants. No endogenous β -ketothiolase activity was detected in untransformed tobacco plants (less than 0.0001 unit/mg plant protein; Table I). The chloroplast transgenic lines showed enzyme activity in the range of 14.08 to 14.71 unit/mg plant protein during normal photoperiod (16 h light/8 h dark; Table I). After 5 d of continuous illumination, the enzyme activity slightly increased in both transgenic lines (Table I). Thus, β -ketothiolase activity remained unchanged from light/dark photoperiod to continuous illumination, even though the *phaA* gene is under the control of strong *psbA* regulatory elements that should enhance translation in the light. The high levels of

enzyme activity correlated well with the high amounts of protein detected by the Coomassie-stained gel and western-blot analyses performed on total plant extracts; these results suggested that the enzyme was in its biologically active form (homotetramer).

Characterization of Male Sterility

Among the 10 T_0 transgenic lines expressing β -ketothiolase, 100% of the flowers produced by transgenic plants failed to develop fruit capsules and seeds, finally senescing and falling off (Fig. 4, A–C). The male-sterile phenotype was maintained in T_1 generation transgenic lines. T_0 (Fig. 5A) and T_1 (Fig. 5B) generation transgenic plants showed no difference in growth and development when compared to untransformed tobacco plants under the same growth conditions. Like the parental line, the T_1 transgenic lines were not affected by hyperexpression of *phaA* and were phenotypically indistinguishable, with the exception of being male sterile, from untransformed control plants (Fig. 5B). Analysis of chlorophyll content of three independent T_1 transgenic lines showed average chlorophyll content of 1.90 ± 0.20 mg/g fresh weight. The chlorophyll content of three wild-type plants averaged 1.92 ± 0.12 mg/g fresh weight. These results showed that the chlorophyll levels in the leaves of the transgenic lines expressing β -ketothiolase were similar to the levels in wild-type tobacco plants, confirming normal chloroplast biosynthetic functions and integrity of the thylakoid membranes, although β -ketothiolase was hyperexpressed.

However, the chloroplast transgenic lines showed specific defects in anther development and failed to produce viable pollen. The anthers were characterized by the lack of pollen grains (Fig. 4H), or when pollen grains were formed, they were abnormal with a

Table I. β -Ketothiolase activity as a function of illumination

Enzyme activity for transgenic lines 4A and 4B and untransformed tobacco plants at the respective illumination periods are shown. Protein samples were obtained by grinding 1 g of leaf tissue in liquid nitrogen, followed by the addition of β -ketothiolase extraction buffer (100 mM Tris-HCl, pH 8.1, 50 mM MgCl₂, 5 mM BME) and homogenization. Total plant protein concentration was determined by Bradford assay. Ten microliters of crude plant extract was used per assay. β -Ketothiolase activity was measured spectrophotometrically at 304 nm for 60 s in the thiolysis direction. na, Not applicable.

Plant Line	Illumination Period Light/Dark	Enzyme Activity Units
	<i>h</i>	μ mol/min
Untransformed	16 h/8 h	<0.0001
4A	16 h/8 h	0.215
4B	16 h/8 h	0.259
Untransformed	5 d of light	<0.0001
4A	5 d of light	0.247
4B	5 d of light	0.239
Purified bacterial β -ketothiolase	na	0.017

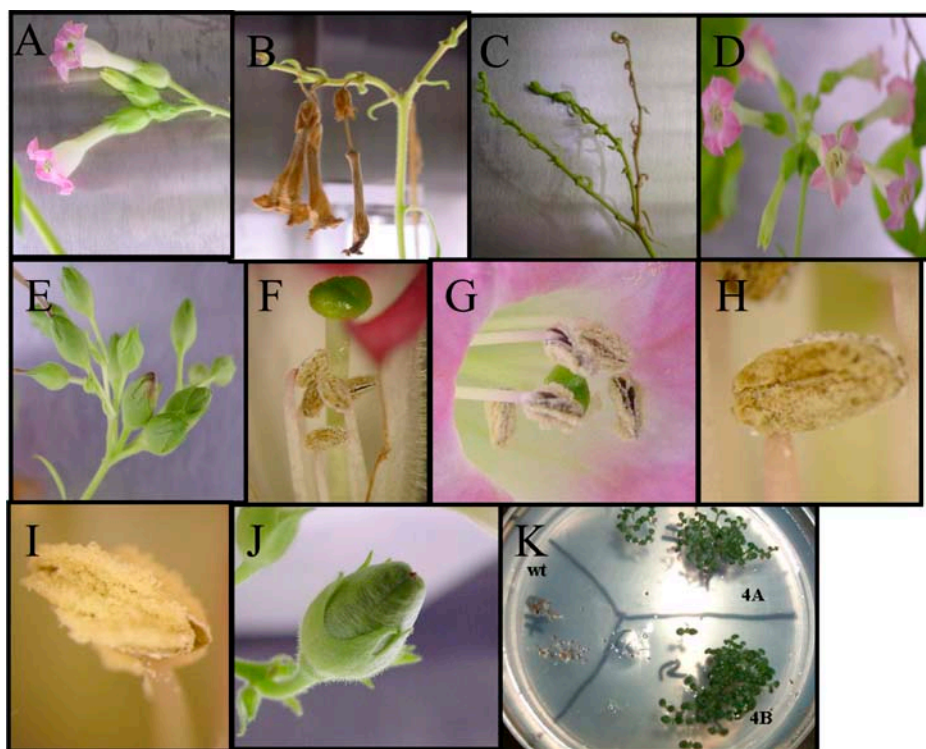


Figure 4. Characterization of the male-sterile phenotype. A to C, Flowers from transgenic lines; note the absence of fruit capsules and fallen flowers. D and E, Untransformed tobacco flowers and fruit capsules. Comparison of stamens and stigma: note shorter stamens in transgenic lines (F) compared to untransformed (G). Comparison of mature anthers: note abundant pollen in untransformed anther (I) and the lack of pollen in transformed anther (H). J, Transgenic fruit capsule with seeds after pollination of transgenic stigma with untransformed pollen. K, Germination and growth of T_1 seedlings on Murashige and Skoog medium with 500 $\mu\text{g}/\text{mL}$ spectinomycin. wt, Untransformed; 4A, T_1 transgenic line 4A; 4B, T_1 transgenic line 4B, obtained after pollination with untransformed pollen.

