# Production of a Biodegradable Plastic Polymer, Poly-β-Hydroxybutyrate, in Transgenic Alfalfa

Purev Saruul, Friedrich Srienc, David A. Somers, and Deborah A. Samac*

## ABSTRACT

**Alfalfa (*Medicago sativa* L.) is primarily used as a source of forage and animal feed. Improving the economic value of alfalfa could be achieved by developing new uses of this perennial crop. To investigate the potential of alfalfa as a source of industrial materials, we employed a genetic transformation approach to produce a biodegradable plastic, poly-β-hydroxybutyrate (PHB), in the leaves of alfalfa plants. Three genes from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) encoding the enzymes for synthesis of PHB (*phb*A, *phb*B, *phb*C) and a copolymer of PHB and polyhydroxyvalerate (polyhydroxybutyrate-*co*-hydroxyvalerate, or PHB/V) (*bkt*B, *phb*B, *phb*C) engineered for plastid targeting were introduced into alfalfa by *Agrobacterium*-mediated transformation. DNA and RNA blot analyses of transgenic plants indicated integration and expression of the PHB biosynthetic pathway genes. Polyhydroxybutyrate content in the leaves of transgenic plants ranged from ≈0.025 to 1.8 g kg$^{-1}$ dry weight (DW). Agglomerations of PHB granules 0.2 to 0.4 μm in diameter, similar to bacterial PHB, were located in the chloroplasts of transgenic plants, demonstrating that *phb* gene products were targeted into the plastids of transgenic alfalfa. Transgenic plants exhibited growth similar to untransformed plants, suggesting that expression of PHB biosynthetic pathway genes at current levels and accumulation of product in the plastids had no deleterious effect on growth and fertility. $F_1$ hybrid progeny, obtained from crosses of PHB transgenic plants with elite alfalfa germplasm, exhibited leaf PHB levels similar to the transgenic parental line, demonstrating that PHB production in alfalfa is a stable and dominantly inherited trait.**

Alfalfa is one of the most important forage crops because of its high biomass production, good nutritional qualities, and adaptation to a wide range of environments. Genetic engineering has the potential to improve the economic value of alfalfa by introducing genes to improve its adaptability and agronomic characteristics, and to increase its utilization in nontraditional areas such as in phytoremediation and production of industrial raw materials. One attractive approach is to engineer alfalfa to produce biodegradable plastics as a value-added component.

Many species of bacteria accumulate polyhydroxyalkanoates (PHA) as energy storage compounds in response to nutritional stress (Anderson and Dawes, 1990). Some PHA polymers are commercially valuable as biodegradable plastics. Polyhydroxybutyrate is a PHA produced in *R. eutropha* (Yabuuchi et al., 1995) via a three-enzyme biosynthetic pathway consisting of β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase. These enzymes are encoded by *phb*A, *phb*B, and *phb*C, respectively (Slater et al., 1988). The monomeric composition of PHA polymers is determined by the substrate specificities of β-ketothiolase and PHB synthase and by the availability of acetyl-CoA and propionyl-CoA. In the case of 4- and 5-carbon PHA precursors, when β-ketothiolase is encoded by *phb*A, a homopolymer of PHB is produced. When β-ketothiolase is encoded by *bkt*B, a copolymer of PHB/V is produced (Slater et al., 1998). Because the PHB/V copolymer has better physical properties than PHB, it is preferred for most commercial applications (Anderson and Dawes, 1990). Presently, the bacterially produced polymers are not cost-competitive with nonbiodegradable plastic polymers derived from petroleum. By genetically engineering crop plants to produce PHA, a less expensive source of these polymers could become available.

PHB production in plants was first demonstrated in transgenic *Arabidopsis thaliana* (L.) Heynh. (Poirier et al., 1992). Poly-β-hydroxybutyrate is tolerated by plants if it is synthesized in chloroplasts and PHB levels ranging between 0.1 and 14% DW have been reported with minimal effects on plant growth (Nawrath et al., 1994). Although various approaches for producing PHB in plants have been reported, most of these utilize *Arabidopsis*. Recently, production of PHB/V was achieved in the seed of oilseed rape (*Brassica napus* L.), demonstrating that it is possible to produce PHA in crop plants (Slater et al., 1999).

At present, the synthesis of PHB as a single end-product in crops is economically challenging. Poly-β-hydroxybutyrate levels of 15% DW are needed for practical large-scale commercial production (Valentin et al., 1999). Recently, *Arabidopsis* plants with PHB levels of 40% DW were obtained, but the plants were stunted and infertile (Bohmert et al., 2000). However, plants producing PHA at levels compatible with growth could be used for multiple purposes to increase overall crop value. Production of bioplastics in alfalfa leaves could be combined with use of alfalfa for feed and energy. In such a system, alfalfa leaves could be harvested to produce PHB, leaf by-product could be processed into a feed, and stems could be used for producing either electricity by gasification or ethanol by fermentation. As a start towards this goal, the objectives of this study were to genetically modify alfalfa to produce PHB and

P. Saruul, Dep. of Plant Pathology, 495 Borlaug Hall; F. Srienc, Biological Process Technol. Inst., 240 Gortner Laboratories; D.A. Somers, Dept. of Agronomy and Plant Genetics, 411 Borlaug Hall; D.A. Samac, USDA-ARS-Plant Science Research Unit and Dep. of Plant Pathology, 495 Borlaug Hall, Univ. of Minnesota, St. Paul, MN 55108-6030. Funding for this project was provided by the Consortium for Plant Biotechnology Research no. CR22072-66 and Monsanto Company. Received 12 June 2001. *Corresponding author (debbys@puccini.cdl.umn.edu).

Published in Crop Sci. 42:919–927 (2002).

**Abbreviations:** DW, dry weight; GC, gas chromatography; GC-MS, gas chromatography–mass-spectrometry; 3-HB, 3-hydroxybutyrate; $^1$H-NMR, proton-nuclear magnetic resonance spectrometry; 3-HV, 3-hydroxyvalerate; NPTII, neomycin phosphotransferase II; PCR, polymerase chain reaction; PHA, polyhydroxyalkanoates; PHB, poly-β-hydroxybutyrate; PHB/V, polyhydroxybutyrate-*co*-hydroxyvalerate; RT-PCR, reverse transcriptase-polymerase chain reaction.

to initiate transfer of this trait into germplasm developed for high biomass production.

## MATERIALS AND METHODS

### Plant Transformation

For synthesis of PHB and PHB/V in alfalfa, two types of transgenic plants were produced. Vectors pMON25948 and pMON25949 (generously provided by K. Gruys, Monsanto Co., St. Louis, MO) carry the entire PHB biosynthetic pathway, but differ in the β-ketothiolase gene used. pMON25948 contains *phb*A, *phb*B, and *phb*C, each under the control of the CaMV 35S promoter and fused to the *Arabidopsis* ribulose biphosphate carboxylase (RuBisCo) small subunit 1a transit peptide. pMON25949 is identical except *phb*A was replaced with *bkt*B. Both vectors use a 35S-neomycin phosphotransferase II (NPTII) gene for selection of transformed plants. The plasmids were mobilized into *Agrobacterium tumefaciens* strain LBA4044 by triparental mating and were used to transform a highly regenerable clone selected from Regen-SY (Bingham, 1991). Transformation was carried out as described (Austin et al., 1995), with the exception that selective media contained 50 μg/mL kanamycin instead of 25 μg/mL. Approximately 160 explants were cocultivated with *A. tumefaciens* LBA4404 containing either pMON25948 or pMON25949. Only one plant from each explant was selected to ensure that each plant arose from an independent transformation event. Plants were transferred into soil and were grown in the greenhouse at 22 to 28°C under a 14-h daylength. Putative transformants were initially screened for presence of *nptII* by polymerase chain reaction (PCR) by means of the primers NPTII upper (5′-GAT ATGACTGGGCACAACAGAC-3′) and NPTII lower (5′-CG TCAAGAAGGCGATAGAAGG-3′). Each reaction contained 1 × *Taq* DNA polymerase reaction buffer (Promega, Madison, WI), 2 m*M* $MgCl_2$, 0.25 m*M* each dNTP, 5 pmol each primer, 1 unit *Taq* polymerase (Promega), and ≈1 μg plant DNA. Polymerase chain reaction was conducted for 30 cycles of 94°C for 30 sec, 55°C for 1 min, and 72°C for 1 min. Polymerase chain reaction products (20 μL) were electrophoresed on a 1% (w/v) agarose gel and visualized by staining with ethidium bromide.

### RNA and DNA Blot Analyses

RNA and genomic DNA from alfalfa leaves were isolated by means of Trizol[1] reagent (Life Technologies, Inc., Rockville, MD) and the Puregene kit (Gentra System, Inc., Minneapolis, MN), respectively, following the manufacturers' instructions. Genomic DNA (20 μg) was digested to completion with restriction enzymes and electrophoresed through 0.8% (w/v) agarose gels. Total RNA (10 μg) was electrophoretically separated through 1% (w/v) formaldehyde/agarose gels. Equal loading of lanes was confirmed by ethidium bromide staining of gels prior to blotting. Gels were blotted to a Zeta-Probe membrane (Bio-Rad, Hercules, CA), prehybridized, hybridized, and washed as recommended by the manufacturer. Probes were made from the coding regions of *phb*A and *phb*B labeled with $^{32}P$ by means of a random priming kit (Roche Laboratories, Nutley, NJ).

[1] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

### Reverse Transcriptase-Polymerase Chain Reaction Analysis

Two 30-mer oligonucleotides (5′-ATTTTCGATGACTG ACGTTGCATCGTATC-3′ and 5′-CTGGCAGTCGAGCGC AAATAAGAATTCTTA-3′), specific to the *phb*A gene from *R. eutropha*, were used as primers in reverse transcriptase-PCR (RT-PCR) analysis by means of the Access RT-PCR System (Promega) and total alfalfa RNA. The 1.3-kilobase pair DNA product was purified by means of a QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Nucleotide sequence data were obtained by means of the same primers as for RT-PCR. Sequences were analyzed and compared by the BLAST program of the Wisconsin Genetic Computer Group software package (Accelrys, San Diego, CA).

### Gas Chromatography and GC-Mass Spectrometry Analysis

Transgenic plants were grown in the greenhouse for 9 wk until the oldest leaves started to senesce to allow for maximal accumulation of PHB. Foliage was harvested, frozen at −80°C, and lyophilized until dry (≈4 d). From each transgenic line, ≈50 mg of dried alfalfa leaves were analyzed for PHB content two times. Whole leaflets were extracted at 95°C for 4 h with a mixture of 1 mL of chloroform and 1 mL of methanol:sulfuric acid (85:15, v/v) containing 100 μg/mL benzoic acid as an internal standard. Following the acid methanolysis, samples were extracted with 1 mL of 0.9 *M* NaCl, and the organic phase was subjected to GC. Chromatography was carried out with a Hewlett Packard 5890 fitted with a 30 m DB Wax 30W column (J&W Scientific, Inc., Folsum, CA). The temperature profile used was 60°C for 0.5 min, increasing at a rate of 10°C $min^{-1}$ for 14 min, and 200°C for 5 min. As a standard, bacterial PHB (Polysciences, Inc., Warrington, PA) was used. For GC–mass-spectrometry (GC-MS), a 1-μL aliquot of the methyl ester derivatives of PHB prepared as described above was injected into a Varian Saturn-3 GC-mass spectrometer and the electron impact mass spectrum was collected at 70 eV. The mass fragmentation profile was compared with that of the PHB standard.

### Nuclear Magnetic Resonance Spectroscopy

Dehydrated alfalfa leaves (2 g) were ground to a fine powder with a coffee grinder, extracted with methanol for 1 d in a Soxhlet apparatus (Kontes Glass Company, Vineland, NJ), and dried for ≈6 h at room temperature. Polymer was extracted from the plant material with hot chloroform in a Soxhlet apparatus for 1 d. The polymer in chloroform solution was concentrated by evaporation and purified by reprecipitation with methanol (chloroform:methanol, 1:5 v/v). Following an overnight precipitation, samples were centrifuged at 4000 × g for 15 min. The pellet was resuspended in 0.5 mL of deuterated-chloroform and analyzed with a 500-MHz Nicolet (Warwick, UK) NT-300WB FT-NMR.

### Visualization of Polyhydroxybutyrate

For visualization by epifluorescence, leaf tissues from 60-d-old transgenic and control plants were stained with Nile Blue A, as described by Ostle and Holt (1982), and specimens were viewed under an excitation wavelength of 546 nm with a Nikon (Tokyo, Japan) Eclipse E800 photomicroscope equipped with epifluorescence illumination with standard UV. A 550-nm filter was used to reduce autofluorescence from chlorophyll. Digital images were collected with a CoolCam liquid-cooled,

three-chip color charge-coupled device camera (Cool Camera Company, Decatur, GA).

Optical sections of the specimens stained with Nile Blue A were viewed with a BioRad MRC-1024 confocal microscope attached to a Nikon Diaphot inverted microscope equipped with a 15-mW Krypton/Argon laser. Digital single images were collected by projection of the optical sections using LaserSharp version 3.2 software (Bio-Rad Microscience Ltd., Hemel Hempstead, UK) and analyzed using NIH Image version 1.62 (National Instiutes of Health, Bethesda, MD).

For transmission electron microscopic analysis, alfalfa plants were kept in darkness for 3 d to deplete starch and samples were prepared as described by Poirier et al. (1992). For immunolocalization, mature leaves were fixed with 3% (w/v) paraformaldehyde:0.25% glutaraldehyde in 0.1 *M* sodium phosphate buffer (pH 7.2) for 2 h at room temperature, then washed in 0.1 *M* sodium phosphate buffer (pH 7.2). Samples were dehydrated in a graded ethanol series and embedded in LR White resin (Sigma-Aldrich, St. Louis, MO). The resin was polymerized for 1 h in a microwave oven initially at 45°C for 10 min, then the temperature was increased 10°C at 10-min intervals until reaching 95°C. The resulting blocks were sectioned with a microtome RMC-7000. Ultra-thin sections (55 nm) were mounted on formvar-coated 300-mesh nickel grids (Electron Microscopy Sciences, Fort Washington, PA) and blocked with 5% (v/v) normal goat serum, 5% (v/v) glycerol, and 0.04% (w/v) sodium azide in 10 m*M* potassium phosphate (pH 7.2) overnight at room temperature. Grids were incubated overnight at 4°C with antiserum against PHB synthase (generously provided by Y. Poirier) diluted in the blocking solution (1:100). Following washing, grids were incubated with a 1:50 dilution of gold-conjugated goat anti-rabbit antibodies (10 nm gold particles) in blocking buffer for 4 h at 37°C. Specimens were double-stained with uranyl acetate and lead citrate and examined with a FEI CM-12 transmission electron microscope (FEI Company, Hillsboro, OR).