collapsed phenotype (Fig. 6). Additionally, the stamens were shorter (Fig. 4F) than in wild-type plants (Fig. 4G), adding a degree of severity to the male-sterile phenotype. To investigate that plants were sterile due to lack of pollen or nonviable pollen, a total of 15 emasculated wild-type flowers from three untransformed plants (five flowers from each untransformed plant) were pollinated with pollen collected from anthers from transgenic lines 4A, 4B, and 4C. Pollen from each transgenic line was used to pollinate a total of five untransformed emasculated flowers. All of these crosses failed to produce seeds. The shorter stamens may be a consequence of the failure of the cells to elongate in the central and upper parts of the anther filament. To investigate the possibility of female infertility affecting the transgenic plants, the plants were fertilized with pollen from untransformed plants. The transgenic plants developed normal fruit capsules (Fig. 4J) and normal seeds that were able to germinate and develop normally (Fig. 4K). The T_1 seedlings grew well in the medium supplemented with 500 $\mu\text{g}/\text{mL}$ spectinomycin and were identical to the parental line (T_0), and Southern-blot analysis showed the presence of the gene construct (Fig. 2, D and E).

Scanning electron microscopy (SEM) was performed on transgenic anthers as well as untransformed anthers to further characterize male sterility in transgenic plants. The SEM revealed that the pollen grains in the transgenic anthers exhibited collapsed morphology and consisted of a heterogeneous population with respect to size and shape (Fig. 6, D–F). Wild-type anthers showed a homogenous population of pollen grains of

uniform size and shape (Fig. 6, A–C). The apparent lack of turgidity of the transgenic pollen may be produced by lack of intracellular material, resulting in the distorted and collapsed morphology. The aberrant pollen morphology observed under the SEM may account for the inability of the transgenic plant to produce seeds.

β -Ketothiolase Expression in Anthers

Plastids in anthers may be in low abundance when compared to the numbers in leaves, but they produce enough β -ketothiolase to affect pollen development in anthers. As shown in Figure 7A, flowers and specifically anthers were green during anther development (stage 1 of flower development is shown). This means that chloroplasts are present and are metabolically active. Northern-blot analysis of leaves showed that the mRNAs coding for β -ketothiolase are present in monocistronic and polycistronic forms, the monocistron form being the most abundant transcript (Fig. 2F). The same pattern was maintained in the *phaA* transcripts in flowers and anthers (Fig. 7B). Northern-blot analysis of transgenic flowers and anthers showed that transcription of the *phaA* gene occurs in the flower and anther; this was expected because *Prrm* and *psbA* promoters are constitutive promoters, allowing transcription to occur in both photosynthetically active as well as nonphotosynthetic plastids (Fig. 7B). The translation of the *phaA* monocistron is under the light-regulated *psbA* 5' UTR because the flowers as well as anthers are green, containing photosynthetically active chloroplasts; translation was quite efficient (Fig. 7C).



Figure 5. Comparison of growth and development. A, Untransformed plant (WT) and T_0 generation transgenic (T) line (4A) grown for 2 months in soil. B, Untransformed plant (WT) and T_1 generation independent transgenic lines 4A, 4B, 4C, and 4D, 1.5 months after germination.

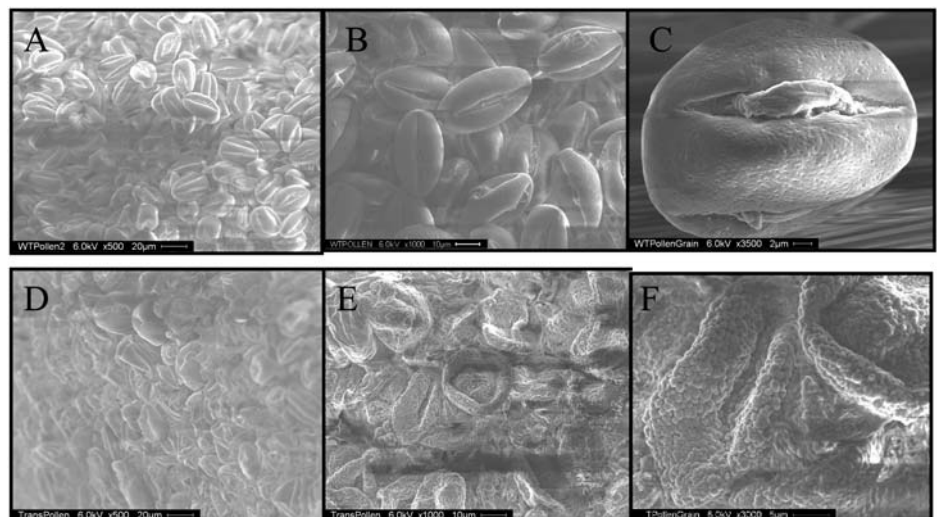
β -Ketothiolase was detected by western blot in both whole flowers and anthers from transgenic plants, confirming that the β -ketothiolase was present during anther development and should play a significant role in the male-sterile phenotype (Fig. 7C).