### Introgression into Elite Germplasm

Selected transgenic lines were cross-pollinated manually with two elite alfalfa germplasms, UMN 3145 and UMN 2966 (provided by J.F.S. Lamb, USDA-ARS, St. Paul, MN), without emasculation as described by Barnes (1980). Fifteen plants from UMN 3145, an alfalfa germplasm developed for high biomass, were pollinated with five original transgenic lines ranging in PHB amounts from 0.34 to 1.8 g $kg^{-1}$. Two plants from UMN 2966, alfalfa germplasm adapted for conditions in the midwestern USA, were cross-pollinated with seven individual transgenic lines with a range of PHB levels from 0.11 to 0.53 g $kg^{-1}$. Seeds from the individual crosses were collected separately and a subset of $F_1$ hybrids from a reciprocal cross (Line 863 with UMN 2966) was grown in the greenhouse for progeny analysis.

## RESULTS AND DISCUSSION

### Expression of Polyhydroxybutyrate Biosynthetic Pathway in Transgenic Alfalfa

From a total of 114 plants putatively transformed with pMON25948 and 98 plants transformed with pMON25949, 100 (88%) and 85 (86%) were positive for the presence of *nptII*, respectively. Integration of genes encoding the PHB biosynthetic pathway into the alfalfa genome was confirmed by DNA blot analysis. Genomic DNA from three transgenic lines, 863, 864 (pMON85948), and 913 (pMON85949), was digested with *Eco*R1 and *Eco*RV, and blots hybridized with probes of *phb*A or *phb*B (Fig. 1). For Line 864, a single high molecular weight band was observed with the *phb*A probe of *Eco*R1-digested DNA, and in Lines 864 and 913, the *phb*B probe hybridized to a single band in *Eco*RV-digested DNA. Because one restriction site is located in the T-DNA and the other presumably in genomic DNA, this indicates a single locus T-DNA insertion event in Lines 864 and 913. Multiple bands were observed for Line 863, indicating insertion in more than one locus. The *phb*A probe did not cross-hybridize with the *bkt*B gene in Line 913, and no distinct bands were observed with any of the probes in lanes containing genomic DNA from untransformed control plants.

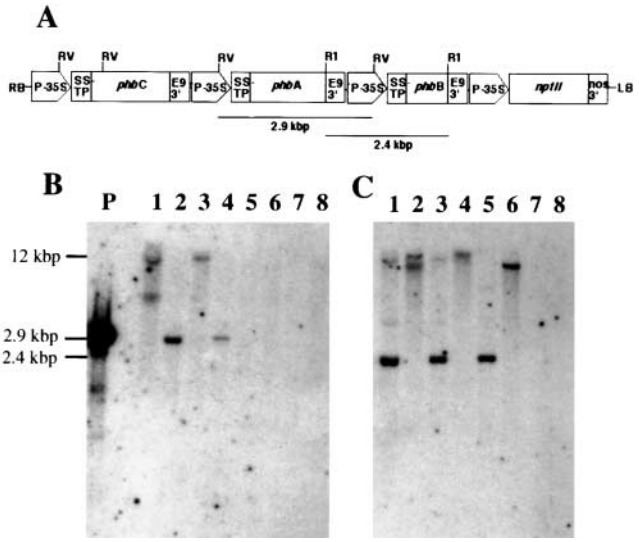


**Fig. 1. DNA Southern blot analysis of transgenic alfalfa plants. Restriction enzyme-digested genomic DNA was hybridized with $^{32}$P-labeled *phb*A (Panel B) or *phb*B (Panel C). (A) Restriction map of the T-DNA from pMON25948. Bars underneath the map indicate sizes of restriction fragments containing *phb*A or *phb*B. R1, *Eco*R1 site; RV, *Eco*RV site; RB and LB, right and left T-DNA border, respectively; P-35S, CaMV 35S promoter; SS-TP, transit peptide of the small subunit of Rubisco. E9-3′ and nos 3′, terminator region of soybean E9 and *Agrobacterium* nopaline synthase, respectively. (B and C) Lane P, *Eco*RV-digested pMON25948; Lane 1, Line 863 digested with *Eco*R1; Lane 2, Line 863 digested with *Eco*RV; Lane 3, Line 864 digested with *Eco*R1; Lane 4, Line 864 digested with *Eco*RV; Lane 5, Line 916 digested with *Eco*R1; Lane 6, Line 916 digested with *Eco*RV; Lane 7, untransformed control digested with *Eco*R1; Lane 8, untransformed control digested with *Eco*RV.**

Expression of each of the *phb* genes in transgenic alfalfa was tested by RNA blot analysis. RNA from mature leaves of transgenic Lines 864 and 913 was probed with the individual *phb* genes. Transcripts of the expected sizes for all three *phb* genes were detected, indicating that alfalfa transformants contained transcriptionally active genes of the PHB biosynthetic pathway (Fig. 2). Additional tissues from transgenic Line 864 were analyzed for expression of each of the *phb* genes. All three genes of the PHB biosynthetic pathway were constitutively expressed in the leaf, stem, root and root-nodule tissues of transgenic alfalfa (data not shown). As a control in RNA blotting experiments, we included RNA from untransformed alfalfa leaves on

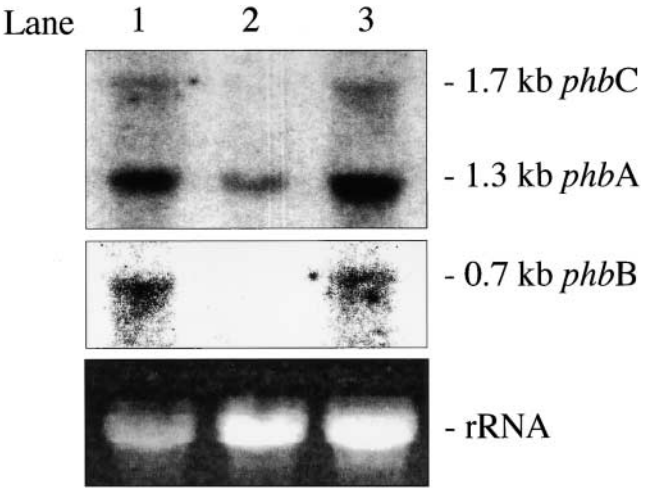


**Fig. 2. RNA northern blot analysis of transgenic alfalfa plants. Total RNA from mature leaves of transgenic lines and untransformed alfalfa were hybridized with individual *phb* genes. RNA loaded in each lane is shown by ethidium bromide staining of 28S rRNA. Lane 1, Line 864; Lane 2, untransformed alfalfa; Lane 3, Line 913.**

the blots and hybridized with probes made from the individual *phb* genes (Fig. 2). The *phb*A probe hybridized to a 1.3-kb band under highly stringent conditions in repeated experiments with separate preparations of RNA from untransformed alfalfa plants, whereas hybridization with the *phb*B or *phb*C gene probes was not detected. To further investigate the transcript hybridizing with *phb*A in untransformed alfalfa, we cloned the transcript by RT-PCR using *phb*A gene-specific primers and sequenced 982-bp from the single 1.3-kb product obtained (GenBank accession: AF410849). Surprisingly, the DNA sequence analysis revealed that *R. eutropha phb*A (Slater et al., 1988) and the untransformed alfalfa sequence share 92% nucleotide identity. This was confirmed by DNA blot analysis, which showed that the 1.3 kb RT-PCR fragment from untransformed alfalfa had strong hybridization with the bacterial *phb*A gene (data not shown). The BLAST search analysis of the untransformed alfalfa sequence revealed 55 to 60% nucleotide identity with 3-ketoacyl-CoA thiolase genes from *Arabidopsis* (Hayashi et al., 1998), canola [*B. rapa* L. subsp. *oleifera* (DC.) Metzg.; Olesen and Brandt, 1996], cucumber (*Cucumis sativus* var. *sativus*; Preisig-Muller and Kindl, 1993), and pumpkin (*Cucurbita pepo* L.; Kato et al., 1996). Given the high nucleotide conservation of the RT-PCR product with *R. eutropha phb*A (92%), and lack of a distinct hybridization signal when genomic DNA from untransformed alfalfa was probed with *phb*A, it is possible that the *phb*A-hybridizing transcript in untransformed alfalfa originated from endophytic or epiphytic bacteria.

## Detection of Polyhydroxybutyrate

Gas chromatography analyses were performed to measure levels of PHB in all transgenic lines. Mature and presenescing leaves of 9-wk-old plants were harvested, and the leaf extracts were analyzed for the presence of PHB constituent monomers 3-hydroxybutyrate (3-HB). We found that 82 out of 100 PHB plants analyzed had a peak corresponding to the same elution time as the methyl 3-HB standard (Fig. 3A). Thus, 82% of the transgenic plants produced detectable amounts of the monomers that comprise PHB. Methyl 3-HB was not detected in untransformed control alfalfa plants. The transgenic plants varied widely in their PHB content, from ≈0.025 to 1.8 g $kg^{-1}$ DW (Line 864) (Fig. 3A). These concentrations are lower than the amounts of PHB previously reported in cotton (0.34% DW) (John and Keller, 1996), *Arabidopsis* (7–40% DW) (Bohmert et al., 2000; Nawrath et al., 1994; Valentin et al., 1999), and *Brassica* (<3% DW) (Slater et al., 1999). The reasons for low amounts of PHB produced in our study are unclear. The PHB levels produced in various transgenic plants cited above were achieved using different genetic strategies to express the PHB biosynthetic pathway, which ultimately could influence the yield of PHB. However, from the various approaches reported, transformation with a single vector containing all genes in the PHB pathway targeted for chloroplast localization was most efficient in achieving high amounts of PHB in *Arabidopsis* (Bohmert et al., 2000; Valentin et al., 1999). Although we used the same strategy, the transgenic alfalfa plants we generated did not produce the amounts of PHB reported in *Arabidopsis* (7–40% DW).

The degree to which PHB accumulates in transgenic plants may depend on host plant and tissue. A high flux of carbon through acetyl-CoA, which is particularly enhanced in seeds of oil-accumulating plants such as *Arabidopsis* and *Brassica*, could result in a higher rate of PHB synthesis than in alfalfa leaves. As shown in Fig. 2, Line 864, with the highest accumulation of PHB, had relatively low amounts of transcript accumulation from the *phb*ABC genes, suggesting poor mRNA stability. Alternatively, the presence of four 35S promoters driving expression of the *phb* genes and the selectable marker gene could lead to T-DNA rearrangements or gene silencing. An increase in PHB production may require use of more active promoters or an increase in gene copy number through insertion of transgenes into the chloroplast genome. Some of the highest levels of transgene product accumulation have been obtained via transformation of the chloroplast genome (De Cosa et al., 2001; Lutz et al., 2001). Plastome transformation is particularly attractive because accumulation of PHB in chloroplasts is well tolerated by plants. In addition, the polycistronic nature of the chloroplast protein synthesis apparatus makes the expression of multiple genes theoretically easier than with nuclear transformation. Recently, stable expression of a biodegradable bio-elastic protein polymer was obtained by transformation of tobacco chloroplasts (Guda et al., 2000). Finally, PHB extraction from plant cells remains challenging. Mittendorf et al. (1998) reported that ≈60 to 75% of the PHB remained in *Arabidopsis* plant material after extraction procedures. It is possible that the PHB detected in alfalfa plants accounts for only the fraction of PHB polymers that were able to pass through the cell wall matrix, while PHB remaining trapped inside the cells may have escaped GC detection.