Anther Development and Male Sterility

Analysis of anther development revealed that the anthers of the transgenic lines followed an accelerated pace in their development and maturation, resulting in

aberrant tissue patterns (Fig. 8). Flower developmental stages were determined as described by Koltunov et al. (1990). At stage -3 of flower development, the untransformed plants showed a normal pattern of tissue development, where all major tissues were differentiated, the anther had acquired its characteristic shape, all tissues were interconnected, and there was callose deposition between microspore mother cells. This pattern was not followed in the transgenic lines, which at stage -3 showed characteristics of a more advanced stage. The transgenic anthers at stage -3 showed the microspores in tetrads, with stomium differentiation occurring, and the tapetum was shrunk and broken. This pattern presented characteristics of more advanced stages of flower development, which included stages $+1$ and $+2$. Additionally, the transgenic anthers showed abnormal development of the epidermis and endothecium, probably resulting in the aberrant shape of anthers. The anthers of the transgenic lines at stage -2 were also advanced in the aberrant phenotype with the microspores already separated, a developmental step that should have been observed at stage $+2$. Again the tapetal layer was broken. We noticed that the transgenic anthers at stage -5 , which is a very early stage of flower development, showed great similarities with stage -2 in the untransformed plants, but aberrant pattern of tissue development could still be observed. At stage -1 in transgenic lines, the tapetum was shrunk and discontinuous, and formation of pollen grains was evident at stage 1. These morphological changes observed in stages -1 and $+1$ should be observed at much later stages, $+3$ and $+4$. At early stages of floral development (stages -5 to $+1$), transgenic lines showed accelerated anther development, which averaged $+3$ stages ahead from the wild-type plants. At late stages of floral development, accelerated phenotype increased even more, at an average of four to six stages ahead of untransformed plants. At stage $+2$ in the transgenic lines, cells adjacent to the stomium had

Figure 6. SEM pictures of pollen grains in anthers of untransformed (A–C) and transgenic (D–F) plants at different magnifications. A and D, $\times 500$; B and E, $\times 1,000$; C, $\times 3,500$; F, $\times 3,000$.



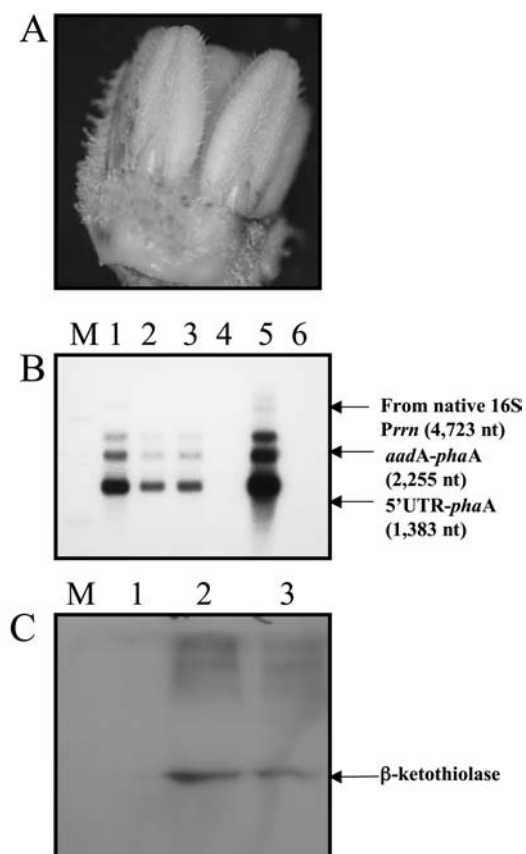


Figure 7. β -Ketothiolase expression in anthers. A, Pigmentation of anthers during flower development in transgenic plants. Stage 1 of flower development is shown. B, Northern-blot analysis of flowers and anthers. Three micrograms of total plant RNA was loaded per lane, and the *phaA* probe was used. M, Marker; 1, transgenic flower; 2 and 3, transgenic anthers from line 4A and 4B, respectively; 4, untransformed flower; 5, transgenic leaf; 6, untransformed leaf. C, β -Ketothiolase expression in transgenic flowers and anthers detected by western-blot analysis in lanes 2 and 3, respectively. RNA and protein samples used per lane were the product of the combined extraction from flowers or anthers from stages 1 and 3.

degenerated, and only remnants of the tapetum were observed. The thickening of the outer wall was accompanied by enlarged endothecium and vacuolation. The irregular shape and collapsed phenotype of pollen grains might be a synergistic effect of the reduced locule space and the apparent lack of turgidity of the transgenic pollen produced by insufficient intracellular material. Lack of intracellular material can be attributed to the lack of tapetum in the transgenic lines. The developmental changes observed in the transgenic anthers at stage +2, although aberrant, were similar to the ones observed in untransformed at stage +6. In the untransformed anthers, abundant normal pollen grains were observed. Almost complete degradation of the connective tissue that separates the pollen sac occurred at stage +3 in transgenic anthers, while this occurred at stage +9 in untransformed plants. Finally, both untransformed and transgenic anthers were bilocular and connective tissue was absent, but the

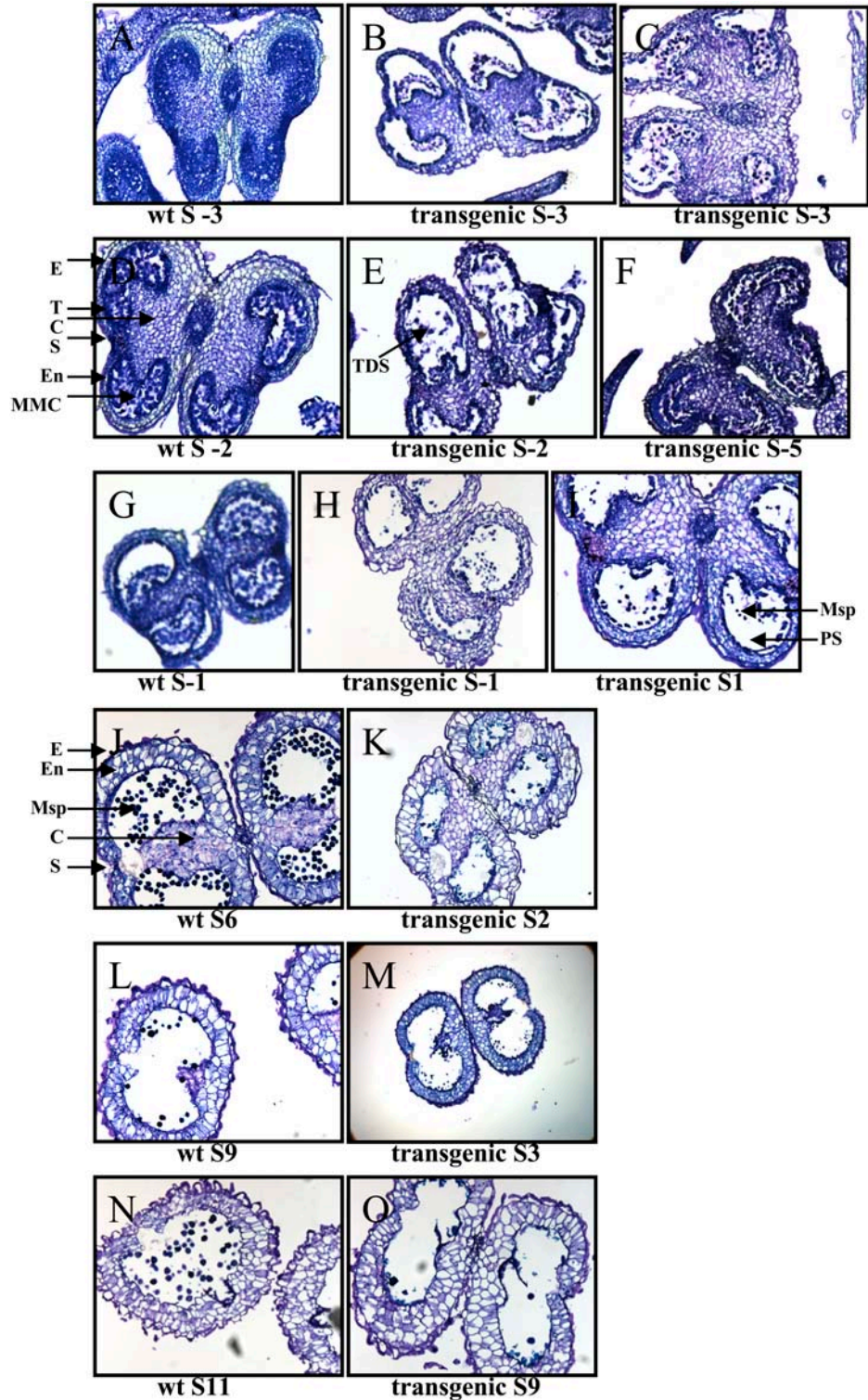
major difference was that abundant pollen was present in untransformed (stage +11) but not in transgenic anthers (transgenic, stage +9). Additionally, the pollen grains formed in transgenic anthers were collapsed.

Anther development is a very complex process involving the coordination of several genes and the specific development and maturation of several tissues and cells (Yui et al., 2003); any defect in these well-coordinated processes may lead to dysfunctional pollen. Many male-sterility systems produced by mutations or nuclear expressions of foreign proteins have shown to interfere with the function or differentiation of tapetum, indicating that this tissue is essential for the production of viable pollen (Goetz et al., 2001). Here, we observed that the tapetum of the transgenic lines was severely impaired. The tapetum is critical for the development of pollen by secreting essential substances such as proteins (Yui et al., 2003), carbohydrates (Goetz et al., 2001), and lipids (Zheng et al., 2003) into the locules. Developing microspores and the surrounding tapetal cells have been shown to be particularly active in lipid metabolism (Zheng et al., 2003). The precise differentiation and maturation of tapetum with respect to microspore development is of major importance for the successful production of pollen. Here, we observed almost total dysfunction in the anther tissue differentiation patterns, which may be caused by an alteration in chloroplast fatty acid metabolism in the transgenic lines expressing the *phaA* gene, thereby affecting the development of pollen grains.

Reversibility of Male Fertility

To test whether the acetyl-CoA pool destined for de novo fatty acid biosynthesis in chloroplast is being diverted by β -ketothiolase to the synthesis of acetoacetyl-CoA, resulting in the male-sterile phenotype, we explored whether continuous illumination would revert male sterility to fertility. The photoperiod experiments represent an indirect test of the proposed basis for male sterility in this system. Acetyl-CoA carboxylase (ACCase) carries the first committed step in fatty acid biosynthesis, which involves the conversion of acetyl-CoA to malonyl-CoA. Because acetyl-CoA is not imported into plastids from the cytoplasm, it should be synthesized in this organelle; the expression of *phaA* in the chloroplast should increase the competition for the same pool. Therefore, if β -ketothiolase and ACCase are competing for the same acetyl-CoA pool, β -ketothiolase will outcompete ACCase when it is hyperexpressed in transgenic chloroplasts; this could decrease the supply of acetyl-CoA for fatty acid biosynthesis. Therefore, conditions that could divert the acetyl-CoA pool back to the fatty acid biosynthesis pathway might restore the fatty acid biosynthesis. It has been shown that the intermediates of fatty acid biosynthesis change during the transition to darkness in leaves and in chloroplasts in a manner consistent with control at the level of ACCase (Post-Beittenmiller et al., 1991).

Figure 8. Studies on anther development. Bright-field photographs of untransformed (wt) and transgenic anthers at different developmental stages (S). Anthers at the designated stages were fixed, embedded with paraffin, and sliced into 5- and 10- μ m transverse sections. The fixed sections were stained with toluidine blue and visualized under the light microscope at a magnification of $\times 100$. C, Connective tissue; E, epidermis; En, endothecium; MMC, microspore mother cells; Msp, microspores; PS, pollen sac; S, stomium; T, tapetum; TDS, tetrads.



The chloroplast uses photosynthesis as the mechanism to generate the reducing power and ATP for diverse metabolic reactions, including fatty acid biosynthesis. De novo fatty acid biosynthesis in the chloroplast has been shown to be regulated by the

response of ACCase to the redox state of the plastid, ensuring that carbon metabolism is linked to the energy status of this organelle (Rawsthorne, 2002). Recent reports have shown light-dependent regulation of ACCase by the redox status of the plastid, whereby the

enzyme is more active under the reducing conditions observed in light (Page et al., 1994; Kozaki et al., 2000).