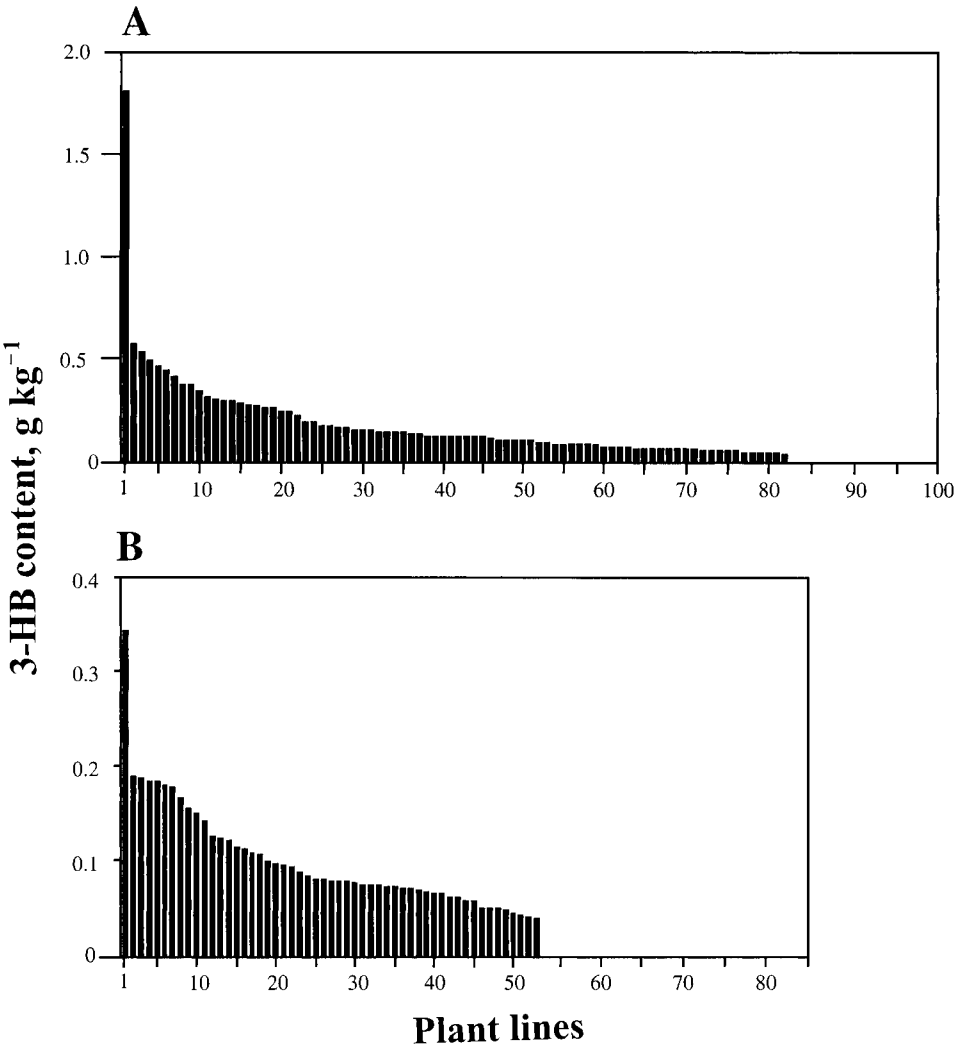


**Fig. 3. Accumulation of 3-hydroxybutyrate (3-HB) in alfalfa plants. (A) Plants transformed with Vector pMON25948 (*phb*ABC). (B) Plants transformed with Vector pMON25949 (*bkt*B, *phb*BC).**

Alfalfa plants transformed with the construct for PHB/V synthesis were screened for the presence of both 3-HB and 3-hydroxyvalerate (3-HV) monomers. On the basis of GC analysis, 3-HB monomers were detected in 53 plants from a total of 85 (Fig. 3B). However, only one transgenic line (913) had detectable amounts of a compound that eluted with the same retention time as the methyl 3-HV standard. To synthesize PHB/V in plants requires sufficient flux of both acetyl-CoA and propionyl-CoA. Unfortunately, plants do not produce large amounts of propionyl-CoA in plastids (Poirier, 1999). Creating a high flux of propionyl-CoA in plastids would require a further modification of metabolic pathways. Polyhydroxybutyrate-*co*-hydroxyvalerate accumulated to 3% DW in *Arabidopsis* leaves and in the seeds of *B. napus*, when threonine deaminase encoded by *ilv*A from *Escherichia coli* for synthesis of propionyl-CoA was expressed along with *bkt*B, *phb*B, and *phb*C (Slater et al., 1999). The transformation construct used in our study did not contain genes needed to boost the pool of precursors in the plastids. Therefore, the absence of detectable amounts of 3-HV is most likely the result of insufficient flux of substrates to synthesize 3-ketovaleryl-CoA for production of PHB/V. Gas chromatography analyses of 3-HB content in the leaf extracts from PHB/V plants varied from ≈0.037 to 0.34 g kg$^{-1}$ (Fig. 3B). The average accumulation of 3-HB in PHB/V plants was two-fold lower than in PHB plants, indicating that expression of the two different β-ketothiolase genes, *phb*A and *bkt*B, has a significant effect on the level of PHB produced in alfalfa leaves. Our results suggest that additional metabolic modification to boost the levels of propionyl substrate in the plastids of alfalfa plants is required for efficient synthesis of PHB/V.

Further identification of the compound from transgenic alfalfa eluting with 3-HB was carried out by GC-MS. Gas chromatography–mass-spectrometry analysis showed that 3-HB from transgenic plants had a mass fragmentation pattern similar to that of the bacterial PHB standard (Fig. 4), indicating the presence of PHB in the transgenic alfalfa plants. However, GC and GC-MS data provide indirect evidence for the presence of PHB in transgenic plants, since both methods detect a derivatized product of the polymer.

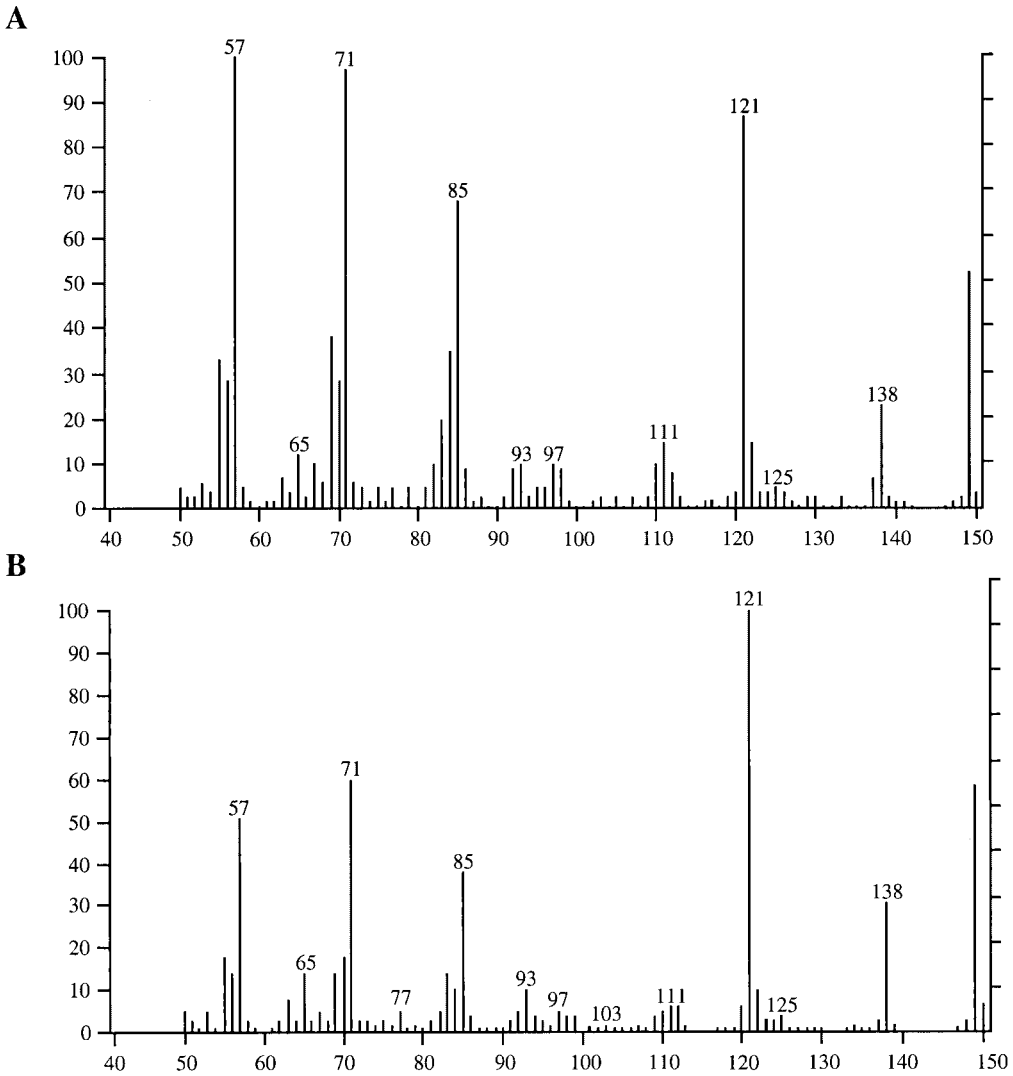


**Fig. 4. Gas chromatography–mass-spectrometry analysis of PHB produced in transgenic alfalfa. (A) Electron impact mass fragmentation spectrum of Line 864. (B) Poly-β-hydroxybutyrate standard.**