Therefore, to test this hypothesis, two independent transgenic lines 4A and 4B were exposed to continuous light for a period of 10 d. These plants have previously been characterized and shown to be 100% male sterile, unable to produce any fruit capsules or seeds. These plants were isolated from all other plants (transgenic and wild types) in a growth chamber at the first indication of flower bud development (before any flower opened), and the flowers were covered with transparent plastic bags to avoid cross-pollination. We observed that from a total of 20 flowers produced during the 10 d of illumination by the two transgenic plants, four flowers were able to produce pollen (Fig. 9A), produce normal-length anther filaments (Fig. 9A), develop fruit capsules (Fig. 9B), and produce seeds (Fig. 9C). Transgenic line 4A produced three male-fertile flowers during the 10-d continuous-light photoperiod; all of them were produced between days 8 and 10. Transgenic line 4B produced one viable flower on day 9 and was able to produce additional viable flowers 3 d after the 10-d assay was completed. The seeds recovered (Fig. 9C) from the reverted male-fertility study were able to germinate in a medium with 500 $\mu\text{g}/\text{mL}$ spectinomycin (Fig. 9D), indicating that these seedlings were transgenic and contained the transgene; untransformed tobacco seedlings were bleached (Fig. 9E). These findings support the hypo-

thesis that an increase in ACCase activity outcompetes, at least partially, the removal of acetyl-CoA by β -ketothiolase. Additionally, this line of evidence supports the hypothesis that male sterility is associated with β -ketothiolase expression in the chloroplast. Based on the lack of increase in β -ketothiolase activity (Table I) but increased ACCase activity during continuous illumination (see "Discussion"), de novo fatty acid biosynthesis should be enhanced in transgenic lines and this should reverse male sterility. Monitoring ACCase activity in different tissues and determining various metabolic pools would further test this hypothesis.

DISCUSSION

We have shown that the hyperexpression of β -ketothiolase via chloroplast transformation resulted in normal growth and pigmentation, even when the activity of the enzyme was very high. Successful expression of β -ketothiolase in transgenic plants showed that this enzyme could be safely expressed in the chloroplast and that the complete PHB pathway should be expressed to cause the stunted phenotype. Although no growth reduction was observed in the chloroplast transgenic lines expressing β -ketothiolase, 100% male sterility was observed. This was interesting and worthy of further investigation because of the importance of the production of male-sterile lines in gene containment and hybrid seed production (Perez-Prat and van Lookeren Campagne, 2002). Because the expression of β -ketothiolase did not disrupt growth and normal development, with the exception of the lack of pollen formation, the expression of β -ketothiolase may be used as a mechanism to generate male-sterile lines.

Here, we also demonstrate that it is possible to revert the chloroplast transgenic lines to male fertility by continuous illumination. This supports the hypothesis that β -ketothiolase may deplete the pool of acetyl-CoA in the chloroplast, and through increasing ACCase activity by continuous light (Post-Beittenmiller et al., 1991; Page et al., 1994; Kozaki et al., 2000), ACCase is able to compete more effectively for acetyl-CoA, thereby increasing the levels of plastidic fatty acid biosynthesis. Developing microspores and surrounding tapetal cells have been shown to be particularly active in lipid metabolism (Fatland et al., 2002; Yui et al., 2003), which is especially needed for the formation of the exine pollen wall from sporopollenin (Wang et al., 2002; Ariizumi et al., 2004).

Support for the normal growth and development observed in the chloroplast transgenic lines expressing *phaA* is provided from studies where a mutant plant with only 10% activity in acyl-CoA synthase (which catalyzes the formation of fatty acyl-CoA in chloroplast from free fatty acid, ATP, and CoA) showed normal content of lipids in leaves and normal growth (Schnurr et al., 2002), indicating that under normal

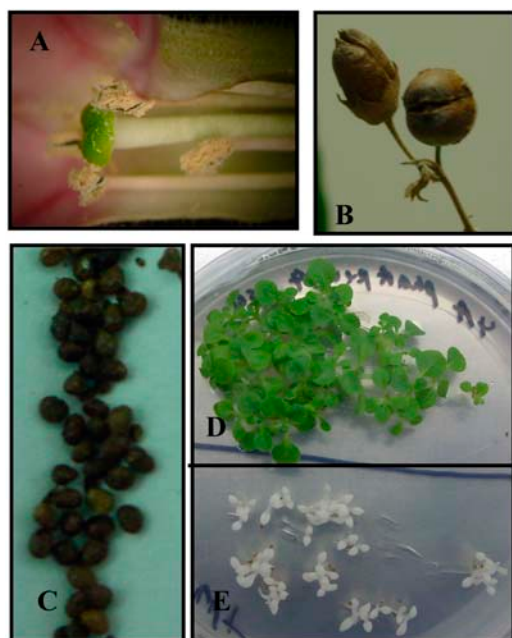


Figure 9. Reversibility of male sterility after 10 d under continuous illumination. A, Transgenic flower after 9 d in continuous light; note normal length of the anther filaments and pollen grains. B, Fully developed fruit capsules containing seeds from reversed transgenic lines. C, Abundant seeds from a transgenic fruit capsule. D, Seedlings produced via the reversibility to male fertility. Transgenic seeds germinated in Murashige and Skoog medium supplied were with 500 $\mu\text{g}/\text{mL}$ spectinomycin. E, Bleached wild-type tobacco seedlings.

growth conditions even plants severely impaired in the fatty acid biosynthesis pathway were able to grow normally. Furthermore, Nikolau et al. (2000) reported that constitutive expression of an antisense construct of acetyl-CoA synthase failed to produce any detrimental morphological phenotype or change in total plant fatty acid composition in the transgenic plant; acetyl-CoA synthase is the enzyme responsible for supplying acetyl-CoA from acetate in chloroplast. However, they observed that the use of acetate as a source for acetyl-CoA decreased in proportion to the degree of down-regulation of the enzyme in the antisense lines. Therefore, under normal developmental conditions, which do not have an excessive demand for de novo fatty acid synthesis, alternative mechanisms can compensate for the decrease in activity of key enzymes of the de novo fatty acid biosynthesis pathway. However, during high fatty acid-driven processes such as tapetum and pollen development, any such alternative mechanism might not be adequate to meet the high demand. This same scenario could apply if acetyl-CoA is in short supply and ACCase cannot synthesize enough malonyl-CoA to drive the synthesis of fatty acids.