To verify the presence of PHB polymer, extracts from transgenic plants were analyzed by proton-nuclear magnetic resonance spectrometry ($^1$H-NMR). Because this method requires a relatively large amount of material, transgenic plants were propagated by vegetative stem cuttings and leaves from multiple plants were harvested after 56 d of growth. Solvent extracts of PHB Lines 863 and 864, PHB/V Line 913 and a nontransformed control prepared as described (Mittendorf et al., 1998) were subjected to $^1$H-NMR. The $^1$H-NMR spectrum obtained from the two PHB plants showed that both transgenic lines had the characteristic peaks at 1.2, 2.5, and 5.2 ppm found in the PHB standard (Yoshie et al., 1992) and an example of the analysis is shown on Fig. 5. For the PHB/V line, the $^1$H-NMR spectrum showed that it contained PHB polymer, but peaks corresponding to 3HV were not apparent, partly due to interference by contaminants in plant cell extracts. It is also possible that 3HV synthesized in plant cells are below the detection levels. Extracts from control nontransformed alfalfa leaves contained no peaks characteristic of the PHB standard. The results from the $^1$H-NMR analysis demonstrated that insertion of *phb*ABC into alfalfa results in synthesis of polymeric PHB.

## Accumulation of Polyhydroxybutyrate in Plant Cells

We employed several microscopy-based methods to visualize PHB accumulation inside the plant cells. The dye, Nile Blue A, is used to stain bacterial PHB (Ostle and Holt, 1982). Stained PHB granules emit red fluorescence at an excitation wavelength of 546 nm. Tissues from the leaf, stem, and root nodules of both wild type and transgenic alfalfa stained with Nile Blue A were observed by epifluorescent microscopy. We detected similar bright fluorescing granules in the leaves, stems, and root nodules of transgenic plants. The diameter of fluorescing granules was ≈0.3 μm. Single images projected from 24 consecutive optical sections from leaves of untransformed and transgenic alfalfa are shown in Fig. 6A, B. Fluorescing particles were observed in all

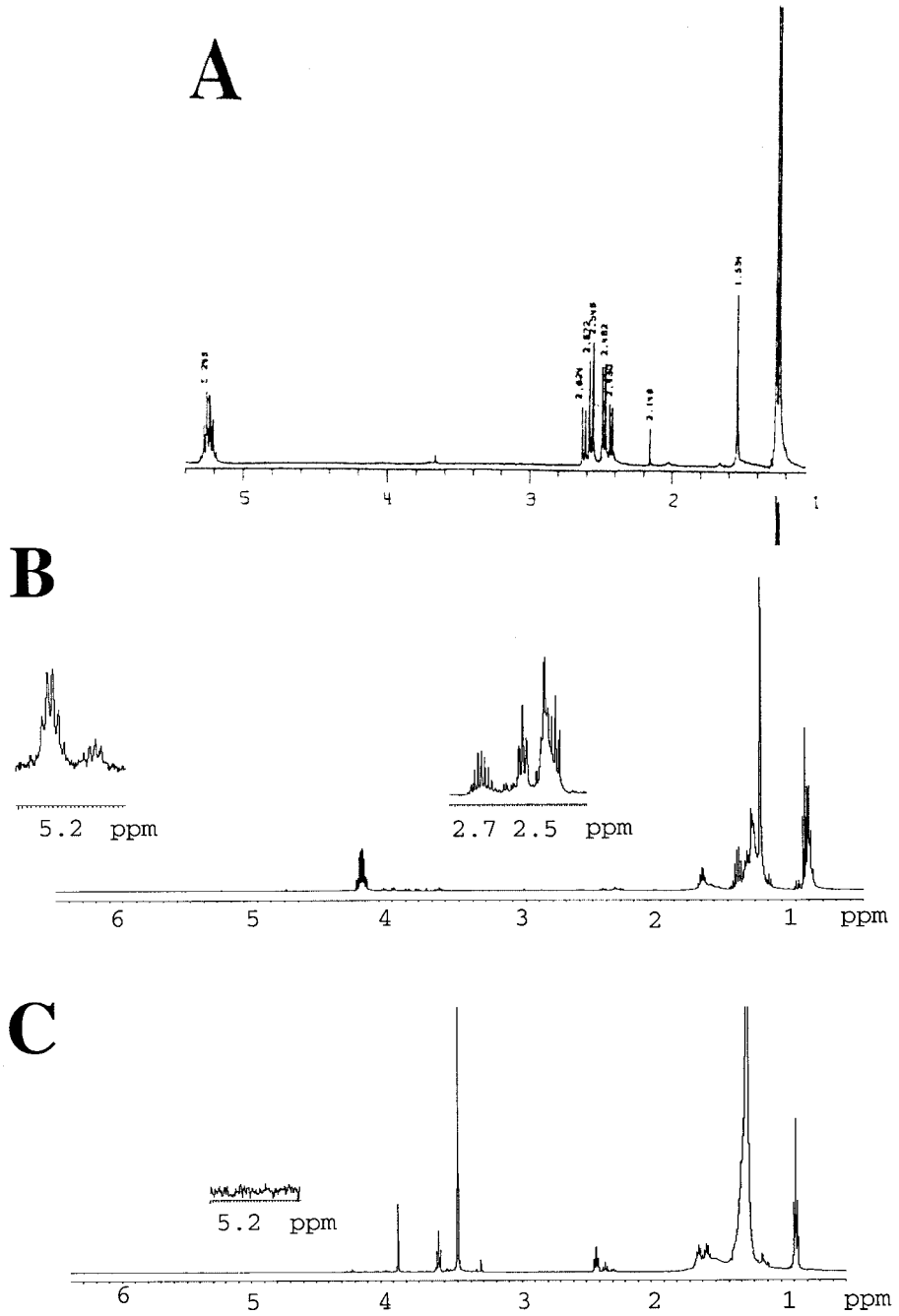


**Fig. 5. Proton-nuclear magnetic resonance spectrometry ($^1$H-NMR) analysis of alfalfa leaf extracts for poly-β-hydroxybutyrate (PHB). (A) 500-MHz $^1$H-NMR spectrum of PHB standard; (B) Line 864 transformed with *phb*ABC genes; (C) Control, untransformed alfalfa. Note the expanded spectrum containing PHB peaks (2.5 and 5.2 ppm) present in transgenic plants.**

chloroplast-containg leaf and stems cells of transgenic plants, but fluorescing particles were not seen in the untransformed control. Root nodules of both transgenic and control plants contained numerous fluorescent spots. Although *Sinorhizobium meliloti* cells naturally produce PHB as free-living bacteria, they do not produce PHB when in symbiotic association with alfalfa (Willis and Walker, 1998). Thus, the fluorescence observed was not due to PHB in bacteroids. Optical sectioning of the nodules by confocal microscopy revealed that intercellular spaces of untransformed root nodules appeared to contain red fluorescing loci, most likely stained lipids, which resemble the PHB staining reaction (Menschik, 1953). Optical sections clearly showed that only transgenic alfalfa contained fluorescing granules, likely to be PHB, within plastids in nodule cells.

To study the cellular localization of PHB granules, we analyzed ultra-thin sections of alfalfa leaf cells. Transmission electron microscopy of Line 864, expressing all three genes, revealed that PHB accumulated as groups of electron-lucent granules (Fig. 6D). The size of PHB-associated granules ranged between 0.2 and 0.4

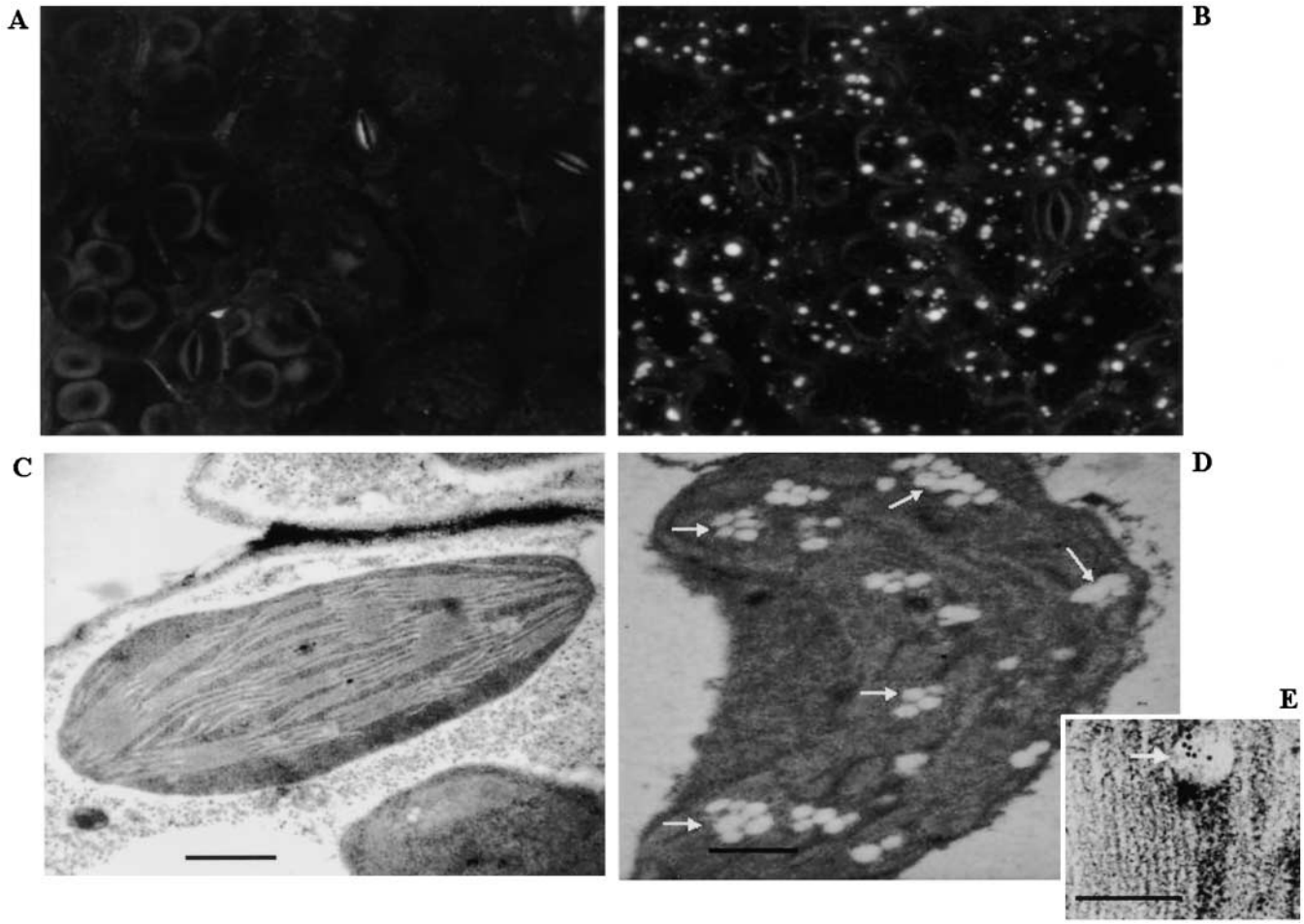


**Fig. 6. Visualization of poly-β-hydroxybutyrate (PHB) granule accumulation in alfalfa Line 864 transformed with *phb*ABC genes. Leaf tissues stained with Nile Blue A and viewed by confocal microscopy. (A) Untransformed alfalfa; (B) Line 864. Chloroplasts from mature leaves of alfalfa viewed by transmission electron microscopy. (C) Untransformed alfalfa, bar = 1 μm; (D) Transgenic alfalfa with agglomerations of electron-lucent PHB granules (indicated with arrows), bar = 1 μm; (E) Immuno-gold labeling of granules with antibodies against PHB synthase, bar = 0.5 μm.**

μm in diameter. Inclusions of PHB were located only in chloroplasts, which suggests plastid-targeting of all enzymes. Transmission electron microscope observations also revealed that some chloroplasts appeared to accumulate fewer granules, ranging from a single granule to groups of two or three per chloroplast, indicating different rates of PHB accumulation. Not all chloroplasts from transgenic plants accumulated PHB granules. A similar observation was found in PHB-producing *Arabidopsis* (Nawrath et al., 1994).

In previous studies, bacterial PHB synthase was found bound to the surface of bacterial granules (Haywood et al., 1989). Immuno-gold labeling with anti-PHB synthase antibodies showed preferential binding of gold particles to a number of chloroplast structures. Gold particles were clearly bound to the surface of PHB-granules, indicating that PHB synthase is present in chloroplasts of transgenic Line 864 (Fig. 6E). The additional structures to which gold particles were attached resembled small granular shapes, apparently the surface of PHB granules, which had been sectioned during sample preparation. A limited amount of nonspecific binding was observed on sections of control nontransformed plants. This study further supports evidence that the enzymes for PHB synthesis are targeted to alfalfa chloroplasts, where they catalyze the production of PHB that accumulates in the stroma as granules.

## Progeny Analysis of Polyhydroxybutyrate-Producing Plants

Transgenic plants exhibited no specific phenotypic features that could be directly linked to the presence of PHB transgenes. Transgenic plants developed normal flowers and seeds, suggesting that expression of the PHB biosynthetic pathway genes at current levels in the plastids had no deleterious effect on growth and fertility. Cross-pollination with nontransformed plants was necessary due to the obligately outcrossing nature of alfalfa in which selfing leads to severe inbreeding depression. Cross-pollination was also performed to transfer the PHB transgenes into an elite alfalfa background, because the Regen-SY alfalfa genotype used for transformation lacks qualities desirable for breeding purposes and agronomic use.

A total of 56 seedlings, 28 from the cross 863 × UMN2966 and 28 from the reciprocal cross, were ana-

lyzed for the presence of *nptII*. Polymerase chain reaction analysis showed 22 progeny plants (78%) from each cross were *nptII* positive, indicating transfer of the marker gene to $F_1$ progeny and supporting the presence of multiple T-DNA insertions into Line 863. Gas chromatography analysis of mature leaves from 44 *nptII*-positive $F_1$ plants showed a detectable level of 3-HB in the leaf extracts of all plants tested. Poly-β-hydroxybutyrate content in individual plants ranged from 0.14 to 0.34 g $kg^{-1}$. Variation in the amounts of 3-HB detected from replicate leaf extracts indicated heterogeneity of PHB content within plants. However, PHB content in all plants was similar to or greater than that obtained from Line 863, which had an average PHB content of 0.11 g $kg^{-1}$.