An alternative explanation for the male-sterile phenotype might be that the formation of acetoacetyl-CoA or β -ketothiolase itself may be producing a detrimental effect on chloroplast metabolism. This explanation is difficult to support from our reversibility studies because under continuous light no decrease in β -ketothiolase level or activity was observed; this excludes a possible decrease in acetoacetyl-CoA or detrimental effect caused by β -ketothiolase. The results of this study clearly show that the expression of *phaA* in the chloroplast does not cause observable severe pleiotropic effects (with the exception of male sterility). This is in sharp contrast with previous reports, where attempts to insert and express the *phaA* via the nuclear genome failed to recover any transgenic plants; this effect was attributed to the putative deleterious effect of *phaA* (Bohmert et al., 2002). Compartmentalization of proteins in chloroplasts has been shown to avoid pleiotropic effects, as previously reported for cholera toxin B subunit (Daniell et al., 2001a), trehalose (Lee et al., 2003), and xylanase (Leelavathi et al., 2003).

In addition, the lack of detrimental effect can be explained by the enzymatic mechanism of β -ketothiolase in the chloroplast. β -Ketothiolase is an enzyme that can generate a carbon-carbon bond in a biological Claisen condensation (Davis et al., 1987). β -Ketothiolase interacts with two identical acetyl-CoA molecules differently, by using one as a carbanion and the other as an electrophile, to produce a head-to-tail condensation. Alternatively, under physiological conditions in bacteria, β -ketothiolase can also undergo the CoASH-mediated thiolysis of acetoacetyl-CoA (Davis et al., 1987); in chloroplast, there is no known endogenous pool of acetoacetyl-CoA. It is possible that the detrimental effects observed during the expression of the entire PHB pathway in chloroplast (Lossl et al., 2003)

were due to the depletion of the chloroplast acetyl-CoA pool by the consecutive action of *phaA*, *phaB*, and *phaC* to produce PHB, which would act as a sink because PHB cannot be metabolized by plants. However, when only *phaA* is expressed in the chloroplast, acetyl-CoA will be converted to acetoacetyl-CoA, which cannot be metabolized by any known chloroplast enzyme. Accumulation of acetoacetyl-CoA will drive the kinetics of the reaction toward β -ketothiolase-mediated thiolysis. This ping-pong effect caused by β -ketothiolase could cause an imbalance between acetyl-CoA removal and production that might be enough to affect a steady supply of acetyl-CoA to the fatty acid biosynthesis pathway. The effect of such an imbalance could be more pronounced under conditions that require high de novo fatty acid biosynthesis, such as in the case of tapetum and pollen development.

Chloroplast genetic engineering approach offers a number of attractive advantages, including high-level transgene expression (De Cosa et al., 2001; Daniell et al., 2005a, 2005b), multigene engineering in a single transformation event (De Cosa et al., 2001; Daniell and Dhingra, 2002; Lossl et al., 2003; Ruiz et al., 2003), transgene containment via maternal inheritance (Daniell, 2002; Hagemann, 2004), lack of gene silencing (De Cosa et al., 2001; Lee et al., 2003; Dhingra et al., 2004), position effect due to site-specific transgene integration (Daniell et al., 2002), and lack of pleiotropic effects due to subcellular compartmentalization of transgene products (Daniell et al., 2001a; Lee et al., 2003; Leelavathi et al., 2003). Because of these advantages, the chloroplast genome has been engineered to confer several useful agronomic traits, including herbicide resistance (Daniell et al., 1998), insect resistance (McBride et al., 1995; Kota et al., 1999), disease resistance (DeGray et al., 2001), drought tolerance (Lee et al., 2003), salt tolerance (Kumar et al., 2004a), and phytoremediation (Ruiz et al., 2003). The chloroplast genome has also been used in molecular farming to express human therapeutic proteins (Guda et al., 2000; Staub et al., 2000; Fernandez-San Millan et al., 2003; Leelavathi and Reddy, 2003; Daniell et al., 2004a, 2004b, 2004c), vaccines for human (Daniell et al., 2001a, 2004c; Daniell, 2004; Watson et al., 2004; Chebolu and Daniell, 2005) or animal use (Molina et al., 2004), and biomaterials (Guda et al., 2000; Lossl et al., 2003; Vitanen et al., 2004). Genetically engineered CMS via the chloroplast genome may be used for the safe integration of foreign genes via the nuclear genome and in those rare cases where plastids genomes are paternally or biparentally transmitted (Hagemann, 2004). Recently, plastid transformation was demonstrated in carrot (*Daucus carota*; Kumar et al., 2004a), showing hyperexpression of the transgene in non-green plastids to levels of up to 75% the expression observed in leaf chloroplasts. Additionally, plastid transformation of recalcitrant crops such as cotton (*Gossypium hirsutum*; Kumar et al., 2004b) and soybean (*Glycine max*; Dufourmantel et al., 2004) allows the

application of the cytoplasmic male-sterile system to commercially important crops.

An important consideration for hybrid development using CMS systems is the requirement (or not) for male-fertility restoration. For vegetable, fruit, or forage crops, restoration of male fertility in the hybrid is not necessary. This simplifies the production of hybrids because effort can concentrate on maintainer line development, without concern for whether the pollinator restores male fertility in the hybrid. For crops with seeds as the economically important product, such as canola, sunflower (*Helianthus annuus*), or maize, one or both of the hybrid's parents must bring in male-fertility restoration factors or the male-sterile hybrid seed must be blended with male-fertile hybrid seed (Havey, 2004). In currently available cytoplasmic male-sterile lines, nuclear genome controls various restoration factors, and such factors are often located at multiple loci. However, we report here restoration of male fertility by changing conditions of illumination. Also, in the case of β -ketothiolase-induced male sterility, fertility can be obtained by the use of regulatory or inducible elements instead of constitutive expression. Thus, this is a novel approach for creating male-sterile transgenic plants, which may help advance the field of plant biotechnology through effective transgene containment.