## CONCLUSIONS

This is the first demonstration of PHB production in a perennial legume crop species. Production of PHB and other PHAs in alfalfa has advantages compared with other crops because of its perennial growth habit and N fixation capacity. Poly-β-hydroxybutyrate production costs would also likely be lower than those of other crops because multiple harvests would occur each year with little fertilization or management costs. The PHB production levels in the transgenic alfalfa plants were lower than those reported in some model plants and would need to be increased to make PHB production in alfalfa economically feasible. Greater PHB gene expression might be attained with different promoter sequences or through expression in chloroplasts. Furthermore, more efficient extraction methods may need to be developed to extract the full amount of PHB produced by these plants. Our results also indicate the need for further modifications of plant metabolism to produce greater levels of PHB/V copolymer, the more valuable PHA.

### ACKNOWLEDGMENTS

We thank G. Ahlstrand, and Dr. M. Sanders from the Imaging Center, Univ. of Minnesota for excellent technical assistance with microscopic analyses. We wish to thank Dr. J.F.S. Lamb, Dr. A. Smith, and Dr. H. Jung for critical review and valuable suggestions.

## REFERENCES

Anderson, A.J., and E.A. Dawes. 1990. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.

Austin, S., E.T. Bingham, D.E. Mathews, M.N. Shahan, J. Will, and R.R. Burgess. 1995. Production and field performance of transgenic alfalfa (*Medicago sativa* L.) expressing alpha-amylase and manganese-dependent lignin peroxidase. Euphytica 85:381–393.

Barnes, D.K. 1980. Alfalfa. p. 177–188. *In* W.R. Fehr and H.H. Hadley (ed.) Hybridization of crop plants. ASA and CSSA, Madison, WI.

Bingham, E.T. 1991. Registration of alfalfa hybrid Regen-SY germplasm for tissue culture and transformation research. Crop Sci. 31: 1098.

Bohmert, K., I. Balbo, J. Kopka, V. Mittendorf, C. Nawrath, Y. Poirier, G. Tischendorf, R.N. Trethewey, and L. Willmitzer. 2000. Transgenic *Arabidopsis* plants can accumulate polyhydroxybutyrate to up to 4% of their fresh weight. Planta 211:841–5.

De Cosa, B., W. Moar, S.-B. Lee, M. Miller, and H. Daniell. 2001. Overexpression of the *Bt*Cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nat. Biotechnol. 19:1–4.

Guda, C., S.-B. Lee, and H. Daniell. 2000. Stable expression of a biodegradable protein-based polymer in tobacco chloroplasts. Plant Cell Rep. 19:257–262.

Hayashi, M., K. Toriyama, M. Kondo, and M. Nishimura. 1998. 2,4-Dichlorophenoxybutyric acid-resistant mutants of *Arabidopsis* have defects in glyoxysomal fatty acid beta-oxidation. Plant Cell 10:183–195.

Haywood, G.W., A.J. Anderson, and E.A. Dawes. 1989. The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by *Alcaligenis eutrophus*. FEMS Microbiol. Lett. 57:1–6.

John, M.E., and G. Keller. 1996. Metabolic pathway engineering in cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. (USA) 93:12768–12773.

Kato, A., M. Hayashi, Y. Takeuchi, and M. Nishimura. 1996. cDNA cloning and expression of a gene for 3-ketoacyl-CoA thiolase in pumpkin cotyledons. Plant Mol. Biol. 31:843–852.

Lutz, K.A., J.E. Knapp, and P. Malinga. 2001. Expression of *bar* in the plastid genome confers herbicide resistance. Plant Physiol. 125: 1585–1590.

Menschik, Z. 1953. Nile Blue histochemical method for phospholipids. Stain Technol. 28:13–17.

Mittendorf, V., E.J. Robertson, R.M. Leech, N. Kruger, A. Steinbuchel, and Y. Poirier. 1998. Synthesis of medium-chain-length polyhyroxyalkanoates in *Arabidopsis thaliana* using intermediates of peroxisomal fatty acid β-oxidation. Proc. Natl. Acad. Sci. (USA) 95:13397–13402.

Nawrath, C., Y. Poirier, and C. Somerville. 1994. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. Proc. Natl. Acad. Sci. (USA) 91:12760–12764.

Olesen, C., and A. Brandt. 1996. A full length cDNA encoding 3-ketoacyl-CoA thiolase from *Brassica napus*. Plant Physiol. 110:714.

Ostle, A.G., and J.G. Holt. 1982. Nile Blue A as a fluorescent stain for poly-beta-hydroxybutyrate. Appl. Environ. Microbiol. 44:238–241.

Poirier, Y., C. Nawrath, and C. Somerville. 1992. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science (Washington, DC) 256:520–523.

Poirier, Y. 1999. Green chemistry yields a better plastic. Nat. Biotechnol. 17:960–961.

Preisig-Muller, R., and H. Kindl. 1993. Thiolase mRNA translated *in vitro* yields a peptide with a putative N-terminal presequence. Plant Mol. Biol. 22:59–66.

Slater, S., K.L. Houmiel, M. Tran, T.A. Mitsky, N.B. Taylor, S.R. Padgette, and K.J. Gruys. 1998. Multiple β-ketothiolases mediate poly (β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J. Bacteriol. 180:1979–1987.

Slater, S., T.A. Mitsky, K.L. Houmiel, M. Hao, S.E. Reiser, N.B. Taylor, M. Tran, H.E. Valentin, D.J. Rodriguez, D.A. Stone, S.R. Padgette, G. Kishore, and K.J. Gruys. 1999. Metabolic engineering of *Arabidopsis* and *Brassica* for poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer production. Nat. Biotechnol. 17:1011–1016.

Slater, S.C., W.H. Vogue, and D.E. Dennis. 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. 170:4431–4436.

Valentin, H.E., D.L. Broyles, L.A. Casagrande, S.M. Colburn, W.L. Creely, P.A. DeLaquil, H.M. Felton, K.A. Gonzalez, K.L. Houmiel, K. Lutke, D.A. Mahadeo, T.A. Mitsky, S.R. Padgette, S.E. Reiser, S. Slater, D.M. Stark, R.T. Stock, D.A. Stone, N.B. Taylor, G.M. Thorne, M. Tran, and K.J. Gruys. 1999. PHA production, from bacteria to plants. Int. J. Biol. Macromol. 25:303–306.

Willis, L.B., and G.C. Walker. 1998. The phbC (poly-β-hydroxybutyrate synthase) gene of *Rhizobium* (*Sinorhizobium*) *meliloti* and characterization of *phb*C mutants. Can. J. Microbiol. 44:554–564.

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacerarum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. Microbiol. Immunol. 39:897–904.

Yoshie, N., Y. Goto, M. Sakurai, Y. Inoue, R. Chujo, and Y. Doi. 1992. Biosynthesis and N.M.R. studies of deuterated poly(3-hydroxybutyrate) produced by *Alcaligenes eutrophus* H16. Int. J. Biol. Macromol. 14:81–6.