Havey (2004) documents the worldwide use of CMS to produce competitive hybrid cultivars. Major investments of time and resources are required to backcross a male-sterility-inducing cytoplasm into elite lines. These generations of backcrossing could be avoided by transformation of an organellar genome of the elite male-fertile inbred to produce female inbred lines for hybrid seed production. Because the male-fertile parental and male-sterile transformed lines would be developed from the same inbred, they should be highly uniform and possess the same nuclear genotype, excluding mutations and residual heterozygosity (Havey, 2004). Therefore, the male-fertile parental line becomes the maintainer line to seed-propagate the newly transformed male-sterile line (Havey, 2004). A few generations of seed increases would produce a CMS-maintainer pair for hybrid seed production. An additional advantage of organellar transformation would be the diversification of CMS sources used in commercial hybrid-seed production. Transformation of the chloroplast genome would allow breeders to introduce different male-sterility-inducing factors into superior inbred lines. Introduction of a male-sterility-inducing transgene into one of the organellar genomes of a higher plant would be a major breakthrough in the production of male-sterile inbred lines (Havey, 2004). This technique would be of great potential importance in the production of hybrid crops by avoiding generations of backcrossing, an approach especially advantageous for crop plants with longer generation times (Havey, 2004).

MATERIALS AND METHODS

Chloroplast Vector Construction

Plasmid DNA from *Acinetobacter* sp. coding for the *phaA* gene (pJKD 1425) was kindly provided by Metabolix (Cambridge, MA). Isolation and amplification of the *phaA* gene from the native plasmid were performed by PCR using *phaA*-specific 5' and 3' flanking DNA primers. All primers were designed using the QUICKPRI program of the DNASTAR software (Madison, WI). The PCR product was cloned into the vector pCR2.1-5'UTR*psbA*, which contained the functional *psbA* gene promoter and 5' regulatory sequence, by directional cloning after *NdeI* and *NotI* restriction digestion of the PCR product and vector. The *phaA* gene and the 5'UTR*psbA* regions were sequenced and subsequently cloned into the chloroplast transformation vector pLDR-ctv, by directional insertion using appropriate restriction sites.

Chloroplast Transformation and Selection of Transgenic Plants

The delivery of the pLDR-5'UTR-*phaA*-3'UTR vector to the chloroplast by particle bombardment and the subsequent selection process of the transgenic tobacco (*Nicotiana tabacum* var Petit Havana) lines were performed essentially as described previously (Daniell, 1997; Daniell et al., 2004a; Kumar and Daniell, 2004). Tobacco leaves were bombarded using the biolistic device PDS-1000/He (Bio-Rad, Hercules, CA). After bombardment, leaves were placed on regeneration medium of plants, supplied with 500 $\mu\text{g mL}^{-1}$ spectinomycin for two rounds of selection on plates, and subsequently moved to jars on Murashige and Skoog medium containing 500 $\mu\text{g mL}^{-1}$ spectinomycin. Finally, homoplasmic plants were transferred to high nutrient soil and grown in controlled growth chambers at 26°C in a 16-h-light/8-h-dark photoperiod.

Confirmation of Chloroplast Integration by PCR

Total plant DNA from untransformed and transgenic plants was isolated using the DNeasy plant mini kit (Qiagen, Valencia, CA) and used as the template for PCR reactions. The PCR primer pairs 3P-3M and 4P-4M were used to confirm the integration of the gene cassette into the chloroplast genome, essentially as described previously (Daniell et al., 2004a). Primer pairs 5P-2M, 5P-*phaA*internal, and 5P-3'*phaA* were used to confirm the presence of the *phaA* gene. PCR analysis was performed using the Gene Amp PCR System 2400 (Perkin-Elmer, Chicago).

Southern-Blot Analysis

Total plant DNA was obtained from T_0 and T_1 transgenic plants as well as from untransformed tobacco plants using the DNeasy plant mini kit (Qiagen) and protocol. Southern-blot analyses were performed essentially as described previously (Daniell et al., 2004a). Two micrograms of plant DNA was digested with *BamHI* and resolved on a 0.8% (w/v) agarose gel at 50 V for 2 h. The gel was soaked in 0.25 N HCl for 15 min and then was rinsed two times with water. The gel was then soaked in transfer buffer (0.4 N NaOH and 1 M NaCl) for 20 min, and the denatured DNA was transferred overnight to a nitrocellulose membrane by capillarity. The next day the membrane was rinsed twice in 2 \times SSC (0.3 M NaCl and 0.03 M sodium citrate), dried on Whatman paper, and then cross-linked in the GS GeneLinker (Bio-Rad) at setting C3 (150 nJoules). The flanking sequence probe was obtained by *BglIII/BamHI* restriction digestion of plasmid pUC-ct, which contains the chloroplast flanking sequence (*trnI* and *trnA* genes). The *phaA* probe was obtained by *NdeI/NotI* restriction digestion of plasmid pCR2.1-5'UTR-*phaA*. Probes were radiolabeled with ^{32}P dCTP by using Ready Mix and Quant G-50 microcolumns for purification (Amersham, Arlington Heights, IL). Prehybridization and hybridization were performed using the Quick-Hyb solution (Stratagene, La Jolla, CA). The membrane was washed twice for 15 min at room temperature in 2 \times SSC with 0.1% (w/v) SDS, followed by two additional washes at 60°C (to increase the stringency) for 15 min with 0.1 \times SSC with 0.1% (w/v) SDS. Radiolabeled blots were exposed to x-ray films and then developed in the Mini-Medical Series x-ray film processor (AFP Imaging, Elmsford, NY).

Northern-Blot Analysis

Total plant RNA from untransformed and chloroplast transgenic plants was isolated using the RNeasy mini kit (Qiagen) and protocol. Northern-blot

analyses were performed essentially as described previously (Ruiz et al., 2003). Total RNA (2.5 μg) per plant sample was resolved in a 1.2% (w/v) agarose/formaldehyde gel. The *phaA* probe generation, labeling reaction, prehybridization/hybridization, membrane washing steps, and autoradiography were performed essentially as explained above in the Southern-blot section. The *aadA* probe was amplified by PCR from the pLD-ctv-*aadA*.

Western-Blot Analysis

Protein samples were obtained from 100 mg of leaf material from untransformed plants and transgenic lines by grinding the tissue to a fine powder in liquid nitrogen, subsequent homogenization in 200 μL of plant protein extraction buffer (100 mM NaCl, 10 mM EDTA, 200 mM Tris-HCl, 0.05% [w/v] Tween 20, 0.1% [w/v] SDS, 14 mM BME, 400 mM Suc, and 2 mM phenylmethylsulfonyl fluoride), and a centrifugation step at 15.7g for 1 min to remove solids. Protein concentrations were determined by Bradford assay (Bio-Rad Protein Assay) with bovine serum albumin as the protein standard. Proteins were resolved by electrophoresis in a 12% (v/v) SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked for 1 h with PTM buffer: 1 \times PBS (phosphate-buffered solution), 0.05% (v/v) Tween 20, and 3% (w/v) nonfat dry milk. The membrane was probed for 2 h with rabbit anti- β -ketothiolase antibody (Metabolix) in a dilution of 1:1,000, then rinsed with water twice and probed with alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit; Sigma, St. Louis) for 1.5 h in a 1:20,000 dilution. Finally, the membrane was washed three times for 10 min with PT buffer (1 \times PBS, 0.05% [v/v] Tween 20) and one time with 1 \times PBS, followed by incubation in Lumi-phos WB (Pierce, Rockford, IL) reagent for the alkaline phosphatase reaction. Film exposure took place for 3 min.

β -Ketothiolase Activity Assay

Protein samples were obtained by grinding 1 g of leaf tissue to a fine powder in liquid nitrogen, followed by the addition of 2 mL of ice-cold β -ketothiolase extraction buffer (100 mM Tris-HCl, pH 8.1, 50 mM MgCl_2 , 5 mM BME) and homogenization. The homogenates were centrifuged for 10 min at 4°C at 5,000g, and the supernatant was passed through PD-10 columns (Amersham) containing Sephadex G-25 M for desalting, and elution was optimized for the recovery of proteins of size range 25 to 60 kD. Protein concentration was determined by a Bradford assay. β -Ketothiolase activity was measured spectrophotometrically at 304 nm in the thiolysis direction (breaking down acetoacetyl-CoA to acetyl-CoA) by monitoring the disappearance of acetoacetyl-CoA for 60 s, which in the presence of magnesium ion forms a magnesium enolate with A_{304} ; this protocol is an adaptation of the protocol by Senior and Dawes (1973). The reaction took place in a total volume of 1 mL containing 62.4 mM Tris-HCl, pH 8.1, 50 mM MgCl_2 , 62.5 μM CoA, 62.5 μM acetoacetyl-CoA (substrate is dissolved in 50 mM phosphate buffer, pH 4.7), 10 μL of plant extract (β -ketothiolase sample), and bringing the volume with distilled water to 1 mL. The plant extract containing the β -ketothiolase was added at the end immediately before the sample reading. In this assay, the enzyme specific activity is given in units per milligram of total plant protein, and 1 unit is defined as the degradation of 1 $\mu\text{mol}/\text{min}$ of acetoacetyl-CoA under standard reaction conditions.

Scanning Electron Microscopy

SEM was performed at the AMPAC facility at the University of Central Florida. Anthers and pollen samples were gold coated on a Sputter Coater (Emitech, Houston) with a gold film thickness of 150 Å. SEM pictures were produced using the scanning electron microscope model JSM-6400F (JEOL, Peabody, MA) and the x-ray energy dispersive spectrometer (Edax, Mahwah, NY) at an acceleration voltage of 6 kV.

Histological Analysis of Anthers

Anthers at relevant developmental stages were dissected from flower buds and fixed in 3% (v/v) glutaraldehyde in phosphate buffer for 12 h at room temperature, applying a continuous vacuum for the first 3 h of incubation and degassing (by bringing the vacuum up and down slowly) for 10 min at 1, 2, and 3 h. The fixed anthers were dehydrated in an ethanol series (5%, 10% to 80% in increments of 10, 95% and 100%) for 30 min per gradient treatment. Samples were kept overnight in fresh 100% ethanol and were washed twice the next day for 1 h in 100% ethanol. Samples were then treated with a gradient

(25% to 100%) of Citro Solv clearing (Fisher, Pittsburgh) reagent for 30 min per gradient treatment, and finally embedded in Paraplast Plus (Fisher). Tissue embedding was performed in molten paraffin for 3 d, changing the molten paraffin every 8 to 12 h. Paraffin-treated tissue was finally embedded into paraffin blocks by using the Leica EG 1160 paraffin embedding station (Leica, Solms, Germany). A metal blade microtome, model HM 315 (MICROM, Walldorf, Germany), was used for tissue embedded sectioning. Finally, tissue sections were put onto Superfrost/Plus microscope slides, followed by a rehydration step and tissue staining with 0.05% (w/v) toluidine blue. Tissue slides were observed under the Olympus BX60F5 light microscope and Olympus U-CMAD-2 camera (Olympus, Melville, NY). Flower developmental stages were characterized following the procedure described by Koltunov et al. (1990).

Reversibility of Male Fertility

Two independent transgenic plants were moved to a separate growth chamber after the first indication of flower bud formation and were kept away from any contact with untransformed plants and other transgenic lines; the flowers were covered with thin transparent plastic bags to inhibit any possibility of cross-pollination. Bags were only removed to take pictures. Transgenic plants were kept under continuous illumination for 10 d with a photon flux density of 11,250 $\mu\text{E m}^{-2}$ supplied throughout this period. The number of flowers developed was counted daily throughout these 10 d, while newly formed flowers, senescent flowers, and fallen flowers were recorded. Fruit capsules and seeds that developed were also counted. After the 10 d, a 16-h-light/8-h-dark photoperiod was reestablished, while the plants were kept from contact with any other plant for 20 d to allow maturation of the fruit capsules and to harvest seeds produced during continuous illumination.

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